Pesticide residues in food — 2019

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2019

Part II – Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food – 2019

Toxicological evaluations

Sponsored jointly by FAO and WHO

Joint Meeting of the
FAO Panel of Experts on Pesticide Residues
in Food and the Environment
and the
WHO Core Assessment Group on Pesticide Residues

Geneva, Switzerland, 17–26 September 2019

Food and Agriculture Organization of the United Nations

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* First full evaluation

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2018 Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

Geneva, 17–26 September 2019

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Abbreviations and acronyms

AChE	acetylcholinesterase
AD	administered dose
ADI	acceptable daily intake
ADME	a , distribution, metabolism and excretion
AFC	antibody-forming cell (assay)
A/G A:G	albumin:globulin ratio
ALP	alkaline phosphatase
ALT	alanine transaminase
APTT	activated partial thromboplastin time
AR	applied radioactivity
AR LBD	androgen receptor-ligand binding domain
ARfD	acute reference dose
AR	androgen receptor
ARE	antioxidant response element
AST	aspartate transaminase
AUC	Area under the concentration-time curve
BMD	benchmark dose
$BMDL_X$	Lower confidence limit on the BMD for a X% response
BUN	blood urea nitrogen
bw	body weight
ca	circa
CCPR	Codex Committee on Pesticide Residues
ChE	cholinesterase
CHO	Chinese Hamster ovary
C_{max}	maximum concentration
CMC	carboxymethyl cellulose
CNS	central nervous system
CP	cyclophophamide
DAPI	4′,6-diamidino-2-phenylindole
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	2,4-dinitrochlorobenzene
ECG	electrocardiograph(ic)
EC	erythrocyte count
EDTA	ethylene diamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
E:P ratio	ratio of 17β-estradiol to progesterone
EROD	ethoxyresorufin-*O*-deethylase
FAO	Food and Agriculture Organization of the United Nations
FCA	Freund's complete adjuvent
FOB	functional observational battery
GC–MS	gas chromatography–mass spectrometry
GD	gestation day
GEF	global evaluation factor
GGTP	γ-glutamyl transpeptidase/transferase
GI	gastrointestinal
GIT	gastrointestinal tract
GLP	good laboratory practice
GSH	glutathione
HBI	haemoglobin binding index
Hb	haemoglobin
HCA	α-hexylcinnamaldehyde
HCD	historical control data
hERα/β	human estrogen receptor α/β
HPC	hydroxypropyl cellulose
HPLC	high-performance liquid chromatography
Ht	haematocrit
IC_{50}	Half-maximal inhibitory concentration
ICH	International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
i.p.	intraperitonial, intraperitonially
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
i.v.	intravenous, intravenously
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
K_M	Michaelis constant
LC_{50}	median lethal concentration
LC-MS	Liquid chromatography–mass spectrometry
LD_{50}	median lethal dose
LD	lactation day
LDH	lactate dehydrogenase
LH	luteinizing hormone
LOAEC	lowest-observed-adverse-effect concentration
LOAEL	lowest-observed-adverse-effect level
LSC	liquid scintillation counting
MA	motor activity
MCH	mean corpuscular/cell haemoglobin

MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular (cell) volume
MetHb	methaemoglobin
MPE	mean photo effect
MMAD	mass median aerodynamic diameter
MN(T)	micronucleus (test)
MOA	mode of action
MOAEC	maximum-observed-adverse-effect concentration
MRL	maximum residue limits
MS	mass spectroscopy/spectrometry
MTD	maximum tolerated dose
NBF	neutral buffered formalin
NCA	nicotinic acid
NCE	normochromic erythrocyte
NMR	nuclear magnetic resonance
NNG	net nuclear grain
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NTE	neuropathy target esterase
OECD	Organisation for Economic Co-operation and Development
PAS	periodic acid–Schiff stain
PCE	polychromatic erythrocyte
PCN	pregnenolone-16α-carbonitrile
PCV	packed cell volume
PFC	plaque-forming cell (assay)
PIF	photo irritation factor
PND	postnatal day
PROD	pentoxyresorufin-*O*-deethylase
PT	prothrombin time
PVDF	polyvinylidene difluoride
QA	quality assurance
QSAR	quantitative structure–activity relationship
RAC	repetitive accumulation ratio
RBC	red blood cell
RNA	ribonucleic acid
ROS	reactive oxygen species
RTG	relative total growth
RT-PCR	reverse transcription polymer chain reaction
s.c.	subcutaneous/ly
SCE	sister chromatid exchange
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel
SI	stimulation index
sRBC	Sheep red blood cell
T.Bil	total bilirubin
$t_{½}$	half-life
T_3	triiodothyronine
T_4	thyroxine
TDAR	T-lymphocyte-dependent antibody response
TFT	5-trifluorothymidine
TG	triglycerides
TK	toxicokinetic
TLC	thin-layer chromatography
T_{max}	time to reach maximum concentration
TOCP	tri-o-cresyl phosphate
TP	testosterone propionate
TPO	thyroid peroxidase
TRR	total radioactive residue
TSH	thyroid-stimulating hormone
TTC	threshold of toxological concern
UDP-GT	uridine diphosphate lucuronosyltransferase
UDS	unscheduled DNA synthesis
V_{max}	maximum rate of reaction
WBC	white blood cell/leucocyte count
WHO	World Health Organization
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

Introduction

A Joint Meeting of the Food and Agriculture Organization of the United Nations (FAO) Panel of experts on Pesticide Residues in Food and the Environment and the World Health Organization (WHO) Core assessment Group on Pesticide Residues (JMPR) was held in Geneva, Switzerland, from 17 to 26 September 2019. The FAO Panel Members met in preparatory sessions from 12 to 16 September.

The WHO Director of Food Safety and Zoonoses, Dr Kazuaki Miyagishima, welcomed all the experts and colleagues from FAO. Dr Miyagishima remarked that the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) is an excellent example of how WHO and FAO can jointly mobilise some of the best expertise from around the world, in this case, in the interest of protecting public health from adverse effects of pesticide residues in food.

Dr Miyagishima reflected on the fact that the JMPR has met on an annual basis since 1963 to provide scientific advice to the Codex Alimentarius Commission and the Codex Committee on Pesticide Residues (CCPR).The high demand for scientific advice on pesticide residues had resulted in an extraordinary JMPR meeting earlier this year that was held in Ottawa in May. The ordinary JMPR meeting is now about to begin with a full agenda.

Dr Miyagishima appreciated the hard work of the experts prior to the meeting and intensive discussion and critical review during the meeting. This engagement assures that the scientific output from the meeting will meet the highest possible standard. This way of working is essential in maintaining the consistent high quality of the scientific advice provided by FAO and WHO to the Codex – and to the countries of the world. As a result, the advice from the JMPR is respected and widely used around the world through application of Codex standards for food in international trade and directly by national authorities.

On behalf of WHO and FAO, Dr Miyagishima conveyed a deep appreciation for the efforts and commitment to the JMPR by the experts. Without these expert inputs, the organisations would not be able to deliver this necessary expert advice and – consequently – the safety of food around the world would suffer. Finally, Dr Miyagishima wished all the participants a fruitful meeting over the next two weeks.

During the meeting, the FAO Panel of Experts on Pesticide Residues in Food was responsible for reviewing residue and analytical aspects of the pesticides under consideration, including data on their metabolism, fate in the environment and use patterns, and for estimating the maximum levels of residues that might occur as a result of use of the pesticides according to good agricultural practice. The methodologies are described in detail in the FAO Manual on the submission and evaluation of pesticide residue data for the estimation of maximum residue levels in food and feed (2016) hereafter referred to as the FAO manual. The WHO Core Assessment Group on Pesticide Residues was responsible for reviewing toxicological and related data in order to establish acceptable daily intakes (ADIs) and acute reference doses (ARfDs), where necessary and possible.

The Meeting evaluated 30 pesticides, including eight new compounds and three compounds that were re-evaluated for toxicity or residues, or both, within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). The Meeting established ADIs and ARfDs, estimated maximum residue levels and recommended them for use by CCPR, and estimated supervised trials median residue (STMR) and highest residue (HR) levels as a basis for estimating dietary exposures.

The Meeting also estimated the dietary exposures (both acute and long-term) to the pesticides reviewed and, on this basis, performed a dietary risk assessment in relation to the relevant ADI and where necessary the ARfD. Cases in which ADIs or ARfDs may be exceeded, if they occur, are clearly indicated in order to facilitate the decision-making process by CCPR.

The Meeting considered a number of general issues addressing procedures for the evaluation and risk assessment of pesticide residues.

TOXICOLOGICAL MONOGRAPHS AND MONOGRAPH ADDENDA

AFIDOPYROPEN

First draft prepared by
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Explanation

Afidopyropen is the ISO-approved common name for [(1*S*,2*S*,5*S*,6*R*,7*R*,9*S*,10*S*,18*R*)-5-(cyclopropanecarbonyloxy)-9,18-dihydroxy-2,6,10-trimethyl-16-oxo-14-pyridin-3-yl-11,15-dioxatetracyclo[8.8.0.$0^{2,7}$.$0^{12,17}$]octadeca-12(17),13-dien-6-yl]methyl cyclopropanecarboxylate, with the CAS number 915972-17-7.

Afidopyropen is a pyripyropene-derivative insecticide and represents a novel class of pesticides. The proposed pesticidal mode of action (MOA) for afidopyropen is gate disruption of transient receptor potential vanilloid (TRPV) channel complexes in insect chordotonal stretch receptor organs. In insects,

these organs are critical for hearing, balance, and proprioception, among other functions. The TRP channels play an important role in cilia-dependent function. Although humans lack these organs, there are human homologues of proteins that make up these channels.

Afidopyropen has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of Codex Committee onPesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of afidopyropen (Fig. 1), as well as its toxicokinetics, have been investigated in F344 and Wistar rats. Summaries of the relevant data are presented below.

The ADME of afidopyropen were investigated using nicotinic acid-labelled and pyranone-labelled afidopyropen (NCA-labelled, Fig. 2; PYA-labelled, Fig. 3, respectively). The test item was a mixture of labelled and unlabelled afidopyropen. The study design is summarized in Table 1.

Figure 1. Afidopyropen

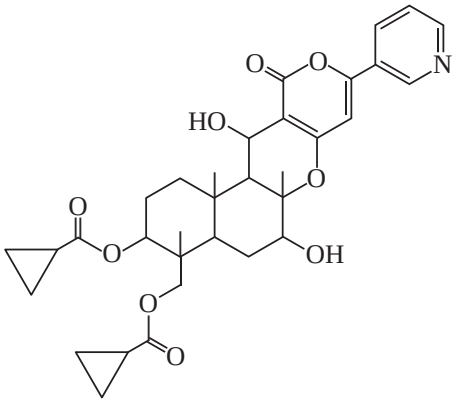


Figure 2. [NCA ^{14}C]-afidopyropen ([9-^{14}C])

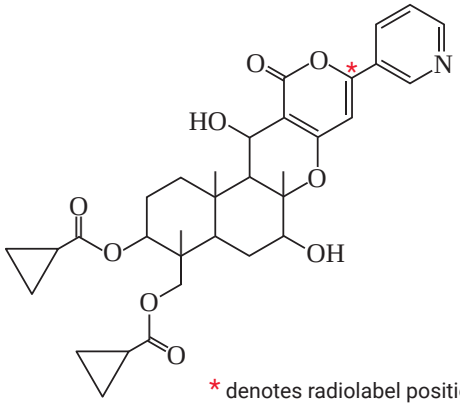


Figure 3. [PYA-4 ^{14}C]-afidopyropen

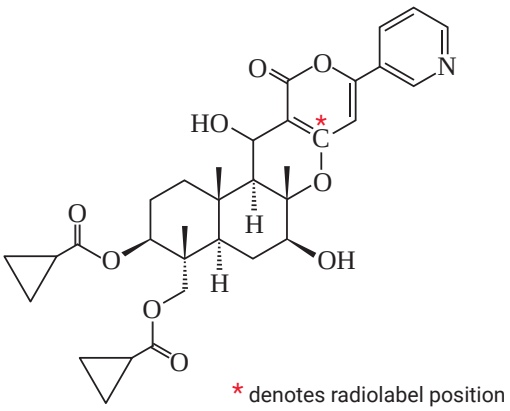


Table 1. Dosing groups for ADME experiments with ^{14}C-labelled afidopyropen

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Preliminary study F344/DuCrlCrlj rats – low dose	3 (oral)	1 M, 1 F	Single-dose mixture of [NCA ^{14}C] afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Excreta, blood (plasma and RBCs), expired air, tissues, volatiles and time-course plasma collected.	Ohyama, 2012
Preliminary study F344/DuCrlCrlj rats – high dose	300 (oral)	1 M, 1 F	Single-dose mixture of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Excreta, blood (plasma and RBCs), expired air, tissues, volatiles and time-course plasma collected.	Ohyama, 2012
Excretion study F344/DuCrlCrlj rats – low dose – excretion/mass balance	3 (oral)	4 M, 4 F	Single-dose mixture of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 96 hours, urine collection at 6, 12, 24, 48, 72 and 96 h, faeces collection at 24, 48, 72 and 96 h.	Ohyama, 2015
Excretion study F344/DuCrlCrlj rats – high dose – excretion/mass balance	300 (oral)	4 M, 4 F	Single-dose mixture of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 96 h, urine collection at 6, 12, 24, 48, 72 and 96 h, faeces collection at 24, 48, 72 and 96 h.	Ohyama, 2015
Excretion study F344/DuCrlCrlj rats – low dose – biliary excretion	3 (oral)	4 M, 4 F	Single-dose mixture of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 48 h, bile collection at 6, 24 and 48 h, urine collection at 6, 24 and 48 h, faeces collection at 24 and 48 h.	Ohyama, 2015
Excretion study F344/DuCrlCrlj rats – high dose – biliaryexcretion	300 (oral)	4 M, 4 F	Single-dose mixture of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 48 h, bile collection at 6, 24 and 48 h, urine collection at 6, 24 and 48 hours, faeces collection at 24 and 48 h.	Ohyama, 2015
Toxicokinetics rats – blood/plasma concentration	300 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous HPC.	Fabian & Landsiedel, (2015)

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Toxicokinetics – rats – blood/ plasma concentration	3 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous HPC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – blood/ plasma concentration	30 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43-3.67 MBq/mg in 3% aqueous HPC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – blood/ plasma concentration	0.5 (intravenous)	4 M, 5 F	Single-dose mixture of [pyranone-4-^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – balance/ excretion	300 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4-^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – balance/ excretion	3 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4-^{14}C] afidopyropen, [pyranone-4-^{13}C]afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – balance/ excretion	300 (oral)	4 M, 4 F	Repeat-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – tissue distribution	300 (oral)	12 M, 12 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – tissue distribution	3 (oral)	12 M, 12 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – bile excretion	300 (oral)	6 M, 6 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015
Toxicokinetics – rats – bile excretion	3 (oral)	6 M, 6 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, and unlabelled afidopyropen with target radioactivity of 3.43–3.67 MBq/mg in 3% aqueous CMC.	Fabian & Landsiedel, 2015

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Definitive study Crl:WI (Han) rats – metabolism (liver/kidney/ plasma quantification)	3 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, [pyranone-6 ^{13}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 48 h, bile collection at 6, 24 and 48 hours, urine collection at 6, 24 and 48 h, faeces collection at 24 and 48 h.	Thiaener & Glaessgen, 2011
Definitive study Crl:WI (Han) rats – metabolism (liver/kidney/ plasma quantification)	300 (oral)	4 M, 4 F	Single-dose mixture of [pyranone-4 ^{14}C]-afidopyropen, [pyranone-4 ^{13}C]-afidopyropen, [pyranone-6 ^{13}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg in 3% aqueous HPC. Killed at 48 h, bile collection at 6, 24 and 48 h, urine collection at 6, 24 and 48 hours, faeces collection at 24 and 48 h.	Thiaener & Glaessgen, 2011
Metabolic fate of ME5343-T7 (dimer of afidopyropen) – rats	0 (oral)	1 M	Single dose of vehicle Killed at 72 h post dosing, urine and faecal collection at 24, 48 and 72 h.	Ohyama, 2013
Metabolic fate of ME5343-T7 (dimer of afidopyropen) – rats	300 (oral)	1 M	Single dose of unlabelled ME5343-T7 Killed at 72 h post dosing, urine and faecal collection at 24, 48 and 72 h.	Ohyama, 2013
Nicotinic acid study – pilot TK study – rats	3 (oral)	4 M, 4 F	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg. Killed at 120 h post dosing.	McClanahan, 2015
Nicotinic acid study – pilot TK study – rats	300 (oral)	4 M, 4 F	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg Killed at 120 h post dosing.	McClanahan, 2015
Nicotinic acid study – pharmacokinetic study – rats	3 (oral)	5 M, 5 F	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg. Killed at 72 h post dosing.	McClanahan, 2015
Nicotinic acid study – pharmacokinetic study – rats	300 (oral)	5 M, 5 F	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg. Killed at 72 h post dosing.	McClanahan, 2015
Nicotinic acid study – Tissue distribution study– rats	3 (oral)	3 M, 3 F per time period	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg. Three animals per group killed at 0.5, 8, 96 h post dosing.	McClanahan, 2015
Nicotinic acid study – Tissue distribution study – rats	300 (oral)	3 M, 3 F per time period	Single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 3.29 MBq/mg. Three animals per group killed at 2, 24, 96 h post dosing.	McClanahan, 2015

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Cancer pharmacokinetics – rats – excretion kinetics	3 (oral)	4 F	Repeat-dose mixture of unlabelled afidopyropen followed by single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 4.47 MBq/mg. Days 1–14 @ 36 ppm (3 mg/kg bw per day) in diet. Day 15 @ 3 mg/kg bw per day gavage. Blood, urine and faeces collected. Killed at 72 h post dosing.	Capello, 2016
Cancer pharmacokinetics – rats – excretion kinetics	50 (oral)	4 F	Repeat-dose mixture of unlabelled afidopyropen followed by single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 4.47 MBq/mg. Days 1–14 @ 600 ppm (44 mg/kg bw per day) in diet. Day 15 @ 50 mg/kg bw per day gavage. Blood, urine and faeces collected. Killed at 72 h post dosing.	Capello, 2016
Cancer pharmacokinetics – rats – excretion kinetics	15 (oral)	4 F	Repeat-dose mixture of unlabelled afidopyropen followed by single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 4.47 MBq/mg. Days 1–14 @ 180 ppm (14 mg/kg bw per day) in diet. Day 15 @ 15 mg/kg bw per day gavage. Blood, urine and faeces collected. Killed at 72 h post dosing.	Capello, 2016
Cancer pharmacokinetics – rats – Tissue distribution and metabolite profiling	50 (oral)	4 F	Repeat-dose mixture of unlabelled afidopyropen followed by single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 4.47 MBq/mg. Days 1–14 @ 600 ppm (51 mg/kg bw per day) in diet. Day 15 @ 50 mg/kg bw per day gavage. Blood, plasma, liver and uterus collected. Killed at 1.5 h post dosing.	Capello, 2016
Cancer pharmacokinetics – rats – Tissue distribution and metabolite profiling	15 (oral)	4 F	Repeat-dose mixture of unlabelled afidopyropen followed by single-dose mixture of [nicotinic acid ^{14}C]-afidopyropen and unlabelled afidopyropen with target radioactivity of 4.47 MBq/mg. Days 1–14 @ 180 ppm (14 mg/kg bw per day) in diet. Day 15 @ 15 mg/kg bw per day gavage Blood, plasma, liver and uterus collected. Killed at 1.5 h post dosing.	Capello, 2016

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Dermal excretion, retention and tissue concentrations	500 µg/cm^2 (dermal)	4 males per time point	Single dose mixture of [pyranone-6-C^{14}] afidopyropen with a target radioactivity of 10 MBq/mL. Killed at 8, 32 and 128 hours post dosing.	Fabian & Landsiedel, 2014
Dermal excretion, retention and tissue concentrations	5.0 µg/cm^2 (dermal)	4 males per time point	Single dose mixture of [pyranone-6 C^{14}]-afidopyropen with a target radioactivity of 1.85 MBq/mL. Killed at 8, 32 and 128 h post dosing.	Fabian & Landsiedel, 2014
Dermal excretion, retention and tissue concentrations	2.5 µg/cm^2 (dermal)	4 males per time point	Single dose mixture of [pyranone-6 C^{14}]-afidopyropen with a target radioactivity of 0.93 MBq/mL. Killed at 8, 32 and 128 h post dosing.	Fabian & Landsiedel, 2014

HPC Hydroxypropyl cellulose CMC Carboxymethyl cellulose

RBCs Red blood cells

bw Body weight

C_{max}: maximum concentration

1.1 Absorption, distribution and excretion

(a) Oral route

Preliminary ADME assays were performed on one Fischer 344 rats per sex per dose at 3 and 300 mg/kg bw. Labelled afidopyropen was readily excreted in the urine and faeces with faecal excretion the predominant route of excretion regardless of sex or dose. No significant radioactivity was found in expired air. At 96 h post dose radioactivity remaining in the carcass and gastrointestinal tract (GIT) was between 0.48 and 1.03% of the administered dose (AD). Recovery of the AD was 94–95%. The highest residue levels were found in the liver at low and high doses. At the low dose this was followed by the adrenals, heart muscle and kidneys, with no other tissues with levels greater than 0.02 mg eq/kg. At the high dose, liver residues were followed by heart muscle, adrenals and kidneys with no other tissues with levels greater than 2 mg eq/kg. Plasma levels were significantly below other tissues at 96 h. (Ohyama, 2012).

In an excretion assay performed on eight Fischer 344 rats per dose at 3 and 300 mg/kg bw, four rats per sex per dose were examined for excretion/mass balance and four rats per sex per dose were examined for biliary excretion. Within 96 h, 94–96% of the AD was excreted with 5.5–20.4% excreted in the urine and 73.4–87.4% excreted in the faeces. In the biliary excretion study at 48 h, 92–97% of the AD was recovered within 48 h of dosing. Between 67 and 72% of the AD was absorbed, based on the sum of radioactivity found in bile, urine and residual carcass. There were no dose or sex differences in absorption, and bile was the predominant manner of excretion in all groups, however, there was a shift towards urinary excretion at 300 mg/kg bw (Ohyama, 2015; see Table 2).

Table 2. Percentage recovery of the administered radioactivity after a single oral administration of [NCA ^{14}C]-afidopyropen to bile duct-cannulated rats – biliary excretion experiment

		Percentage recovery of the administered dose			
Dose rate		**3 mg/kg bw**		**300 mg/kg bw**	
Sex		**Male**	**Female**	**Male**	**Female**
Bile	0–6 h	41.58 ± 13.86	39.56 ± 13.05	6.81 ± 0.52	5.23 ± 1.78
	6–24 h	10.71 ± 3.72	13.17 ± 6.62	23.73 ± 2.96	23.06 ± 3.41
	24–48 h	0.81 ± 0.42	0.55 ± 0.24	9.61 ± 1.55	12.16 ± 7.33
	sub-total	53.10 ± 10.94	53.28 ± 10.13	40.16 ± 2.21	40.45 ± 4.75

		Percentage recovery of the administered dose			
Dose rate		**3 mg/kg bw**		**300 mg/kg bw**	
Sex		**Male**	**Female**	**Male**	**Female**
Urine	0–6 h	5.19 ± 4.96	3.51 ± 2.71	1.39 ± 2.08	6.84 ± 1.35
	6–24 h	10.61 ± 6.28	9.09 ± 11.93	20.84 ± 6.72	15.30 ± 1.17
	24–48 h	0.43 ± 0.16	0.36 ± 0.07	7.69 ± 2.93	8.16 ± 4.05
	sub-total	16.23 ± 6.41	12.96 ± 9.45	29.91 ± 6.49	30.30 ± 3.65
Faeces	0–24 h	14.34 ± 4.89	20.26 ± 4.67	1.53 ± 1.94	5.98 ± 8.83
	24–48 h	8.03 ± 2.80	6.47 ± 4.38	24.83 ± 7.58	18.08 ± 6.62
	sub-total	22.37 ± 2.85	26.73 ± 0.60	26.36 ± 7.34	24.05 ± 7.93
Cage wash		0.60 ± 0.31	0.24 ± 0.11	0.65 ± 0.20	0.99 ± 0.19
Total excreted		**92.30 ± 1.70**	**93.22 ± 0.92**	**97.08 ± 0.87**	**95.80 ± 3.79**
GI tract and contents		1.03 ± 0.61	1.05 ± 0.66	2.83 ± 0.81	4.20 ± 2.29
Carcass		1.00 ± 0.07	0.94 ± 0.06	1.07 ± 0.08	1.26 ± 0.24
Total		**94.34 ± 1.50**	**95.20 ± 1.23**	**100.97 ± 1.53**	**101.26 ± 2.24**
Absorbed		**70.34 ± 4.57**	**67.19 ± 1.43**	**71.13 ± 7.53**	**72.01 ± 5.87**

Source: Ohyama, 2015

In a biokinetics, excretion and tissue distribution study performed on Crl:WI (Han) rats, animals were allocated to one of the following treatments:

- a single gavage dose of 3, 30, or 300 mg/kg bw [pyranone-4 $C^{13/14}$]-afidopyropen (four rats/sex per dose)
- a single intravenous (i.v.) dose of 0.5 mg/kg bw [pyranone-4 $C^{13/14}$]-afidopyropen (four males and five females) in a blood/plasma concentration assay
- a single dose of 3 or 300 mg/kg bw [pyranone-4 $C^{13/14}$]-afidopyropen (four rats/sex per dose)
- 14 doses of unlabelled afidopyropen at 300 mg/kg bw per day, followed by a single dose of 300 mg/kg bw [pyranone-4 $C^{13/14}$]-afidopyropen (four rats/sex), in a balance/excretion assay
- a single dose of 3 or 300 mg/kg bw of [pyranone-4 $C^{13/14}$]-afidopyropen (four rats/sex per dose at each time point) in a tissue distribution assay
- a single dose of 3 or 300 mg/kg bw of [pyranone-4 $C^{13/14}$]-afidopyropen (six males/dose or 6–12 females/dose) in a bile excretion assay.

In the pharmacokinetics assay, it was determined that absorption was rapid but that females had higher exposures than males at all but the lowest dose levels. (Table 3) Values for the females in the 30 mg/kg bw dose group indicate either supralinear kinetics or an issue with data collection, in that T_{max} and intial half-life values were inconsistent with the low- and high-dose group and the mid-dose group in males.

Table 3. Pharmacokinetic parameters of radioactivity in plasma after single oral administration to male and female rats of ^{14}C-BAS 440 I at dose levels of 300, 30 and 3 mg/kg bw

Sex	Dose (mg/kg bw)	C_{max} (µg equiv./g)	T_{max} (h)	Initial half-life (h)	Terminal half-life (h)	AUC_{0-168} (µg equiv. × h/g)	$AUC_{0-\infty}$ (µg equiv. × h/g)
Male	300 p.o.	45.66	4	7.14	92.02	756.3	783.6
	30 p.o.	6.24	1	3.63	82.88	47.2	49.7
	3 p.o.	0.39	1	1.22	18.7	2.1	2.1
	0.5 i.v.	1.52	§	0.34	61.91	4.1	4.5
Female	300 p.o.	61.11	4	8.16	81.57	982.1	1003.3
	30 p.o.	11.83	8	18.74	38.98	531.1	543.9
	3 p.o.	0.4	1	1.11	16.8	2.2	2.2
	0.5 i.v.	0.65	§	0.46	48.68	3.6	3.8

AUC Area under the concentration–time curve

p.o. Per oral i.v. Intravenously

§ Directly after administration

Males

(AUC/dose) 3mg/kgbw p.o./ (AUC/dose)0.5mg/kgbw i.v.**: 9.0 %**

(AUC/dose) 300mg/kgbw p.o./ (AUC/dose) 0.5mg/kgbw i.v.: **33.8 %**

Females

(AUC/dose) 3mg/kgbw p.o./ (AUC/dose)0.5mg/kgbw i.v.**: 11.2 %**

(AUC/dose) 300mg/kgbw p.o./ (AUC/dose) 0.5mg/kgbw i.v.: **50.8 %**

Source: Fabian & Landsiedel (2015)

In the balance/excretion assay, it was determined that afidopyropen was primarily excreted via the faeces with 72–78% of the administered radioactivity recovered in the faeces when exposed to either single or repeat dosing at 300 mg/kg bw. In the animals given 3 mg/kg bw, faecal excretion accounted for 86–87% of the administered dose. Urinary excretion accounted for 20–21 % of the administered dose recovered in the single high-dose group, and 15–18% in the repeat high-dose group. Urinary excretion in the low-dose group accounted for 5–6% of the administered dose.

Insufficient bile flow in the bile excretion assay necessitated the addition of a second female high-dose group to attain sufficient samples. At the low dose, within 72 h, mean excretion in the bile was 39–46%, and mean total excretion in the urine 17 and 11% in males and females respectively. Based on the radioactivity excreted in the bile and urine, as well as cage and carcass residues, oral absorption was approximately 57% in males and females. At 300 mg/kg bw mean excretion was 41 and 36% in males and females respectively, within 72 h of dosing. Mean total excretion in the urine was 15 and 22% in males and females respectively. Absorption based on urine, bile, cage wash and carcass residues was 57 and 60% in males and females respectively. Oral absorption was considered independent of dose.

Tissue distribution was extensive and included the gonads, uterus and brain. Tissue concentrations in the testes were lower than plasma up until the final time point; however, tissue concentrations in the uterus and ovaries were higher than plasma at all time points (Tables 4 and 5). The highest concentrations were in the GI tract and contents in males and females at both low and high doses. Other tissues exhibiting high concentrations of radioactivity compared to plasma include the liver, adrenal glands, kidneys, heart, spleen, lungs, thyroid and pancreas, in addition to ovaries, uterus and brain tissues noted above. For both low and high doses, radioactive residue concentrations generally declined in organs and tissues parallel to the radioactive residues in plasma (Van Cott & Fabian 2016).

Table 4. Mean tissue concentration of radioactivity after single oral administration to male and female rats of ^{14}C-BAS 440 I at a dose level of 3 mg/kg bw (µg equiv./g tissue)

Time after administration (h)	Male				Female			
	1	2	3.5	8	1	2	3.5	8
Blood cells	0.12	0.11	0.09	0.01	0.19	0.15	0.05	0.02
Plasma	0.28	0.25	0.16	0.02	0.24	0.17	0.05	0.01
Lung	0.45	0.44	0.29	0.04	0.41	0.30	0.08	0.02
Heart	0.71	0.59	0.37	0.11	0.50	0.37	0.08	0.05
Spleen	0.55	0.48	0.32	0.03	0.46	0.32	0.12	0.02
Kidney	1.18	1.24	1.08	0.10	0.85	0.57	0.15	0.03
Adrenal glands	2.96	2.68	1.69	0.33	1.90	1.25	0.27	0.11
Testes/Ovaries	0.08	0.14	0.13	0.04	8.30	4.84	8.44	0.40
Uterus	-	-	-	-	0.81	0.65	0.74	0.06
Muscle	0.36	0.39	0.28	0.04	0.36	0.30	0.09	0.02
Brain	0.03	0.03	0.03	0.02	0.03	0.02	0.01	0.00
Adipose tissue	0.50	0.91	0.83	0.09	0.56	0.62	0.25	0.06
Bone	0.13	0.20	0.16	0.01	0.17	0.13	0.04	0.01
Bone marrow	0.40	0.35	0.25	0.05	0.40	0.30	0.10	0.05
Thyroid	1.14	1.46	0.82	0.38	0.83	0.66	0.52	0.12
Pancreas	1.04	1.05	0.65	0.10	1.79	0.80	0.23	0.06
Stomach content	78.12	71.36	5.53	9.05	107.01	93.35	41.64	4.99
Stomach	15.76	20.85	2.60	1.09	18.90	35.67	6.18	0.82
Gut content	14.29	30.01	43.89	39.45	16.88	26.42	41.17	44.21
Gut	4.53	6.17	12.03	2.05	14.42	15.01	14.82	3.77
Liver	4.61	3.63	2.49	0.44	4.50	3.04	0.78	0.31
Skin	0.36	0.37	0.25	0.04	0.31	0.31	0.09	0.02
Carcass	0.36	0.44	0.34	0.06	0.45	0.40	0.21	0.04

Source: Fabian & Landsiedel, 2015

Table 5. Mean tissue concentration of radioactivity after single oral administration to male and female rats of [NCA ^{14}C]-afidopyropen at a dose level of 300 mg/kg bw (µg equiv./g tissue)

Time after administration (h)	Male				Female			
	4	12	24	36	4	14	20	36
Blood cells	14.20	5.40	5.73	1.05	16.00	8.86	11.71	7.08
Plasma	17.91	6.56	6.49	0.26	17.85	7.11	7.93	3.17
Lung	28.61	12.29	11.79	1.12	26.80	13.52	14.86	6.78
Heart	30.85	14.20	17.49	1.43	33.03	14.30	15.27	9.36
Spleen	25.45	9.95	11.64	0.77	26.05	11.25	14.29	6.23
Kidney	46.01	21.54	23.81	0.74	38.31	18.38	19.29	8.31
Adrenal glands	99.49	40.43	42.91	0.98	89.06	42.01	43.74	20.16
Testes/Ovaries	10.82	4.17	4.10	0.39	22.79	10.51	10.22	4.94
Uterus	-	-	-	-	32.68	13.81	13.78	8.90
Muscle	23.70	9.56	9.51	0.38	23.13	8.84	9.37	4.13
Brain	2.28	1.77	1.26	0.51	2.17	1.15	1.63	0.71
Adipose tissue	39.28	18.63	21.25	0.46	35.64	12.85	14.99	7.49

Time after administration (h)	Male				Female			
	4	12	24	36	4	14	20	36
Bone	10.90	5.57	3.27	0.34	6.93	3.86	3.49	1.21
Bone marrow	26.86	11.95	12.22	3.19	23.96	12.54	9.70	10.02
Thyroid	54.90	25.50	29.38	5.36	60.66	23.71	26.04	18.50
Pancreas	45.60	21.74	24.47	0.95	45.26	19.74	19.89	8.24
Stomach content	3760.06	3993.00	3307.81	0.55	4762.89	3000.79	2560.71	1873.81
Stomach	1170.47	577.91	572.83	2.63	768.31	592.95	296.54	407.53
Gut content	3157.81	1779.02	1128.98	136.38	3955.58	2539.97	2044.09	820.40
Gut	170.89	104.63	105.60	5.85	182.15	139.54	78.66	43.81
Liver	100.69	49.81	63.01	3.15	81.49	45.59	52.73	22.96
Skin	21.78	8.74	10.38	0.89	22.81	9.72	9.48	4.18
Carcass	31.54	12.80	13.10	2.47	21.63	26.00	14.04	7.77

Source: Fabian & Landsiedel, 2015

In an excretion and metabolism study performed on four Wistar rats/sex per group as single oral doses of 3 and 300 mg/kg bw of afidopyropen, or a single labelled dose of [pyranone-4 C^{14}]-afidopyropen and [pyranone-4 C^{13}]-afidopyropen following 14 doses of unlabelled afidopyropen, animals were examined for metabolism. Additionally, samples taken from the Fabian & Landsiedel (2015) study were analysed for metabolite patterns.

In the absorption assays it was determined that afidopyropen was rapidly absorbed and excreted quickly, primarily via the urine and faeces. Based on bile excretion experiments, absorption after a single dose was within the range of 53–66% of the AD and similar at both dose levels. This was based on the proportions excreted via the bile and urine, and radioactive residues found in the cage wash and carcass.

In the excretion assays, excretion via the urine and faeces was nearly complete within 96–120 hours of dosing and more than two-thirds was excreted within the first 48 h (Table 6). Between 72 and 86% of the AD was excreted via the faeces and 5–21% in the urine within seven days. Compared to the low dose, excretion was slower following a high dose and a higher proportion was excreted in the urine. The amount of any given metabolite increased with administered dose with the exception of M440I017 in males. Following repeated administration, urinary excretion was slightly lower compared to single high dosing. No significant gender-specific differences were observed, based on route and total rate of excretion. Excretion via the bile was almost complete within 12 h in low-dose groups (39–46%), and 33–51 h in the case of high-dose groups (53–66%) (Thiaener & Glaessgen, 2011)

Table 6. Percentage recovery of the administered radioactivity after a single oral and repeat oral administration of [pyranone-4-^{14}C]afidopyropen and [pyranone-4-^{13}C]afidopyropen to rats

Dose rate		Percentage recovery of the administered dose					
		3 mg/kg bw		300 mg/kg bw		300 mg/kg bw per day (14 unlabelled + 1 labelled)	
		Male	Female	Male	Female	Male	Female
Urine	0–6 h	3.88	4.7	2.57	4.27	4.75	3.74
	6–12 h	0.8	0.49	5.34	6.03	6.82	4.97
	24–48 h	0.49	0.42	7.20	5.54	4.93	4.84
	48–72 h	0.04	0.07	0.23	1.06	0.21	0.14
	72–96 h	0.02	0.03	0.11	0.13	0.12	0.07
	96–120 h	0.02	0.02	0.08	0.08	0.014	0.04
	120–144 h	0.01	0.01	0.07	0.05	0.12	0.03
	144–168 h	0.01	0.01	0.05	0.05	0.09	0.03
	Sub-total	**5.49**	**5.91**	**20.19**	**21.23**	**17.91**	**15.44**

Dose rate		Percentage recovery of the administered dose					
		3 mg/kg bw		300 mg/kg bw		300 mg/kg bw per day (14 unlabelled + 1 labelled)	
		Male	Female	Male	Female	Male	Female
Faeces	0–24 h	58.54	77.45	22.80	16.89	32.96	18.66
	24–48 h	24.73	7.91	42.15	32.50	36.32	51.68
	48–72 h	2.35	0.58	6.40	21.19	2.72	7.03
	72–96 h	0.16	1.43	0.43	3.18	0.16	0.66
	96–120 h	0.05	0.02	1.12	0.53	0.19	0.09
	120–144 h	0.02	0.01	0.05	0.15	0.04	0.04
	144–168 h	0.04	0.02	1.38	0.05	0.03	0.03
	Sub-total	**85.87**	**87.43**	**74.34**	**74.49**	**72.41**	**78.19**
Cage wash		0.06	0.32	0.15	0.25	0.10	0.12
Blood cells		0.01	0.01	0.03	0.02	0.02	0.01
Plasma		0.00	0.00	0.00	0.00	0.00	0.00
Lung		0.00	0.00	0.00	0.00	0.00	0.00
Heart		0.00	0.00	0.00	0.00	0.00	0.00
Spleen		0.00	0.00	0.00	0.00	0.00	0.00
Kidney		0.00	0.00	0.00	0.00	0.00	0.00
Adrenals		0.00	0.00	0.00	0.00	0.00	0.00
Testes/Ovaries		0.00	0.00	0.00	0.00	0.00	0.00
Uterus		0.00	0.00	0.00	0.00	0.00	0.00
Muscle		0.00	0.00	0.00	0.00	0.00	0.00
Brain		0.00	0.00	0.00	0.00	0.00	0.00
Adipose tissue		0.00	0.00	0.00	0.00	0.00	0.00
Bone		0.00	0.00	0.00	0.00	0.00	0.00
Bone marrow		0.00	0.00	0.00	0.00	0.00	0.00
Thyroid		0.00	0.00	0.00	0.00	0.00	0.00
Pancreas		0.00	0.00	0.00	0.00	0.01	0.01
Stomach contents		0.00	0.00	0.00	0.00	0.00	0.00
Stomach		0.00	0.00	0.00	0.00	0.00	0.00
Gut contents		0.01	0.01	0.01	0.01	0.01	0.01
Gut		0.03	0.01	0.01	0.01	0.01	0.00
Liver		0.01	0.00	0.02	0.01	0.02	0.02
Skin		0.02	0.02	0.07	0.04	0.10	0.01
Carcass		0.09	0.12	0.13	0.13	0.14	0.16
Total recovery		**91.60**	**93.84**	**94.96**	**96.20**	**90.74**	**94.00**

Source: Thiaener & Glaessgen, 2011

Toxicokinetics and tissue distribution was investigated in F344/DuCrl rats following administration of a single radiolabelled dose of 3 or 300 mg/kg bw [NCA ^{14}C]-afidopyropen. Four rats/sex per dose were used in the pilot assay, five/sex per dose in the toxicokinetics assay and three/sex per dose in the tissue distribution assay.

In the toxicokinetics assay, pharmacokinetic parameters were calculated from the whole blood, plasma and RBC total radioactivity concentration data (see Table 7). In the pharmacokinetic

study T_{max} occurred between 0.25 h and 1 h following administration of 3 mg/kg, and between 2 h and 4 h following the administration of 300 mg/kg; radioactivity was distributed between the plasma and the RBCs. Elimination half-life in whole blood was 1–2.5 h following administration of 3 mg/kg; it was 15.1–16 h following administration of 300 mg/kg. In plasma and RBCs it was 4.7–4.8 h and 1.2–2.1 h respectively following administration of 3 mg/kg, and 7.9–10.2, and 31.4–43.6 h respectively following administration of 300 mg/kg. There was no evidence of preferential binding to RBCs.

Table 7. Whole blood, plasma, and red blood cell total radioactivity pharmacokinetic parameters for pilot pharmacokinetic experiment

Matrix	Dose (mg/kg)	Sex	T_{max} (h)	R^2	Half-life (h)	C_{max} (µg equiv./mL)	AUC_{last} (h × µg equiv./mL)	$AUC_{0-\infty}$ (h × µg equiv./mL)
Whole blood	3	M	1.0	0.94	5.0	0.257	0.899	1.038
		F	0.5	0.96	2.1	0.259	0.523	0.532
	300	M	4.0	1.00	7.3	20.413	312.838	316.586
		F	4.0	0.98	7.0	20.102	316.770	319.785
Plasma	3	M	1.0	0.97	4.1	0.356	1.097	1.203
		F	0.5	1.00	3.2	0.305	0.602	0.625
	300	M	4.0	1.00	6.8	22.450	292.938	326.473
		F	4.0	0.93	6.0	20.693	297.768	315.841
Red blood cells	3	M	0.25	0.86	3.6	0.181	0.601	0.704
		F	0.5	0.78	2.4	0.197	0.385	0.450
	300	M	4.0	1.00	11.9	17.162	283.968	302.564
		F	4.0	0.95	9.6	19.179	309.243	321.396

Source: McClanahan, 2015

In the tissue distribution study, the test substance distributed rapidly to the tissues and T_{max} occurred at 0.5 h following administration of 3 mg/kg bw, and 2 h (the first sampling point) following administration of 300 mg/kg bw. There was widespread tissue distribution, highest levels occurring in the GI tract and contents, liver, adrenals, kidney, urinary bladder (male only), pancreas, prostate, uterus, ovaries, spleen (females only), pituitary (females only), fat, mesenteric lymph nodes, heart, and lung. Levels were notably reduced after 96 h. Tissues with the highest percent of administered dose at the 2 h and 24 h termination times were GI tract and contents, liver, and residual carcass (McClanahan, 2015).

In a supplemental MOA study , female F344/DuCrlCrj rats were given single oral doses of [NCA ^{14}C]-afidopyropen and unlabelled afidopyropen at 3, 15 or 50 mg/kg bw following 14 days of dosing with approximately 3, 15 or 50 mg/kg bw unlabelled afidopyropen in the diet. This was a mechanistic study conducted with the primary intent of measuring the pharmokinetic properties of BAS 440 I in the strain and sex of rat and at doses that were relevant to the rat carcinogenicity studies conducted with afidopyropen. Additionally, this study measured the pharmacokinetic properties of a major cyclopropanecarboxylic acid-related metabolite (CPCA-carnitine, M440I060) in the rat. (Capello, 2016).

In plasma kinetics, AUC values indicated an internal exposure that is clearly correlated to the dosing regimen of [^{14}C]-afidopyropen. The AUC values of afidopyropen increased with increasing dose level in an nonproportional manner. In plasma kinetics of afidopyropen and its metabolites (bioanalytical data), the AUC values of afidopyropen increased with increasing dose level in an nonproportional manner, which may be suggestive of saturation of elimination. However, when all submitted ADME studies were analyzed, the saturation of elimination was not adequately demonstrated (see Annex 1 for a detailed explanation).

Afidopyropen and the metabolites M440I001 and M440I017 displayed comparable concentration–time curves, while the metabolite CPCA-carnitine displayed a plateau level with a minor decrease after repeated administration.

Over an observation period of about 72 h, 0.9, 1.3 and 1.6% of the dose at 3, 15, and 50 mg/kg, respectively, was excreted via urine. Excretion via faeces was significantly higher and for a period of about 72 hours amounted to 85, 90 and 65% of the dose at 3, 15 and 50 mg/kg bw respectively. The major part of faecal excretion occurred for all groups within 8–48 h. Tissue sampling demonstrated distribution to the liver and, to a lesser extent, the uterus. There were no major differences in metabolism between the Wistar and F344 strains of rats (Capello 2016).

(b) Dermal route

In a dermal absorption study, four male Crl: WI(Han) rats per time point were exposed for eight hours to doses of 0.25, 5.0 or 500 µg/cm^2 of afidopyropen, representing two spray dilutions and the concentrated forms of a product containing 50 g/L afidopyropen, labelled with [pyranone-6 C^{14}]-afidopyropen. Rats were killed at 8, 32 or 128 h. During the exposure period animals were placed in metabolism cages and excreta was collected. Following exposure, the treatment site was washed, a tape strip sample was taken, animals were necropsied and blood, plasma and carcasses analyzed for residual radioactivity.

Between 93 and 99% of the AD was recovered with the vast majority found in the skin washes of all dose groups. The mean absorption in the high-dose group was 0.37% of the AD after 8 h; this increased to 1.22% of the AD in the 128 h group. In the mid-dose group, absorption after 8 h was 2.89% of the AD and 3.48% of the AD in the 128 h dose group. In the low-dose group, absorption after 8 h was 4.25% of the AD, and 5.55% after 128 h. In conclusion, although there was evidence of dermal absorption, it was low compared to oral absorption and decreased with increased concentration (Fabian & Landsiedel, 2015).

1.2 Biotransformation

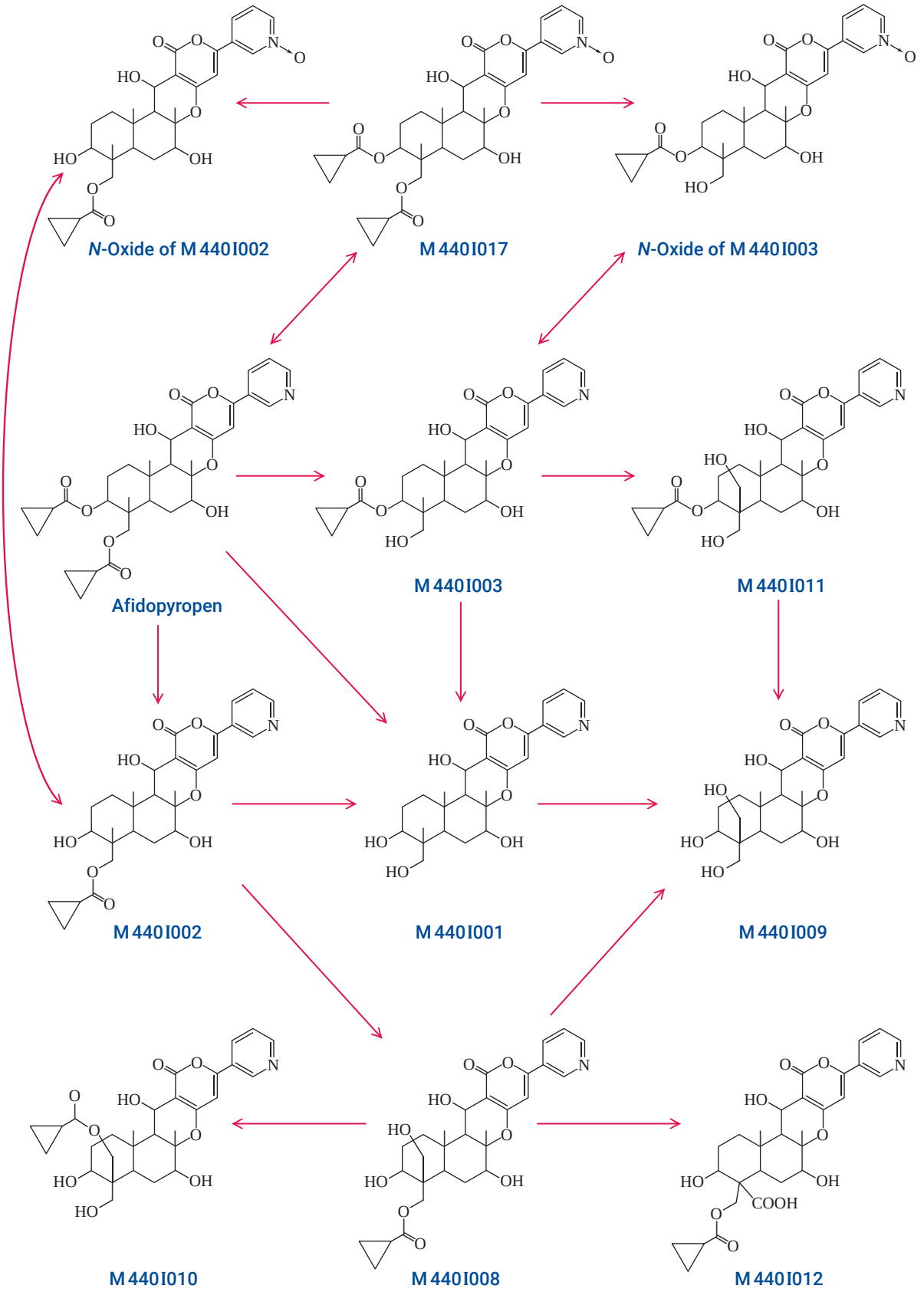
In the preliminary ADME assays, metabolites ME5343-T1, ME5343-T2, ME5343-T8 and an unknown metabolite, HPLC Reg.#25, were found at > 5% of AD in either the urine or faeces. Minor metabolites (ME5343-T9 and other unknown metabolites) were found at < 5% of AD. Metabolite profiles observed were similar in relative terms for urine and faeces, except for the presence of unchanged afidopyropen in the faeces. (Ohyama, 2012)

In the excretion assay within Ohyama (2015) metabolite characterization and quantitation was performed. The main metabolic reactions of afidopyropen (ME5343) are considered to be:

- hydrolytic elimination of both 3- and 4-cyclopropanecarbonyl groups,
- hydroxylation at 4-methyl group,
- *N*-oxidation of the pyridine ring.

The primary reaction is likely to be hydrolytic elimination of the 3-cyclopropanecarbonyl group, then further hydrolysis or hydroxylation. In addition, the *N*-oxidation of the pyridine ring occurs, followed by formation of various hydrolytic products. No significant sex-related difference was observed in excretion route and metabolic pathway of [NCA ^{14}C]-afidopyropen in rats. The proposed pathway according to Ohyama (2015) is shown in Fig. 4.

Figure 4. Proposed metabolic pathway of afidopyropen in rats



Redrawn from Ohyama, 2015

In the metabolite pattern assays from Thiaener & Glaessgen (2011), low levels of unchanged afidopyropen were detected in the urine in all treated groups. The main urinary metabolite in all but the single low-dose groups was M440I001 and the secondary urinary metabolite (primary in the single low-dose group) was M440I002 (Table 8). Slight differences were noted in metabolite patterns for both sexes and in low versus high dose groups. In the faeces, unchanged parent comprised 21–37% of the AD in single low-dose groups and 5–10% of the AD in in the single and repeat high-dose groups. In the low-dose groups, unchanged afidopyropen was the main metabolite, followed by M440I001, whereas in high-dose groups, the main faecal metabolite was M440I001, followed by M440I058. The total identified metabolites (including unchanged parent) in faeces accounted for 69% and 75% AD for the low-dose groups, for 64% and 60% of for the high-dose groups, and for 52% and 64% for the repeat high-dose groups. Slight differences in metabolite patterns between dose groups were noted for both sexes.. In the bile, unchanged afidopyropen was noted at ≤ 1.5% of the AD in the single low-dose males and female and the single high-dose females. It was not detected at all in the single high-dose males. Bile samples contained a notable number, and high proportion, of pyridine-*N*-oxidized derivatives. The main component was metabolite M440I017, the second was M440I019 and a glucuronic acid conjugate of M440I059. The total identified metabolites (including unchanged parent) in bile accounted for 37% and 43% of AD for low-dose groups and for 35% and 28% of the high-dose groups. Metabolite patterns in bile were similar for both sexes (Thiaener & Glaessgen, 2011).

Table 8. Metabolites identified in rat urine, faeces and bile

	Percent of administered dose (urinary metabolites)						
Dose	**Single low dose (3 mg/kg bw)**		**Single high dose (300 mg/kg bw)**		**Repeated high dose (14 + 1 labelled) 300 mg/kg bw)**		**Single high dose (300 mg/kg bw)**
Compound/ Peak [a]	**Male (0–48 h)**	**Female (0–48 h)**	**Male (0–72 h)**	**Female (0–72 h)**	**Male (0–48 h)**	**Female (0–48 h)**	**Male (0–72 h)**
Afidopyropen	0.068	0.041	0.058	0.017	0.036	0.116	0.260
M440I001/ M440I054	2.181	1.892	10.551	10.136	9.548	7.806	5.849
M440I002/ M = 611 u	0.413	2.151	2.245	5.381	0.964	1.491	2.852
M440I003/ M440I026	0.071	0.131	0.198	0.715	0.303	0.461	0.271
M440I008/ M440I043	0.061	0.024	0.823	0.370	1.310	1.313	0.537
M440I017	0.933	0.438	0.371	0.716	0.688	0.426	0.755
M440I018	ND	ND	ND	ND	ND	ND	0.125
M440I019/ M440I059	0.098	0.142	0.697	0.674	0.227	0.408	1.045
M440I035	0.023	0.004	0.054	0.072	0.104	0.131	0.089
M440I036	n.d.	0.062	0.133	0.256	0.028	0.069	0.109
M440I039	0.313	0.024	0.667	0.312	1.101	0.634	0.524
M440I056	0.174	0.017	0.587	0.099	0.025	n.d.	0.406
M440I058	0.105	0.149	1.689	0.870	0.781	0.926	1.158
Total identified	**4.440**	**5.077**	**18.072**	**19.618**	**15.117**	**13.781**	**13.982**
M = 539 u	ND	ND	ND	ND	ND	ND	0.051
Sum of components with one (top) or two (bottom) CPCA esters cleaved	0.771 2.668	2.665 1.934	5.838 11.805	8.337 10.547	3.719 10.674	4.799 8.440	6.237 6.780

	Percent of administered dose (faecal metabolites)						
Dose	**Single low dose (3 mg/kg bw)**		**Single high dose (300 mg/kg bw)**		**Repeated high dose (14 + 1 labelled) 300 mg/kg bw)**		**Single high dose (300 mg/kg bw)**
Compound [a]	**Male (0–72 h)**	**Female (0–96 h)**	**Male [b] (0–168 h)**	**Female (0–96 h)**	**Male (0–72 h)**	**Female (0–72 h)**	**Male (0–72 h)**
Afidopyropen	20.688	36.677	10.291	5.317	7.763	6.697	21.223
M440I001	16.898	20.819	17.956	23.496	14.894	21.382	2.784
M440I002	6.088	10.029	9.809	10.125	3.268	4.925	1.591
M440I003/ M440I026	2.629	3.097	0.720	0.320	0.106	1.018	0.293
M440I008	3.387	0.109	7.235	4.793	6.531	7.856	0.095
M440I012	3.592	0.025	1.207	ND	3.375	1.005	ND
M440I020	0.320	ND	ND	ND	ND	ND	0.574
M440I025	1.090	ND	ND	ND	ND	ND	0.117
M440I034	5.415	ND	0.106	ND	0.500	0.495	ND
M440I039	2.501	0.672	3.095	2.254	4.171	4.460	ND
M440I058	5.916	3.421	13.213	14.067	11.736	15.658	ND
Total identified	**68.523**	**74.849**	**63.633**	**60.371**	**52.345**	**63.495**	**26.676**
M = 487 u	1.496	0.020	0.314	ND	1.558	0.359	ND
Sum of components with one (top) or two (bottom) CPCA esters cleaved [c]	21.922 23.702	16.681 21.522	32.194 23.931	32.820 25.775	25.803 24.774	32.011 28.182	1.978 2.784

	Percent of administered dose (biliary metabolites)			
Dose	**Single low dose (3 mg/kg bw)**		**Single high dose (300 mg/kg bw)**	
Compound [a]	**Male (0–39 h)**	**Female (0–39 h)**	**Male (0–39 h)**	**Female (0–39 h)**
Afidopyropen	0.307	1.381	ND	0.035
M440I001/ M440I040	3.593	2.252	3.456	3.707
M440I002	0.114	0.320	0.136	0.401
M440I008/ M440I043	2.076	0.337	2.875	2.668
M440I012	0.565	0.876	1.842	1.176
M440I017	20.250	28.518	10.433	8.281
M440I018	1.078	0.158	2.673	1.368
M440I019/ M440I059	6.036	6.687	8.865	6.745
M440I027	ND	ND	0.223	0.018
M440I028	0.070	ND	0.291	0.044
M440I032/ M440I035	1.607	2.221	2.456	2.061
M440I037	0.506	ND	0.952	0.716

	Percent of administered dose (biliary metabolites)			
Dose	**Single low dose (3 mg/kg bw)**		**Single high dose (300 mg/kg bw)**	
Compound [a]	**Male (0–39 h)**	**Female (0–39 h)**	**Male (0–39 h)**	**Female (0–39 h)**
M440I038	0.409	0.318	1.131	0.559
Total identified	**36.610**	**43.068**	**35.333**	**27.779**
M = 487 u	0.070	ND	0.357	0.197
M = 537 u	0.688	0.558	1.515	2.905
Sum of components with one (top) or two (bottom) CPCA esters cleaved [c]	12.164 4.647	11.157 2.570	20.362 6.409	17.324 5.241

CPCA Cyclopropanecarboxylic acid

ND Not detected/identified

[a] Metabolites are listed in numerical order of the metabolite code

[b] Faeces sampled within the time intervals 72–96 h and 120–144 h was not analysed by high-performance liquid chromatography (HPLC) due to low level of radioactive residues (were < 1% of the dose)

[c] Cyclopropane carboxylic acid

Source: Thiaener & Glaessgen, 2011

Figure 5a. Proposed metabolic pathway part 1

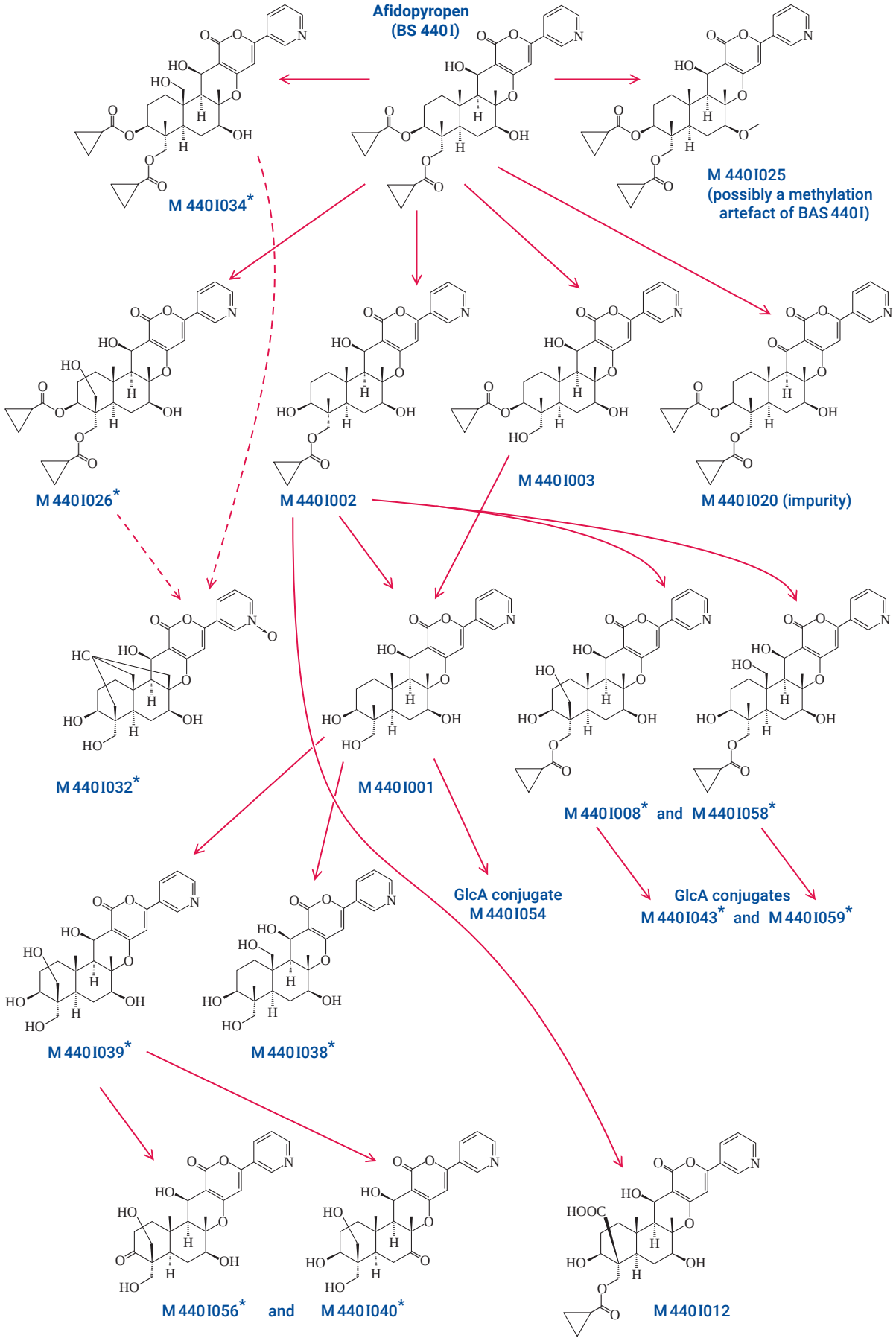
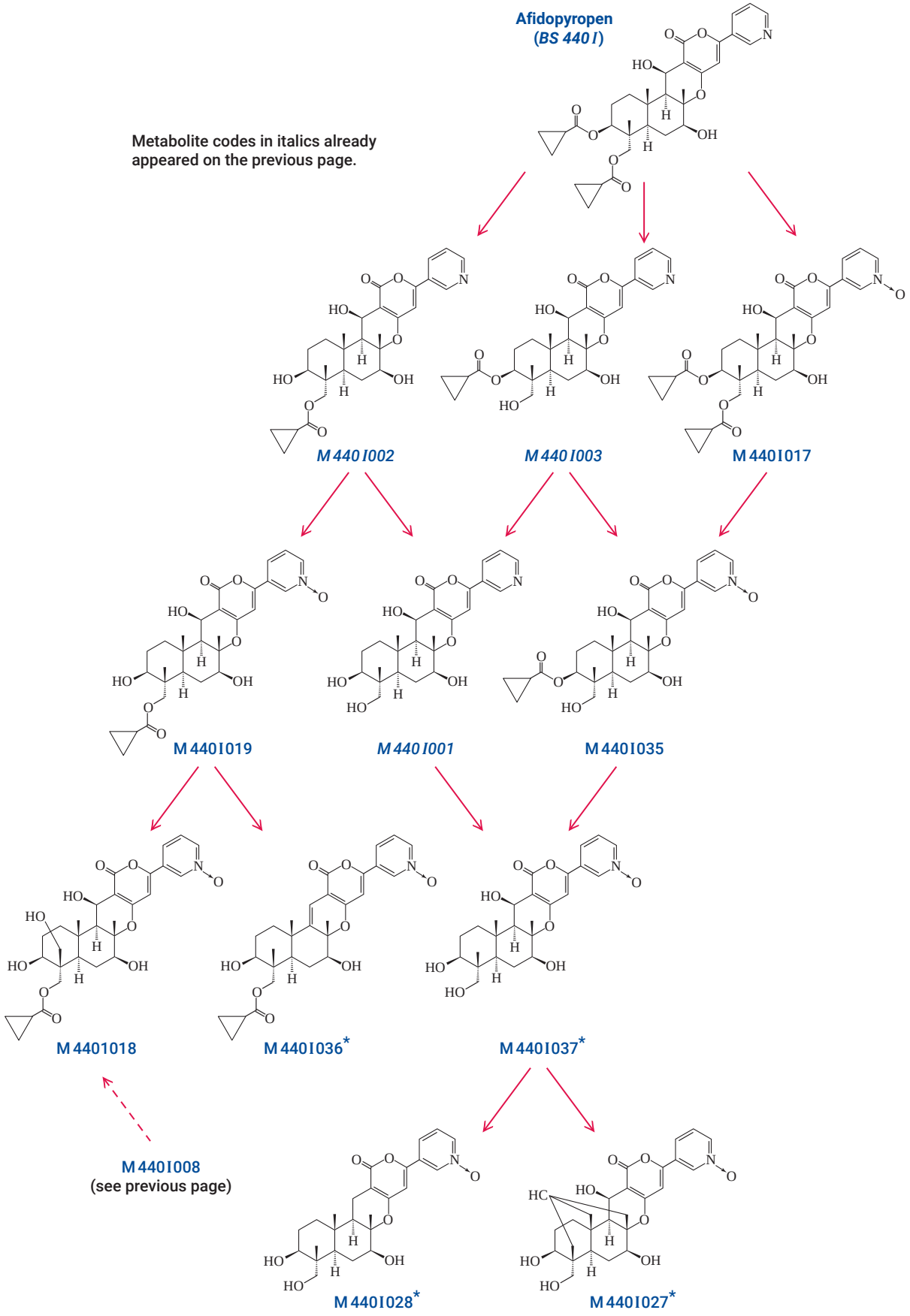


Figure 5b. Proposed metabolic pathway part 2



Redrawn from Thiaener &Glaessgen, 2011

In a special metabolism study on unlabelled M400I007, F344/DuCrlCrlj, SPF/VAF rats (one male/dose) were exposed to 0 or 300 mg/kg bw per day M400I007. Urine and faeces were collected at 24, 48 and 72 h post dosing. M400I007 was excreted only through faeces without extensive transformation to M400I001, M400I002 or M400I003. Afidopyropen was present in the dosing solution as an impurity at levels greater than those seen in the faeces, indicating that the parent compound was not produced though metabolism of the dimer. Therefore there was no evidence to prove that M400I007 biotransforms to afidopyropen. No detectable test substance nor metabolites were noted in the urine of the treated animal. The absorption rate or bioavailability of 400I007 is assumed to be very low; however, contribution of the biliary excretion could not be excluded. (Ohyama, 2013)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In an acute oral toxicity study, rats were given doses of afidopyropen (purity 95.74%) in 0.5% methylcellulose at 300 or 2000 mg/kg bw. There were no mortalities and no clinical signs of toxicity. All animals gained weight throughout the observation period and there were no gross changes at necropsy (Fukuyama, 2009a).

In an acute dermal toxicity study, rats were given a limit dose of afidopyropen (purity 95.74%) in 1% w/v aqueous methylcellulose at 2000 mg/kg bw. There were no mortalities and no signs of systemic toxicity or irritation. All animals gained weight throughout the study and there were no changes at gross necropsy (Fukuyama, 2009b).

In an acute inhalation toxicity study, rats were given a maximum achievable concentration of afidopyrpen (purity 95.74%) at 5.48 mg/L or 1.03 mg/L white carbon for four hours. There were no mortalities. Treated animals exhibited abnormal respiratory sounds for the first four hours following exposure. Body weights were decreased following treatment; however, all animals gained weight throughout the rest of the observation period. There were no gross changes at necropsy (Fukuyama, 2010).

Afidopyropen was therefore considered of low acute oral, dermal and inhalation toxicity in rats (Table 9).

Table 9. Acute toxicity of afidopyropen

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Wistar	Female	Oral	95.74	LD_{50} > 2000 mg/kg bw	Fukuyama (2009a)
Rat	Wistar	Male and female	Dermal	95.74	LD_{50} > 2000 mg/kg bw	Fukuyama (2009b)
Rat	Wistar	Male and female	Inhalation	95.74	LC_{50} > 5.48 mg/L	Fukuyama (2010)

LC_{50} Median lethal concentration LD_{50} Median lethal dose

(b) Dermal irritation

In a dermal irritation study, 0.5 mg of afidopyropen (purity 95.74%) was moistened with deionized water and applied under a 2.5 cm^2 gauze patch to the skin of three female New Zealand White rabbits for four hours. There were no signs of dermal irritation and all scores at the 24-, 48- and 72-hour marks were zero. Afidopyropen was considered to be nonirritating to the skin of rabbits (Ueda, 2009a).

(c) Ocular irritation

In an eye irritation study, 0.1 mg of afidopyropen (purity 95.74%) was instilled into the conjunctival sac of the right eyes of six female New Zealand White rabbits. In three animals, the eyes were washed 30 seconds after instillation. In the other three animals, the eyes were left unwashed. Minor to moderate conjunctival redness, oedema and discharge were noted in both washed and unwashed groups in the first hour following treatment, however, there were no signs of irritation by 24 h post instillation. Afidopyrope onsidered to be transiently irritating to the eyes of rabbits (Ueda, 2009b).

(d) Dermal sensitization

In a dermal sensitization study using a maximization assay, female Hartley guinea pigs were exposed to afidopyropen (purity 95.74%) in the following numbers per group: 20 main test, ten main naïve, ten positive control test, five positive control naïve. Test substance concentrations for the intradermal induction, the topical induction with patch and the challenge with patch were 1% weight/volume (w/v), 50% weight/weight (w/w) and 50% w/w respectively. Skin reaction to the challenge was observed 24 and 48 h after patch removal. There were no signs of irritation following the challenge dose in the main group. A concurrent positive control assay was performed with hexylcinnamaldehyde, which validated the system. Afidopyropen is not considered to be a dermal sensitizer in guinea pigs (Ueda, 2009c).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study1

In a 28-day toxicity study, groups of six Crlj:CD1(ICR) mice received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 300, 1000, 3000 or 10 000 ppm (equal to 0, 44, 145, 435 and 1277 mg/kg bw per day for males, 0, 49, 158, 484 or 1017 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and histopathological examinations were performed on the liver and gall bladders of control and high-dose animals.

Five of six females died at 10 000 ppm. Two were found dead in week 2, one was killed in extremis in week 3, and in week 4 one killed in extremis and another found dead. There were no effects on clinical signs of toxicity in males or in females that survived until study termination. In decedent females, emaciation, lateral position, prone position, decreased spontaneous motor activity, bradypnea and piloerection were noted. Body weights were decreased in 10 000 ppm males and females and body weight gains and feed consumption/efficiency were decreased in 10 000 ppm males.

The surviving female in the 10 000 ppm group exhibited severe anaemia. The surviving female and males at 10 000 ppm had increased RBC distribution width, haemoglobin (Hb) distribution width, reticulocytes and increased spleen weights. Additional haematological changes in the surviving 10 000 ppm female consisted of increased neutrophil, monocyte count and eosinophil count, increased numbers of large unstained cells and decreased lymphocytes and platelets. Total bilirubin was increased in males and females at 300 ppm and above.

Clinical chemistry and liver changes consisted of increased total cholesterol in males at 3000 ppm and above. At 10 000 ppm, liver weights, hepatocellular hypertrophy and hepatocellular fatty change were increased in males and females, triglycerides were increased in males and total cholesterol, inorganic phosphorus and albumin/globulin ratios were increased and there was an increased incidence of enlarged livers in females. Although there was evidence of adverse liver changes, liver marker enzyme activity was not affected to an adverse degree.

Other changes consisted of increased adrenal weights at 1000 ppm and above in males and 3000 ppm and above in females, decreased testicular weights in males, decreased pituitary and uterine weights in females at 3000 ppm and above, decreased brain weights in males and females, increased thymus, decreased epididymis weights in males and decreased ovarian weights in females at 10 000 ppm. Due to the limited examinations, a no-observed-adverse-effect level (NOAEL) was not derived (Takahashi, 2009).

Study2

In a 90-day toxicity study, groups of ten Crlj:CD1(ICR) mice received afidopyropen (purity 95.74%) in their diet at a concentration of 0, 150, 500, 2000 or 6000 ppm (equal to 0, 0, 21, 69, 285 or 819 mg/kg bw per day for males, 0, 25, 83, 327 or 919 mg/kg bw per day for females). Animals were inspected twice

daily for mortality, moribundity and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and histopathological examinations performed for all tissues from the control and high-dose animals, in all dose groups for the submandibular glands and liver as well as in all tissues exhibiting gross lesions.

Five of ten females died in the 6000 ppm group between weeks 2 and 13. There were no effects on clinical signs of toxicity in males or females that survived until study termination. In decedent females, lateral position, decreased spontaneous motor activity, bradypnea, soiled/wetted fur in genital region and piloerection were noted. Food consumption was decreased in males in the first week of treatment and in females in the first four weeks of treatment; however, as there were no effects on body weight, the adversity of the change is unknown.

Changes to the haematological parameters at 2000 ppm and above consisted of increased bilirubin (> 50%) in males and females and increased spleen weights in females (20%). Males and females at 6000 ppm exhibited increased RBC distribution width and decreased platelets and lymphocyte counts. High-dose males exhibited increased Hb distribution width, neutrophils and increased spleen weights. High-dose females exhibited decreased Hb, increased monocyte and eosinophil counts and decreased hematopoiesis in the bone marrow.

At 2000 ppm and above, liver changes consisted of increased triglycerides (40%) in females. At 6000 ppm, both males and females exhibited hepatocellular hypertrophy. Males exhibited increased aspartate transaminase (AST) and alanine transaminase (ALT) activities and single cell necrosis of the hepatocytes. In females, liver changes were limited to increased liver weights and pale colour/ accentuated lobular pattern of the liver.

Other changes at 6000 ppm included an increase in secreted material depletion in granular ducts of the submandibular glands in males and decreased ovarian weights in females. Additionally, in females there was an increase in vacuolation of multiple tissues including cardiac muscle, parietal cell of the glandular stomach, hepatocytes, kidney proximal tubule, urinary bladder mucosal epithelial cells, neuropil of the cerebral cortex, choroid plexus epithelium and glial cell in gray matter of the spinal cord and increased apoptosis in lymphoid tissues (spleen, thymus and lymph nodes). The vacuoles in the heart and liver stained positive for lipid; those in the brain were negative for lipid stains.

The NOAEL was 500 ppm (equal to 69 mg/kg bw per day), based on increased blood bilirubin in males and females and increased blood triglycerides and spleen weights in females at the lowest-observed-adverse-effect level (LOAEL) of 2000 ppm (equal to 285 mg/kg bw per day) (Takahashi, 2010).

Rat

Study 1

In a 28-day toxicity study, groups of six F344/DuCrlCrlj (SPF/VAF) rats received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 500, 750, 1500, 3000 or 6000 ppm (equal to 0, 40, 59, 122, 250 or 479 mg/kg bw per day for males, 0, 41, 63, 128, 244 or 511 mg/kg bw per day for females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues fixed, however no histopathological examinations were performed.

There were no effects on mortality or clinical signs of toxicity. Body weight and body weight gain was decreased at 3000 ppm and above and feed consumption was decreased at 6000 ppm in males. In females, body weight and feed consumption parameters were decreased at 6000 ppm.

Changes at urinalysis were limited to females at 3000 ppm and above with increased urine bilirubin, ketones and urinary proteins.

Haematologically, monocyte counts were increased in males at 3000 ppm and above. At 6000 ppm, Hb was decreased in males and females, RBC and haematocrit (Ht) values were increased in males along with increased spleen weights. In females at 6000 ppm, white blood cell (WBC) counts were increased.

Liver weights were increased in males at all levels except 500 ppm. In females at 1500 ppm and above, blood urea nitrogen (BUN) and liver weights were increased. At 3000 ppm and above BUN was increased in males. In males, total and free cholesterol were increased and inorganic phosphorus was decreased at 3000 ppm, whereas these changes were seen at 6000 ppm in females. Blood potassium was increased in males and females at 6000 ppm.

Thymus weights were increased and pituitary, uterine and ovarian weights decreased in females at 3000 ppm and above. At 6000 ppm, relative kidney weights were increased and absolute heart, testicular and epididymis weights were decreased in males. Absolute lung weights were decreased in females and there was evidence of ovarian and uterine atrophy at 6000 ppm. Due to the limited examinations, no NOAEL was derived (Shibuya, 2010).

Study 2

In a 90-day toxicity study, groups of ten F344/DuCrlCrlj (SPF/VAF) rats received afidopyropen (purity 95.74%; lot 080722) in the diet at a concentration of 0, 150, 300, 1000 or 3000 ppm (equal to 0, 8.9, 18, 61 or 182 mg/kg bw per day for males, 0, 10.2, 20, 68 or 197 mg/kg bw per day for females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. A functional observation battery (FOB) was performed prior to study termination. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues were examined histopathologically in control and high-dose groups, as well as in all tissues with gross lesions and in the hearts and livers of animals from all dose groups. Selected sections of the heart and liver in the control and high-dose (3000 ppm) groups were stained with Oil Red O stain to confirm lipid deposition histopathologically.

There were no effects on mortality, body weights or ophthalmoscopic evaluation, nor clinical signs of toxicity. Feed consumption was decreased at 1000 ppm in females and 3000 ppm in males, but there were no effects on body weight so effects were not considered adverse. Hindlimb grip strength was decreased in 3000 ppm males.

While there was a trend towards haematological changes that would indicate anaemia, the changes were of insufficient magnitude to be considered adverse with the exception of an increase in platelets in males at 3000 ppm (Table 10a). Relative spleen weights were increased in females at 1000 ppm and above and in males at 3000 ppm and absolute spleen weights were increased in males and females at 3000 ppm. Histopathologically, both males and females exhibited congestion of the spleen at 3000 ppm. Although spleens at lower doses were not examined, the absence of any significant changes in spleen weight at lower doses provides some reassurance that there would be no treatment-related, adverse changes to the spleen at 1000 ppm or below (see Table 10a on the next page).

Table 10a. Select haematological, clinical chemistry and urinalysis parameters in the 90-day oral toxicity study in rats

	Males					Females				
Dose (ppm)	**0**	**150**	**300**	**1000**	**3000**	**0**	**150**	**300**	**1000**	**3000**
Haematology										
Red blood cell (10^4/μL)	925 ± 16	908 ± 17	916 ± 18	898** ± 22 (↓3%)	881** ± 10 (↓5%)	860 ± 25	865 ± 20	863 ± 16	839 ± 19	811** ± 20 (↓6%)
Haemoglobin (g/dL)	16.0 ± 0.3	15.6 ± 0.3*	15.7 ± 0.2	15.3** ± 0.3 (↓4%)	14.9** ± 0.3 (↓7%)	15.9 ± 0.4	16.0 ± 0.4	16.0 ± 0.4	15.5 ± 0.3	15.0** ± 0.3 (↓6%)
Haematocrit (%)	49.1 ± 0.9	48.3 ± 1.1	48.4 ± 0.8	47.5** ± 1.0 (↓3%)	46.3** ± 0.8 (↓6%)	48.0 ± 1.4	48.3 ± 1.2	48.1 ± 1.0	46.6* ± 1.0 (↓3%)	44.9** ± 1.1 (↓6%)
Platelet (10^4/μL)	67.0 ± 2.3	68.9 ± 3.5	70.5 ± 5.6	70.9 ± 4.8	74.7** ± 5.2 (↑12%)	73.5 ± 4.5	75.1 ± 2.7	76.1 ± 2.9 (↑4%)	79.9** ± 3.7 (↑9%)	75.5 ± 8.6 (↑3%)
Clinical Chemistry										
Urea nitrogen (mg/dL)	17.4 ± 1.2	16.8 ± 1.2	16.9 ± 1.7	17.9 ± 1.3	21.1** ± 2.1 (↑21%)	18.6 ± 1.4	18.3 ± 1.3	17.7 ± 1.8	21.9** ± 0.8 (↑18%)	27.4** ± 2.3 (↑47%)
Aspartate amino-transferase (IU/L)	94.8 ± 14.3	91.1 ± 7.0	103.5 ± 18.5	94.1 ± 12.9	101.0 ± 18.1	75.6 ± 2.8	77.0 ± 5.9	77.0 ± 7.2	98.6** ± 12.9 (↑30%)	94.8** ± 10.9 (↑25%)
Alanine amino-transferase (IU/L)	59.1 ± 9.0	59.3 ± 3.4	60.3 ± 3.3	58.9 ± 7.8	52.5* ± 2.8 (↓11%)	34.2 ± 2.2	34.4 ± 4.8	37.1 ± 4.8	43.4** ± 5.8 (↑27%)	39.7* ± 2.5 (↑16%)
Alkaline phosphatase (IU/L)	436 ± 19	447 ± 23	442 ± 23	459 ± 26	477** ± 31 (↑9%)	360 ± 41	354 ± 41	358 ± 36	394 ± 39	461** ± 46 (↑28%)
Potassium (m equiv./L)	3.71 ± 0.21	3.67 ± 0.13	3.66 ± 0.14	3.81 ± 0.27	3.76 ± 0.18	3.49 ± 0.21	3.68 ± 0.14	3.72 ± 0.52	3.85* ± 0.17 (↑10%)	4.06** ± 0.15 (↑16%)
Urinalysis										
Urobilinogen 0.1 EU/dL	9	4	5	3*	3*	8	8	7	6	5
Urobilinogen 1.0 EU/dL	1	6	5	7*	7*	2	2	3	4	5
Casts, absent	3	6	5	7	9*	5	4	9	8	9
Casts, ±	4	4	4	3	1*	3	2	1	0	1
Casts, 1+	3	0	1	0	0	2	4	0	2	0

* $p < 0.05$ ** $p < 0.01$

Source: Yamashita, 2010

Relative liver weights were increased in males and females at 1000 ppm and above. Males at the same dose exhibited decreased triglyceride levels. Females exhibited increased BUN, potassium and absolute liver weights and vacuolar fatty change of hepatocytes at 1000 ppm and above, while males exhibited the same changes at 3000 ppm. Liver changes at 3000 ppm in both males and females consisted of increased albumin/globulin ratios, decreased bilirubin and cloudiness of the liver. High-dose females exhibited increased blood glucose and decreased triglycerides (Table 10a).

In males, relative kidney weights and urinary urobilinogen were increased at 1000 ppm and above, but urinary casts were decreased at the high dose.

Cardiac changes consisted of decreased absolute heart weights at 1000 ppm and above and decreased relative heart weights at 3000 ppm in females. There was an increase vacuolar change (fatty change) of the myocardium at 1000 ppm in females and at 3000 ppm in males.

Ovarian and uterine weights were decreased at 3000 ppm and thymus weights were increased at 1000 ppm in females (Table 10b).

Table 10b. Select postmortem parameters in the 90-day oral toxicity study in rats

	Males					Females				
Dose (ppm)	**0**	**150**	**300**	**1000**	**3000**	**0**	**150**	**300**	**1000**	**3000**
Organ weights										
Body weight (g)	317 ± 11	322 ± 16	318 ± 9	320 ± 15	306 ± 17 (↓3.5%)	176 ± 9	173 ± 5	176 ± 5	171 ± 6	173 ± 7
Absolute liver wt (g)	6.8 ± 0.3	7.0 ± 0.4	6.9 ± 0.4	7.2 ± 0.7 (↑6%)	7.8** ± 0.6 (↑15%)	3.9 ± 0.2	3.7 ± 0.1	3.8 ± 0.1	4.3** ± 0.2 (↑10%)	4.7** ± 0.2 (↑21%)
Relative liver wt (%)	2.15 ± 0.05	2.16 ± 0.05	2.17 ± 0.08	2.25* ± 0.11 (↑5%)	2.56** ± 0.07 (↑19%)	2.21 ± 0.08	2.17 ± 0.07	2.17 ± 0.07	2.51** ± 0.11 (↑14%)	2.72** ± 0.15 (↑23%)
Absolute heart wt (g)	0.96 ± 0.06	0.97 ± 0.05	0.95 ± 0.04	0.96 ± 0.05	0.92 ± 0.05	0.62 ± 0.03	0.60 ± 0.02	0.59 ± 0.02	0.58* ± 0.03 (↓7%)	0.56** ± 0.03 (↓10%)
Relative heart wt (%)	0.303 ± 0.010	0.301 ± 0.019	0.298 ± 0.012	0.302 ± 0.009	0.300 ± 0.009	0.351 ± 0.012	0.350 ± 0.016	0.336 ± 0.013	0.337 ± 0.017 (↓4%)	0.324** ± 0.019 (↓8%)
Relative spleen wt (%)	0.200 ± 0.010	0.200 ± 0.011	0.200 ± 0.009	0.209 ± 0.010	0.226** ± 0.009 (↑13%)	0.235 ± 0.016	0.231 ± 0.008	0.231 ± 0.012	0.249* ± 0.010 (↑6%)	0.267** ± 0.014 (↑14%)
Absolute kidney wt (g)	1.94 ± 0.07	1.99 ± 0.14	2.00 ± 0.06	2.06 ± 0.10 (↑6%)	2.05 ± 0.16 (↑6%)	0.62 ± 0.03	0.60 ± 0.02	0.59 ± 0.02	0.58* ± 0.03 (↓7%)	0.56** ± 0.03 (↓10%)
Relative kidney wt (%)	0.612 ± 0.017	0.619 ± 0.032	0.628 ± 0.021	0.645* ± 0.024 (↑5%)	0.671** ± 0.017 (↑10%)	0.351 ± 0.012	0.350 ± 0.016	0.336 ± 0.013	0.337 ± 0.017 (↓4%)	0.324** ± 0.019 (↓8%)
Absolute thymus wt (mg)	206 ± 25	204 ± 33	237 ± 36	225 ± 29	235 ± 42 (↑14%)	166 ± 17	165 ± 12	174 ± 13	181 ± 20 (↑9%)	**205 ± 24 (↑23%)
Relative thymus wt (%)	0.065 ± 0.007	0.063 ± 0.011	0.075 ± 0.011	0.071 ± 0.009	0.077* ± 0.010 (↑18%)	0.095 ± 0.010	0.095 ± 0.007	0.098 ± 0.007	0.106* ± 0.011 (↑12%)	0.118** ± 0.011 (↑24%)

	Males					Females				
Dose (ppm)	**0**	**150**	**300**	**1000**	**3000**	**0**	**150**	**300**	**1000**	**3000**
Relative ovary wt (%)						0.0352 ± 0.0040	0.0338 ± 0.0033	0.0333 ± 0.0041	0.0346 ± 0.0057	0.0289* ± 0.0049 (↓18%)
Relative uterus wt (%)						0.290 ± 0.058	0.315 ± 0.120	0.311 ± 0.090	0.271 ± 0.036	0.168** ± 0.058 (↓42%)
Gross pathology										
Liver: cloudiness	0/10[a]	0/10	0/10	0/10	3/10	0/10	0/10	0/10	0/10	**9/10
						(*Table 10b continued on the following page*)				
Histopathology										
Liver: vacuolar change, hepatocyte, periphery										
slight	0/10	0/10	0/10	0/10	4/10*	0/10	0/10	0/10	2/10	1/10
moderate	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	6/10**	9/10**
total	0/10	0/10	0/10	0/10	4/10*	0/10	0/10	0/10	8/10**	10/10**
Heart: Vacuolar change, myocardium										
slight	0/10	0/10	0/10	0/10	3/10	0/10	0/10	0/10	7/10**	6/10**
Spleen: congestion										
slight	0/10	-	-	-	4/10*	0/10	-	-	-	6/10**

* $p < 0.05$ ** $p < 0.01$

[a] Number of times observed/total number of animals affected

Source: Yamashita, 2010

The NOAEL was 300 ppm (equal to 18 mg/kg bw per day), based on increased relative liver weights in females, increased urobilinogen in males and increased BUN and potassium, and increased vacuolar change (fatty change) of the liver and myocardium in females at the LOAEL of 1000 ppm (equal to 61 mg/kg bw per day) (Yamashita, 2010).

Study 3

In a 90-day toxicity study, groups of ten F344 DuCrlCrlj rats received in the diet afidopyropen (purity 94.54%; batch 080722) at concentrations of 0, 300, 1000 or 4000 ppm (equal to 0, 18.7, 65.5 or 225.6 mg/kg bw per day for males, 0, 20.5, 79.4 or 404.2 mg/kg bw per day for females). An additional control group of ten rats per sex was used for blood sampling and clinical parameters only. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed prior to study termination. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Additional blood samples were taken to measure cardiac troponin I (a marker of myocardial damage) and prolactin. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues fixed, however, histopathological examination was only performed on control and high-dose adrenal glands, heart, liver, pancreas, thyroid glands (with parathyroid) and uterus (with cervix). Estrus cycle determination was made by vaginal smears that were taken between the hours of 14:30 and 15:30 CET on weekdays and 14:30 and 16:00 on weekends and holidays. Females and male rats were housed in the same animal room for this study, however the cage racks with males and females were placed on opposite sides of the animal room.

There were no effects on mortality, ophthalmoscopy or estrus cyclicity. Body weights were decreased in males at 4000 ppm and body weight gains were decreased in males and females at 4000 ppm. Feed consumption was decreased throughout the administration period in 4000 ppm males and in the first two weeks of treatment in 4000 ppm females. In 4000 ppm males, forelimb grip strength was decreased and activity was decreased in the first two intervals of the motor activity assessment.

There were minor, nonadverse decreases in RBC, Hb and Ht values in females starting at 1000 ppm. Platelet counts and reticulocytes were increased in males at 4000 ppm. In females, there was an increase in platelets at 1000 and 4000 ppm but this lacked dose–response.

Clinical chemistry changes at 1000 ppm and above consisted of increased γ-glutamyl transpeptidase (GGTP) in males and females, decreased triglycerides in males and increased urea and cholesterol in females. Liver weights were increased in females at the same dose. At 4000 ppm, inorganic phosphate was decreased and blood potassium was increased in males and females. In males, urea, cholesterol and potassium were increased and creatinine and serum glucose decreased. In 4000 ppm females there was an increase in the incidence and severity of peripheral fatty change in the liver.

Urine volume was increased in females of all dose groups and urinary glucose was increased in 4000 ppm males. Additionally, there was an increase in degeneration of the islet cells of the pancreas in 4000 ppm males.

Cardiac troponin I was increased markedly at 4000 ppm in both sexes (Table 11). At 4000 ppm males and females exhibited a slight increase in the severity of necrosis/fibrosis of the heart.

Prolactin levels were very variable (standard deviation > mean) in control and animals treated at 300 or 1000 ppm; at 4000 ppm prolactin levels were statistically significantly reduced (Table 11).

Table 11. Effects in the 90-day oral toxicity study in rats (Study 3)

	Males				Females			
Dose (ppm)	**0**	**300**	**1000**	**4000**	**0**	**300**	**1000**	**4000**
Clinical chemistry								
GGTP (nkat/L)	1 ± 3	0 ± 0	6 ± 11	10 ± 25	0 ± 0	0 ± 1	10 ± 7**	54 ± 12**
Triglycerides (mmol/L)	0.72 ± 0.09	0.82 ± 0.19	0.53 ± 0.09** (↓26%)	0.53 ± 0.08** (↓26%)	0.52 ± 0.14	0.42 ± 0.09	0.48 ± 0.11	0.48 ± 0.11
Urea (mmol/L)	6.39 ± 0.36	6.94 ± 0.72*	6.66 ± 0.44	8.42 ± 1.50** (↑32%)	7.16 ± 0.72	7.32 ± 1.50	8.21 ± 0.70** (↑15%)	9.79 ± 1.10** (↑37%)
Cholesterol (mmol/L)	1.73 ± 0.10	1.85 ± 0.19	1.70 ± 0.17	2.08 ± 0.29** (↑20%)	2.32 ± 0.15	2.23 ± 0.23	2.58 ± 0.27* (↑11%)	2.68 ± 0.26** (↑16%)
Cardiac troponin I (ng/mL)								
Day 29	0.01 ± 0.00	0.03 ± 0.07	0.02 ± 0.02	0.95 ± 1.66**	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.03	0.32 ± 0.37**
Day 92	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.08 ± 0.13**
Serum prolactin (µg/L)								
Day 92: mean	-	-	-	-	43.27 ± 45.78	168.19 ± 243.31	152.78 ± 245.97	10.88 ± 5.26*
Day 92: median	-	-	-	-	28.73	26.29	58.80	10.86

	Males				Females			
Dose (ppm)	**0**	**300**	**1000**	**4000**	**0**	**300**	**1000**	**4000**
Organ weights, relative (%)								
Heart weight	0.303 ± 0.015	0.294 ± 0.017	0.309 ± 0.013	0.334 ± 0.021** (↑10)	0.35 ± 0.013	0.342 ± 0.01	0.346 ± 0.014	0.34 ± 0.013
Liver weight	2.165 ± 0.067	2.21 ± 0.048	2.3 ± 0.083** (↑6)	2.885 ± 0.335** (↑33)	2.191 ± 0.095	2.209 ± 0.071	2.436 ± 0.109** (↑11)	2.678 ± 0.123** (↑22)
Prostate weight	0.232 ± 0.031	0.188 ± 0.024** (↓19)	0.217 ± 0.024	0.143 ± 0.022** (↓38)	-	-	-	-
					(*Table 11 continued on the following page*)			
Seminal vesicle weight	0.29 ± 0.031	0.253 ± 0.042	0.269 ± 0.041	0.169 ± 0.049** (↓42)	-	-	-	-
Ovary weight	-	-	-	-	0.038 ± 0.004	0.036 ± 0.004	0.034 ± 0.003* (↓11)	0.027 ± 0.003** (↓29)
Uterus weight	-	-	-	-	0.272 ± 0.057	0.324 ± 0.133	0.283 ± 0.056	0.135 ± 0.017** (↓50)
Histopathology								
Adrenal cortex:								
– increased vacuolation	0	NE	NE	3	0	NE	NE	0
– accessory cortical tissue	0	NE	NE	2	1	NE	NE	0
Uterus: atrophy, diffuse	-	-	-		NE	NE	NE	6
Cervix: atrophy, diffuse	-	-	-		NE	NE	NE	4
Heart: necrosis and/ or fibrosis	10	NE	NE	7	5	NE	NE	8
grade 1	9	NE	NE	5	4	NE	NE	6
grade 2	1	NE	NE	0	1	NE	NE	1
grade 3	-	NE	NE	2	-	NE	NE	1

NE Not examined

* $p < 0.05$ ** $p < 0.01$

Source: Flick et al., 2016a

Ovarian weights were decreased starting at 1000 ppm and uterine weights were decreased at 4000 ppm along with serum prolactin and increased atrophy of the cervix and uterus. Prostate and seminal vesicle weights were decreased at 4000 ppm.

Additional changes consisted of increased thymus weights at 1000 ppm and above, and increased thyroid and spleen weights at 4000 ppm in females. There was an increase in vacuolation of the adrenal cortex in males at 4000 ppm. Due to the limited examinations, no NOAEL was derived (Flick et al., 2016a).

Study 4

In a 90-day toxicity study, groups of ten F344 DuCrlCrlj rats received higher purity afidopyropen (purity 97.2%; batch COD-001545) in their diet at concentrations of 0, 300, 1000 or 4000 ppm (equal to 0, 19, 54 or 181 mg/kg bw per day for males, 0, 20, 60 or 361 mg/kg bw per day for females). An additional control group of ten per sex was used for blood sampling and clinical parameters only. Animals were inspected twice daily for mortality and moribundity, daily for clinical signs of toxicity and a detailed clinical observation was performed weekly. A detailed FOB including motor activity was performed at the end of the administration period. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Estrus cyclicity was monitored in the final three weeks of treatment. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Additional blood samples were taken to measure cardial troponin I and rat prolactin. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. All organs were weighed and tissues fixed, however histopathological examinations were performed only on control and high-dose adrenal glands, heart, liver, pancreas, thyroid glands (with parathyroid) and uterus (with cervix).

There were no effects on mortality, clinical signs of toxicity or effect on urinalysis parameters. Forelimb grip strength was decreased in 4000 ppm males and females. Body weights were decreased in males, and body weight gains were decreased in males and females at 4000 ppm. Feed consumption was decreased at 1000 ppm and above in males, however the change was considered adverse at 4000 ppm when accompanied by decreased body weights.

A nonadverse decrease in haematological parameters, indicative of regenerative anaemia, was seen in the rest of the database. Additionally, there were adverse increases in reticulocytes and platelets at 1000 ppm and above in males and females.

Clinical chemistry and liver changes were seen in males and females, starting at 300 ppm. At 300 ppm cholesterol was increased in males, while BUN and GGTP were increased in females. At 1000 ppm, liver weights were increased in both sexes, BUN was increased and triglycerides were decreased in males and cholesterol increased in females. At 4000 ppm, total protein was increased in males, while potassium was increased and inorganic phosphate decreased in females. Additionally, one male exhibited moderate centrilobular liver hypertrophy and females exhibited an increase in the severity of peripheral or diffuse fatty change of the liver.

Changes to the cardiac system consisted of an increase in cardiac troponin I in males at 4000 ppm and in females at 1000 ppm and above. Single animals amongst the 300 ppm males and 1000 ppm females, exhibited increased cardiac tropin I at day 29. Both sexes exhibited a slight increase in the severity of necrosis/fibrosis of the heart at 4000 ppm, the only treated group examined (Table 12, on the following page).

Table 12. Effects in the 90-day oral toxicity study in rats

	Males				Females			
Dose (ppm)	**0**	**300**	**1000**	**4000**	**0**	**300**	**1000**	**4000**
Clinical chemistry								
Cholesterol (mmol/L)	1.64 ± 0.18	1.84 ± 0.12** (↑12%)	1.86 ± 0.13** (↑13%)	1.97 ± 0.11** (↑20%)	1.97 ± 0.26	2.15 ± 0.27	2.35 ± 0.33** (↑19%)	2.38 ± 0.17** (↑21%)
Total protein (g/L)	61.49 ± 2.47	65.18 ± 1.80** (↑6%)	66.01 ± 1.20** (↑7%)	67.66 ± 1.98** (↑10%)	58.45 ± 0.46	58.34 ± 1.46	59.59 ± 2.49	60.73 ± 1.34** (↑4%)
Urea (mmol/L)	6.38 ± 0.48	6.44 ± 0.60	7.24 ± 0.75** (↑13%)	7.45 ± 0.66** (↑17%)	6.74 ± 0.55	7.44 ± 0.51** (↑10%)	8.02 ± 0.46** (↑19%)	9.85 ± 0.70** (↑46%)
GGTP (nkat/L)	0 ± 0	0 ± 1	0 ± 0	0 ± 0	0 ± 0	4 ± 5*	10 ± 10**	56 ± 12**
Potassium (mmol/L)	4.62 ± 0.21	4.60 ± 0.17	4.85 ± 0.16** (↑5%)	4.98 ± 0.21** (↑8%)	4.27 ± 0.19	4.31 ± 0.26	4.55 ± 0.25* (↑7%)	5.00 ± 0.23** (↑17%)
Total protein (g/L)	61.49 ± 2.47	65.18 ± 1.80** (↑6%)	66.01 ± 1.20** (↑7%)	67.66 ± 1.98** (↑10%)	58.45 ± 0.46	58.34 ± 1.46	59.59 ± 2.49	60.73 ± 1.34** (↑4%)

(*Table 12 continued on the following page*)

Cardiac troponin I (ng/mL)								
Day 29	0.01 ± 0.00	0.06 ± 0.14	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.31 ± 0.64	1.89 ± 1.58**
Day 92	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.06	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.03	0.15 ± 0.28**
Serum prolactin (μg/L)								
Day 92: mean	-	-	-	-	70.07 ± 174.51	237.53 ± 326.77	146.44 ± 181.97	27.50 ± 29.83
Day 92: median	-	-	-	-	26.43	55.52	88.79	11.05
Estrus cyclicity								
Number of cycles	-	-	-	-	3.90 ± 0.99	4.00 ± 0.82	3.80 ± 0.79	3.00 ± 1.05
Cycle length (days)	-	-	-	-	4.15 ± 0.82	4.30 ± 0.64	4.60 ± 0.83	5.58 ± 1.72
Organ weights								
Absolute liver wt	5.881 ± 0.48	6.541 ± 0.35** (↑11%)	6.76 ± 0.30** (↑15%)	6.627 ± 0.47** (↑13%)	3.804 ± 0.20	3.842 ± 0.25	4.074 ± 0.21* (↑7%)	4.266 ± 0.21** (↑12%)
Relative liver wt	2.171 ± 0.081	2.201 ± 0.053	2.272 ± 0.052** (↑5%)	2.545 ± 0.113** (↑17%)	2.231 ± 0.094	2.32 ± 0.095* (↑4%)	2.541 ± 0.09** (↑14%)	2.685 ± 0.11** (↑20%)
Relative heart wt					0.358 ± 0.027	0.352 ± 0.014	0.354 ± 0.018	0.344 ± 0.02

	Males				Females			
Dose (ppm)	**0**	**300**	**1000**	**4000**	**0**	**300**	**1000**	**4000**
Relative prostate wt	0.219 ± 0.03	0.216 ± 0.028	0.214 ± 0.037	0.18 ± 0.029** (↓18 %)	-	-	-	-
Relative seminal vesicle wt	0.271 ± 0.059	0.292 ± 0.047	0.28 ± 0.032	0.225 ± 0.032 (↓17%)	-	-	-	-
Relative ovary wt	-	-	-	-	0.037 ± 0.003	0.036 ± 0.004	0.033 ± 0.002** (↓11%)	0.025 ± 0.004** (↓32%)
Relative uterus wt	-	-	-	-	0.325 ± 0.081	0.402 ± 0.157	0.382 ± 0.131	0.161 ± 0.1** (↓51%)
Histopathology								
Uterus: Atrophy, diffuse					–			9
Cervix: atrophy, diffuse					–			9
Heart:								
Necrosis and/ or fibrosis	10	NE	NE	10	7	NE	NE	10
grade 1	5	NE	NE	2	5	NE	NE	5
grade 2	5	NE	NE	7	2	NE	NE	5
grade 3	–	NE	NE	1	–	NE	NE	–
Liver:								
Fatty change, peripheral	–	NE	NE	–	7	NE	NE	8
grade 1	–	NE	NE	–	5	NE	NE	–
grade 2	–	NE	NE	–	2	NE	NE	3
grade 3	–	NE	NE	–	-	NE	NE	5
Fatty change, diffuse	–	NE	NE	–	2	NE	NE	2
grade 1	–	NE	NE		2	NE	NE	–
grade 2	–	NE	NE	–	–	NE	NE	2
Centrilobular hypertrophy	–	NE	NE	1	–	NE	NE	–
Fatty change, centrilobular (grade 2)	–	NE	NE	1	–	NE	NE	–

* $p < 0.05$ ** $p < 0.01$

Source: Flick et al., 2016b

Prolactin levels were decreased at 4000 ppm; however, the data were very variable. At the same dose, there was a biologically, but not statistically significant, decrease in the number of estrus cycles and an increase in estrus cycle length. Ovarian weights were decreased at 1000 ppm and above and uterine weights were decreased at 4000 ppm. At 4000 ppm, there was also a diffuse atrophy of the uterus and cervix. In males, prostate and seminal vesicle weights were decreased at 4000 ppm.

Additional organ weight changes consisted of increased thymus weights in males at 1000 ppm and above, increased adrenal and thyroid weights and increased relative spleen weights in males and increased relative thyroid weights in females at 4000 ppm. Due to the limited examinations, no NOAEL was derived (Flick et al., 2016b).

Study 5

In a 90-day toxicity study, groups of ten Wistar rats received afidopyropen (purity 97.2%; batch COD-001545) in the diet at a concentration of 0, 300, 1000 or 4000 ppm (equal to 0, 20, 79 or 171 mg/kg bw per day for males,0, 26, 98 or 197 mg/kg bw per day for females). An additional control group of ten/sex was used for blood sampling and clinical parameters only. Animals were inspected twice daily for mortality and moribundity, daily for clinical signs of toxicity and a detailed clinical observation was performed weekly. A detailed FOB including motor activity was performed at the end of the administration period. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Estrus cyclicity was monitored in the final three weeks of treatment. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Additional blood samples were taken to measure cardial troponin I. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues fixed, however histopathological examinations were only performed on control and high-dose adrenal glands, heart, liver, pancreas, thyroid glands (with parathyroid) and uterus (with cervix).

There were no effects on mortality, clinical signs of toxicity, ophthalmoscopic evaluation, urinalysis parameters or estrus cyclicity. Motor activity was decreased at 1000 ppm and above in females in the first interval. Body weight was decreased at 4000 ppm in males. Feed consumption was decreased in males and females at 4000 ppm, however other than the effect on compound consumption compared to the previous study at the same doses, the change was only considered adverse in males where there was an effect on body weight.

Values for Ht and Hb were decreased to a nonadverse extent similar to signs of regenerative anaemia in the rest of the database. Reticulocytes were increased in 4000 ppm males and platelets were increased in females at 1000 ppm and above. There was evidence of minimal to slight extramedullary haematopoiesis in the spleen in males at 4000 ppm. Total bilirubin values were decreased in males at 1000 ppm and above but in females only at 1000 ppm. However, in the absence of marked indication of hypoproliferative anaemia, the lower bilirubin levels were not considered to be adverse.

Blood glucose was decreased in males and females at 4000 ppm. In 4000 ppm rats, triglycerides, calcium and inorganic phosphate were decreased in males and potassium was increased in females.

In the heart, there was an increase in cardiac troponin I and a slight increase in incidence and severity of cardiac necrosis/fibrosis in 4000 ppm females (Table 13).

Table 13. Effects in the 90-day oral toxicity study in rats (Study 5)

	Males				Females			
Dose (ppm)	**0**	**300**	**1000**	**4000**	**0**	**300**	**1000**	**4000**
Cardiac troponin I (ng/mL)								
day 29	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.09
day 92	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.25 ± 0.25*
Histopathology								
Heart:								
Necrosis/fibrosis	2	NE	NE	2	1	NE	NE	3
grade 1	2	NE	NE	2	1	NE	NE	2
grade 2	-	NE	NE	-	-	NE	NE	1

NE Not examined

Source: Flick et al., 2016c

* $p < 0.05$

There was a single incident of diffuse atrophy of the uterus in the 4000 ppm females. Due to the limited examinations no NOAEL was derived (Flick et al., 2016c).

Dog

Study 1

In a 28-day toxicity study, one Beagle dog/sex per dose received afidopyropen (purity 95.74%) in capsules at a concentration of 0, 3, 9, 30 or 90 mg/kg bw per day. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues fixed, however only the duodenum, jejunum, and ileum collected from control dogs and dogs administered the 90 mg/kg bw per day dose were examined.

There were no effects on mortality. Vomiting was increased at 90 mg/kg bw per day in both sexes. Body weight losses were noted at 90 mg/kg bw per day in both sexes, and feed consumption of the 90 mg/kg bw per day female was decreased.

Liver and kidney weights were increased in the 90 mg/kg bw per day male. Gross changes were limited to white mucosa in the small intestine. Due to the limited examinations no NOAEL was derived (Yoshida, 2009).

Study 2

In a 90-day toxicity study, groups of four Beagle dogs received afidopyropen (purity 95.74%) in capsules at a concentration of 0, 15, 30 or 90/60 mg/kg bw per day. The high dose was reduced from 90 to 60 mg/kg bw/day at week 7 for males and week 5 for females; dosing was discontinued for two males and three females for up to two weeks between weeks 3 and 9. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to initiation and at weeks 7 and 13. Opthalmoscopic examinations were performed prior to initation and at the end of the study. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues were examined. Additional sections of the cerebrum of one dog per sex from the control and high-dose groups were subjected to Klüver-Barrera's stain to determine vacuolated lesions.

One mortality per sex was noted in the 90→60 mg/kg bw per day groups. One male was found dead and one female was killed in extremis during week 9. The male found moribund exhibited soiled fur and the female exhibited lateral position, inability to stand up and tremors. Other clinical signs of toxicity consisted of vomiting of feed at 30 mg/kg bw per day (starting at week 2–3) and at 90→60 mg/kg bw per day (starting in the first week of treatment) in males and females, along with salivation and absence of faeces at 90→60 mg/kg bw per day in males and females. Body weight and body weight gains were decreased in 90→60 mg/kg bw per day males and females and feed consumption was decreased in the same groups. There were no effects apparent on ophthalmoscopic examination.

White blood cell counts and neutrophils were decreased in males and females at week 7 prior to the decrease in dosage at 90→60 mg/kg bw per day. Reticulocytes were decreased at week 7 in 90→60 mg/kg bw per day males.

In the clinical chemistry assessment, BUN and albumin were increased at 30 mg/kg bw per day in females, however there was no dose response. In males, BUN was increased at 90→60 mg/kg bw per day along with a decrease in potassium and triglycerides. In all three cases with the males, the magnitude of the change was greater prior to the reduction of dose at week 7. At 30 mg/kg bw per day and above there was an increase in hyaline droplet deposition in the hepatocytes in both males and females. Relative liver weights were increased in males at 90→60 mg/kg bw per day.

Changes in urinary parameters were limited to males. At 30 mg/kg bw per day and above there was an increase in haematuria. At 90→60 mg/kg bw per day there was a decrease in urinary volume, urinary pH and an increase in dark-coloured urine, however, there was no further evidence of kidney damage.

In 90→60 mg/kg bw per day males, testicular, epididymis and prostate weights were decreased. Additionally, there was an increase in atrophy of the prostate, atrophy and hypoplasia of the seminiferous tubules and oligospermia in the epididymis.

Other organ weight changes consisted of decreased absolute heart weights, increased adrenal weights and decreased thymus weights in 90→60 mg/kg bw per day males.

Histopathologically, both males and females exhibited increased degeneration and regeneration of the striated muscle fibre, vacuolation of the white matter and neurophil of the cerebrum and slight to moderate decreases in myelin density of the subcortical white matter in the 90→60 mg/kg bw per day groups.

In the two decedent high-dose animals, both male and female exhibited increased liver congestion and centrilobular fatty change of the hepatocytes. The male exhibited lung oedema, haemorrhage of the liver and focal fatty change of the hepatocytes. The female exhibited accumulation of alveolar foamy cells, periportal fatty change of the hepatocytes and deposition of brown pigment in the Kupffer cells.

The NOAEL was 15 mg/kg bw per day based on increased vomiting and hyaline droplet deposition in the hepatocytes of males and females, and increased haematuria in males at the LOAEL of 30 mg/kg bw per day (Yoshida, 2010).

Study 3

In a one-year toxicity study, groups of four Beagle dogs received afidopyropen (purity 95.74%) in capsules at a concentration of 0, 8, 20 or 50/40 mg/kg bw per day. The high dose was reduced from 50 to 40 mg/kg bw per day at week 30 for males and week 29 for females; dosing was discontinued for three males and two females for up to two weeks between weeks 3 and 43 (except for one female for which dosing was discontinued four times between weeks 5 and 42 for one or two weeks at a time). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured weekly. Haematology and clinical chemistry parameters were analysed and urinalysis was performed using samples taken prior to initiation and at weeks 7 and 13. Opthalmoscopic examinations were performed prior to initation and at the end of the study. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues examined. Additional sections of the cerebrum of one dog per sex from the control and high-dose groups were subjected to Klüver-Barrera's stain to determine changes in myelin in the vacuolated brain lesions.

One 50→40 mg/kg bw per day male was killed in a moribund state at week 27 prior to the reduction in dosage. An increase in the frequency of vomiting was noted in 50→40 mg/kg bw per day males; there was an increase in vomiting in the low and mid-dose females but it was not consistent and did not progress with duration of dosing. Clinical signs in 50→40 mg/kg bw per day animals consisted of conjunctival injection in males and females, salivation, tonic convulsion, lateral position, reddish urine and loss of fur in females. Clinical signs in the decedent male consisted of decreased spontaneous motor activity, bradypnea, staggering gait, tremor and lateral position. There were transient periods of body weight loss and decreased food consumption in males and females at 50→40 mg/kg bw per day. There were no effects apparent on ophthalmoscopic evaluation or in urinalysis parameters.

Haematological changes consisted of decreased neutrophil counts in males and females at 20 mg/kg bw per day and above. In females, white blood cell counts were decreased at 50→40 mg/kg bw per day only.

At the top dose BUN values were increased in females. Alkaline phosphatase (ALP) was increased at 50→40 mg/kg bw per day in males and females. In 50→40 mg/kg bw per day females, albumin, albumin/globulin ratios and bilirubin were increased throughout the treatment period and globulin was decreased at study termination. Creatinine was increased in females at 20 mg/kg bw per

day and above. In both males and females at 20 mg/kg bw per day and above, there was an increase in hyaline droplet deposition of the hepatocytes.

Organ weight changes in animals that survived to study termination were limited to a decrease in epididymis weights in the 50→40 mg/kg bw per day groups (Table 14).

Table 14. Effects in the one-year oral toxicity study in dogs (Study 3)

	Males				Females			
Dose (mg/kg bw per day)	**0**	**8**	**20**	**50→40**	**0**	**8**	**20**	**50→40**
Clinical signs of toxicity								
Vomiting								
Incidences per week	0–6	0–2	2–4	1–16 §	0–1	0–17	4–9	5–19
Mean incidences per week	2.5	1.5	2.25	6.75	0.25	7	6.25	11.5
Clinical chemistry								
Creatinine								
Week −1	0.60 ± 0.05	0.54 ± 0.02	0.57 ± 0.12	0.53 ± 0.04	0.56 ± 0.03	0.56 ± 0.04	0.57 ± 0.04	0.62 ± 0.05 (↑11%)
Week 13	0.72 ± 0.03	0.64 ± 0.05	0.72 ± 0.17	0.62 ± 0.04	0.60 ± 0.05	0.65 ± 0.04	0.65 ± 0.05	0.70 ± 0.10 (↑17%)
Week 26	0.72 ± 0.03	0.62 ± 0.10	0.72 ± 0.15	0.68 ± 0.04	0.57 ± 0.05	0.67 ± 0.08	0.73 ± 0.07* (↑28%)	0.71 ± 0.10* (↑25%)
Week 52	0.78 ± 0.05	0.66 ± 0.11	0.74 ± 0.13	0.72 ± 0.04	0.63 ± 0.07	0.75 ± 0.07 (↑19%)	0.73 ± 0.06 (↑16%)	0.76 ± 0.07 (↑21%)
Organ weights								
Final body weight (kg)	12.0 ± 1.0	10.2 ± 0.3** (↓15%)	10.9 ± 0.5 (↓9%)	10.8 ± 0.8 (↓10%)	10.9 ± 1.3	11.5 ± 0.9	10.3 ± 1.2	10.2 ± 1.0
Epididymides – Abs (mg)	3587 ± 358	3166 ± 923	3478 ± 908	2818 ± 384 (↓21%)				
Rel (%)	0.0298 ± 0.0010	0.0312 ± 0.0092	0.0319 ± 0.0075	0.0262 ± 0.0032 (↓12%)				

(***Table 15 continued on the next page***)

	Males				Females			
Dose (mg/kg bw per day)	**0**	**8**	**20**	**50→40**	**0**	**8**	**20**	**50→40**
Histopathology								
Liver								
Hyaline droplets	0	0	3	4**	0	0	1	3
Muscle								
Degeneration, striated muscle fibre	0	0	0	1[#]	0	0	0	0
Cerebrum								
Vacuolation, white matter	0	0	1	4*[#]	0	0	0	3
Vacuolation, neuropil	0	0	2	4*[#]	0	0	0	4*
Medulla oblongata								
Vacuolation, white matter	0	0	0	0	0	0	0	1
Pons								
Vacuolation, white matter	0	0	0	1[#]	0	0	0	0

* $p < 0.05$ ** $p < 0.01$

[§] One animal killed in extremis during week 27

[#] Lesions were noted in the animals that died or were sacrificed moribund.

Source: Yoshida, 2011

Vacuolation of the white matter and neurophil of the cerebrum was noted in males at 20 mg/kg bw per day and above and females at 50→40 mg/kg bw per day. At 50→40 mg/kg bw per day both males and females exhibited a slight to moderate decrease in myelin density of subcortical white matter and corpus callosum and the females exhibited vacuolation of the white matter of the medulla oblongata.

Post-mortem changes in the decedent male consisted of increased liver and kidney weights, degeneration of the striated muscle fibre, liver congestion, diffuse fatty change of the hepatocytes, inflammation of the oesophagus, cyst formation in the cerebellum and vacuolation of the white matter of the pons.

The NOAEL was 8 mg/kg bw per day, based on decreased neutrophils and hyaline droplet deposition of the hepatocytes in males and females, vacuolation of the white matter and neuropil of the cerebrum in males at the LOAEL of 20 mg/kg bw per day (Yoshida, 2011).

(b) Dermal application

In a 28-day dermal toxicity study, groups of ten Crl:WI(Han) Wistar rats received afidopyropen (purity 95.74%) at a concentration of 0, 100, 300 or 1000 mg/kg bw per day in drinking water containing 1% carboxymethyl cellulose to approximately 10% of the body surface area clipped free of fur and covered under semiocclusive dressing for 6 h a day for 28 days. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. Prior to study termination an FOB was performed. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Haematology and clinical chemistry parameters were analysed at terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues examined

histopathologically in control and high-dose groups as well as in all tissues with gross lesions and the livers of animals from all dose groups.

There was an increase in the incidence of multifocal hyperkeratosis of the skin in females at 300 mg/kg bw per day and above and males at 1000 mg/kg bw per day. There were no findings of systemic toxicity.

The NOAEL for systemic toxicity was 1000 mg/kg bw per day, the highest dose tested (Buesen et al., 2017).

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In an 18-month carcinogenicity study, groups of 50 ICR [Crlj:CD1(ICR)] (SPF) mice of each sex received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 120, 700 or 4000 ppm for males (equal to 0, 13, 79 and 445 mg/kg bw per day) and 0, 120, 700 or 4000→3000→2000 ppm, for females (equal to 0, 13, 76 and 333 mg/kg bw per day for females). In females the dose was changed to 3000 ppm at week 24 and then to 2000 ppm at week 44 due to death or moribundity. Animals were monitored daily for mortality, moribundity and for clinical signs of toxicity. Body weight, body weight gain and feed consumption were measured weekly for the first 13 weeks of treatment, monthly thereafter and additionally just prior to scheduled termination. White blood cell counts were analysed from samples taken from the tail vein at 12 months and orbital sinus at 18 months. Gross examinations were performed and organs weighed from ten animals/sex per group. Histopathological examination was performed on animals from the control and high-dose groups, all animals killed in extremis or found dead and on the liver and submandibular gland (males only), heart (females only), and all gross lesions (both sexes) from the animals that were subjected to the terminal kill in the 120 and 700 ppm groups. The limited level of histopathology in the low- and mid-dose groups is considered not to affect the conclusion of the study, as the critical lesions of the brain, spinal cord, kidney and liver were not seen in top-dose animals surviving to termination.

There was an increase in mortality at weeks 21–24 and 41 in high-dose females after which the dose was decreased from 4000 ppm to 3000 ppm and subsequently to 2000 ppm. Survival was over 50% in all groups at 18 months. After the final reduction in dose there were no further differences from control in mortality. There was an increase in prone body position, a decrease in spontaneous motor activity and an increase in bradypnea in high-dose females. There were no effects on clinical signs of toxicity in males. Body weights were decreased slightly in males and markedly in females at the high dose. Overall body weight gain was decreased in both males and females at the high dose. Decreases in feed consumption were limited to the first week of treatment in high-dose females.

Changes in white blood cell parameters were limited to increases in white blood cells, lymphocytes and large unstained cells at the high dose in females.

Although there was an increase in spleen weights in high-dose females, there was also a decrease in haematopoieisis in the bone marrow and atrophy of the spleen in the same group.

There was no evidence of treatment-related increases in tumour incidence.

Changes to the liver were limited to an increase in centrilobular hypertrophy in high-dose males and an increase in pale coloured livers in high-dose females. There was an increase in vacuolation of the hepatocytes in high-dose females as discussed below.

Ovarian weights were increased in high-dose females (see Table 15 on the next page).

Table 15. Effects in the 18-month oral oncogenicity study in mice

	Males				Females			
Dose (ppm)	**0**	**120**	**700**	**4000**	**0**	**120**	**700**	**4000→3000 →2000**
Organ weights								
Final body weight (g)	52.5 ± 7.3	53.0 ± 4.6	46.5* ± 4.5 (↓11%)	47.5 ± 2.6 (↓10%)	50.0 ± 11.3	53.8 ± 7.8	52.2 ± 12.8	44.3 ± 4.5 (↓10%)
Relative spleen wt (%)	0.29 ± 0.13	0.38 ± 0.30	0.26 ± 0.06	0.46 ± 0.30	0.34 ± 0.24	0.30[a] ± 0.09	0.39 ± 0.31 (↑15%)	0.60*[a] ± 0.29 (↑76%)
Absolute ovary wt (mg)					225.5 ± 539.6	472.3 ± 1118.5	293.0 ± 386.8	822.8 ± 2168.1 (↑265%)
Relative ovary wt (%)					0.385 ± 0.867	0.937 ± 2.319	0.510 ± 0.633	1.680 ± 4.289 (↑336%)
Histopathology								
Survival to wk 78 (%)	62	52	56	63	71	77	71	62
Heart: vacuolation, cardiac muscle fibres					0/37 (tk) 0/52 (all)	0/40 (tk) 0/52 (all)	0/37 (tk) 0/52 (all)	0/32 (tk) 9/52** (all)
Heart: fibrosis, cardiac muscle					3/37 (8%) (tk) 6/52 (12%) (all)	1/40 (3%) (tk) 1/52 (2%) (all)	5/37 (14%) (tk) 10/52 (19%) (all)	18/32** (56%) (tk) 28/52** (54%) (all)
Liver: vacuolation, hepatocyte, diffuse					0/37 (tk) 0/52 (all)	0/2 (tk) 0/14[b] (all)	0/2 (tk) 0/17[b] (all)	0/32 (tk) 16/52** (all)
Kidney: vacuolation, proximal tubular cell					0/37 (tk) 0/52 (all)	0/6 (tk) 0/18[b] (all)	0/2 (tk) 0/17[b] (all)	0/32 (tk) 9/52 ** (all)
Urinary bladder: vacuolation, mucosal epithelial cell					0/37 (tk) 0/52 (all)	0/0 (tk) 0/12[b] (all)	0/0 (tk) 0/15[b] (all)	0/32 (tk) 7/52** (all)
Cerebrum: vacuolation, neuropil, cortex					0/37 (tk) 0/52 (all)	0/0[b] (tk) 0/12[b] (all)	0/0[b] (tk) 0/15[b] (all)	0/32 (tk) 4/52 (all)
Cerebrum: vacuolation, choroid plexus epithelium					0/37 (tk) 0/52 (all)	0/0[b] (tk) 0/12[b] (all)	0/0[b] (tk) 0/15[b] (all)	0/32 (tk) 4/52 (all)
Spinal cord (cervical): vacuolation, glial cell, gray matter					0/37 (tk) 0/52 (all)	0/0[b] (tk) 0/12[b] (all)	0/0[b] (tk) 0/15[b] (all)	0/32 (tk) 3/52 (all)

	Males				Females			
Dose (ppm)	**0**	**120**	**700**	**4000**	**0**	**120**	**700**	**4000→3000 →2000**
Spinal cord (thoracic): vacuolation, glial cell, gray matter					0/37 (tk) 0/52 (all)	0/0[b] (tk) 0/12[b] (all)	0/0[b] (tk) 0/15[b] (all)	0/32 (tk) 4/52 (all)
Spinal cord (lumbar): vacuolation, glial cell, gray matter					0/37 (tk) 0/52 (all)	0/0[b] (tk) 0/12[b] (all)	0/0[b] (tk) 0/15[b] (all)	0/32 (tk) 3/52 (all)

(tk) Terminal kill

[a] Recalculated values (by the study authors) with outliers removed

[b] Examined in the animals that showed macroscopic lesions. Not subjected to statistical analysis

* $p < 0.05$ ** $p < 0.01$

Source: Takahashi, 2012

Vacuolation of a number of tissues was noted in high-dose females. These consisted of the cardiac muscle, glandular stomach parietal cells, hepatocytes, kidney proximal tubule cells, urinary bladder mucosal epithelial cells, neuropil in the cortex, and the choroid plexus epithelium in the cerebrum and glial cell in the gray matter in the three levels of the spinal cord (cervical, thoracic and lumbar). Additionally, in the same group, there was fibrosis of the cardiac muscle and apoptosis in lymphocytes in the thymus and lymphoid follicle in the cervical and mesenteric lymph nodes. In high-dose males, there was an increase in secreted material depletion in the granular ducts of the submandibular gland.

The NOAEL was 700 ppm (equal to 76 mg/kg bw per day), based on clinical signs of toxicity culminating in mortality, decreased body weight, decreased feed consumption, increased WBC counts, increased spleen and ovarian weights, increased pale-coloured livers, decreased haematopoieisis in the bone marrow, atrophy of the spleen, myocardial fibrosis, apoptosis of lymphocytes of the thymus and lymph nodes, and vacuolation of various tissues at the LOAEL of 2000 ppm (equal to 333 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 333 mg/kg bw per day) in females and 4000 ppm (equal to 445 mg/kg bw per day) in males, the highest doses tested. (Takahashi, 2012).

Rat

Study 1

In a one-year toxicity study, groups of 24 Fischer 344 rats of each sex received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 75, 150, 300 or 1000 ppm (equal to 0, 3.7, 7.3, 15 and 48 mg/kg bw per day in males, 0, 4.4, 8.9, 18 and 56 mg/kg bw per day in females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed at week 49. Body weight and body weight gain were measured weekly for the first 13 months of treatment and monthly thereafter. Feed consumption was measure once in the first week of treatment, twice weekly from weeks 2–13 and twice every month thereafter. Ophthalmoscopic examinations were performed before the start of treatment for all animals, then at the end of the study for only the control and high-dose groups. Haematology and clinical chemistry parameters were analysed from samples taken from ten animals/sex per group at weeks 14 and 27 and prior to terminal kill. Urinalysis was performed on ten animals/sex per group at weeks 13, 26 and 51. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues examined histopathologically in control and high-dose groups, as well as in all tissues with gross lesions and the hearts and livers of females from all dose groups.

Body weights, body weight gains and feed consumption were decreased to a nonadverse extent in males and females of the 1000 ppm group. There were no clinical signs of toxicity and no effects on mortality, urinalysis or ophthalmoscopic evaluations.

There were changes indicative of regenerative anaemia at 1000 ppm in males and females that did not reach sufficient magnitude to be considered adverse, however platelets were increased in males and females.

Triglycerides were decreased in males and females at 1000 ppm. High-dose females also exhibited a slight vacuolar change (lipid deposition) of the hepatocytes as well as the myocardium (Table 16).

Table 16. Effects in the one-year oral chronic toxicity study in rats

	Males					Females				
Dose (ppm)	**0**	**75**	**150**	**300**	**1000**	**0**	**75**	**150**	**300**	**1000**
Histopathology										
Heart:vacuolar change, myocardium (all slight)	0	0	0	0	0	0	0	0	0	6 (25%)*
Liver: vacuolar change, hepatocyte, periphery (all slight)	0	0	0	0	0	0	0	0	2 (8%)	15 (63%)**

* $p < 0.05$ ** $p < 0.01$

Source: Yamashita, 2011

Uterine weights were decreased in 1000 ppm females; however, there were no associated gross or histopathological changes at that dose.

The NOAEL was 300 ppm (equal to 15 mg/kg bw per day) based on increased platelets and decreased triglycerides in males and females and increased vacuolar change in the liver and myocardium of females at the LOAEL of 1000 ppm (equal to 48 mg/kg bw per day) (Yamashita, 2011).

Study 2

In a one-year toxicity study, groups of 24 Fischer 344 rats of each sex received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 1000 or 3000 ppm (equal to 0, 48 and 143 mg/kg bw per day in males, 0, 57 and 161 mg/kg bw per day in females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed at week 49. Body weight and body weight gain were measured weekly for the first 13 months of treatment and monthly thereafter. Feed consumption was measure once in the first week of treatment, twice weekly from weeks 2–13 and twice every month thereafter. Ophthalmoscopic examinations were performed before the start of treatment for all animals and at the end of the study in the control and high-dose groups. Haematology and clinical chemistry parameters were analysed from samples taken from ten animals/sex per group at weeks 14, 27 and prior to terminal kill. Urinalysis was performed on ten animals/sex per group at weeks 13, 26 and 51. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues examined histopathologically in control and high-dose groups as well as in all tissues with gross lesions, all tissues of animals found dead or killed moribund and in the heart, liver, pancreas and pituitary of females from the 1000 ppm group.

There were no treatment-related effects on mortality, no clinical signs of toxicity and no effects on body weight, body weight gain, feed consumption or ophthalmoscopic evaluation.

At 3000 ppm, changes in RBC, Hb and Ht showed statistically significant increases in males through the administration period and in females just prior to study termination. The magnitude of the change was, however, just under adverse levels in males and did not approach adverse levels in females. Platelet levels were increased in males and females in both treatment groups, however the levels were only considered adverse at 3000 ppm. Reticulocytes were increased in males only at 3000 ppm. Bilirubin was decreased in the blood of females at 1000 ppm and urinary bilirubin was increased in males and females at 3000 ppm. Spleen weights were increased in both sexes in both treatment groups.

Triglycerides were increased in males and females in both treatment groups. Males in the 3000 ppm group exhibited increased serum potassium. In females BUN was increased at 1000 ppm and above and blood glucose was increased in 3000 ppm females. Liver weights were increased in all treated animals. Under gross examination, discolouration of the liver was noted at 3000 ppm in females. Histopathologically, there was a vacuolar change (lipid deposition) of hepatocytes that increased in severity from the 1000 ppm to 3000 ppm in females. Additionally, at 3000 ppm females exhibited slight foci of basophilic or tigroid type cells of the liver.

In the 3000 ppm groups, urine specific gravity was increased in males and females and volume was decreased while urinary protein and ketones were increased in females. Kidney weights were increased in all treated groups.

Testicular, and epididymal weights were increased and uterine weights decreased at 1000 ppm and above; ovarian weights were decreased at 3000 ppm (Table 17).

Table 17. Effects in the one-year oral chronic toxicity study in rats (Study 2)

		Males			Females		
Dose (ppm)		**0**	**1000**	**3000**	**0**	**1000**	**3000**
Organ weights							
Final body weight		420 ± 21	429 ± 20	408 ± 25	213 ± 10	211 ± 8	213 ± 9
Adrenal[§],	absolute (mg)	39.8 ± 4.0	43.7 ± 5.5* (↑10%)	44.1 ± 2.8** (↑11%)	46.6 ± 4.1	49.0 ± 3.9	46.1 ± 4.7
	relative (%)	0.0095 ± 0.0009	0.0102 ± 0.0014 (↑7%)	0.0108 ± 0.0009** (↑14%)	0.0219 ± 0.0016	0.0232 ± 0.0018*	0.0216 ± 0.0021
Heart,	absolute (g)	1.14 ± 0.08	1.18 ± 0.07	1.13 ± 0.08	0.71 ± 0.03	0.68 ± 0.02** (↓4%)	0.67 ± 0.03** (↓6%)
	relative (%)	0.271 ± 0.015	0.275 ± 0.015	0.276 ± 0.013	0.335 ± 0.017	0.322 ± 0.011* (↓4%)	0.313 ± 0.010** (↓7%)
Pituitary,	absolute (mg)	9.6 ± 1.2	10.0 ± 2.1	10.2 ± 1.3	11.6 ± 2.2	12.9 ± 1.8* (↑11%)	14.0 ± 1.5** (↑21%)
	relative (%)	0.0023 ± 0.0003	0.0023 ± 0.0005	0.0025 ± 0.0003	0.0054 ± 0.0010	0.0061 ± 0.0009* (↑13%)	0.0066 ± 0.0008** (↑22%)
Testes[§],	absolute (g)	3.04 ± 0.36	3.30 ± 0.16** (↑9%)	3.44 ± 0.45** (↑13%)	-	-	-
	relative (%)	0.726 ± 0.095	0.771 ± 0.039 (↑6%)	0.844 ± 0.116** (↑16%)	-	-	-
Epididymides[§], absolute (mg)		874 ± 96	945 ± 62* (↑8%)	951 ± 95* (↑9%)	-	-	-
	relative (%)	0.208 ± 0.022	0.221 ± 0.013 (↑6%)	0.233 ± 0.023** (↑12%)	-	-	-

		Males			Females		
Dose (ppm)		**0**	**1000**	**3000**	**0**	**1000**	**3000**
Ovaries[§],	absolute (mg)	-	-	-	53.6 ± 5.0	51.6 ± 5.0	49.7 ± 5.3* (↓7%)
	relative (%)	-	-	-	0.0253 ± 0.0025	0.0245 ± 0.0023	0.0233 ± 0.0027* (↓8%)
Uterus,	absolute (mg)	-	-	-	921 ± 186	813 ± 169 (↓12%)	513 ± 159** (↓44%)
	relative (%)	-	-	-	0.434 ± 0.091	0.386 ± 0.084 (↓11%)	0.241 ± 0.077** (↓44%)
Histopathology							
Heart, vacuolar change, myocardium							
	Slight	0	0	0	0	10(44%)**	17 (81%)**
	Moderate	0	0	0	0	0	4 (19%)*
	Total	0	0	0	0	10(44%)**	21 (100%)**
Pancreas,							
Decreased zymogen granules, acinar cell							
	All slight	0	0	0	4 (17%)	12 (52%)*	18 (86%)**
Pituitary							
Hyperplasia, focal anterior lobe							
	All slight	0	1	0	2 (8%)	1 (4%)	7 (33%)*

[§] Organ weights were determined bilaterally; data represent the values for left- and right-side tissues combined

* $p < 0.05$ ** $p < 0.01$

Source: Oshima, 2015a

Heart weights were decreased in females and there was an increase in slight vacuolar change (lipid deposition) of the myocardium in both dose groups.

Pituitary weights were increased in both dose groups; in females and there was slight focal hyperplasia of the anterior lobe of the pituitary at 3000 ppm.

Adrenal weights were increased in males in both dose groups.

Thyroid weights were increased in both dose groups in males and in the 3000 ppm group in females.

Other histopathological changes consisted of a decrease in the zymogen granules of pancreatic acinar cells in both dose groups and slight hyperplasia of the bile duct in the 3000 ppm female group.

A NOAEL was not established in this study. The LOAEL was 1000 ppm (equal to 48 mg/kg bw per day) based on increased platelets and decreased triglycerides, increased liver, spleen and kidney weights in males and females, increased adrenal, thyroid, testicular and epididymal weights in males and increased BUN, decreased bilirubin, decreased heart weights and increased pituitary and uterine weights, slight vacuolar change of the liver and myocardium and decreased zymogen granules of the pancreatic acinar cells in females (Oshima, 2015a).

Study 3

In a two-year toxicity study, groups of 50 Fischer 344 rats of each sex received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 100, 300 or 1000 ppm (equal to 0, 4.4, 13, and 43 mg/kg bw per day in males, 0, 5.3, 16 and 51 mg/kg bw per day in females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed at week 49. Body weight and body weight gain were

measured weekly for the first 13 months of treatment and monthly thereafter. Feed consumption was measure once in the first week of treatment, twice weekly from weeks 2–13 and twice every month thereafter. Haematology parameters were analysed from samples taken from all animals at weeks 52, 78 and prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed from ten animals/sex per dose and tissues were examined histopathologically in control and high-dose groups as well as in all tissues with gross lesions and in the adrenals of males and uteruses of females in all dose groups.

There were no effects on mortality, clinical signs of toxicity, body weights, feed consumption or haematological parameters.

In 1000 ppm females, uterine weights and adenocarcinomas were increased (Table 18).

Kidney, liver and adrenal weights were increased in males at 1000 ppm. In 1000 ppm females there was an increase in hyperplasia of the bile duct in the liver.

There was an increase in pheochromocytoma of the adrenal glands in 1000 ppm males at termination and when including all animals. However, this finding was not replicated in the subsequent study at higher doses and was not considered treatment-related.

Table 18. Effects in the 2-year oral toxicity and carcinogenicity study in rats

	Males				**Females)**			
Dose (ppm)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Organ weights								
Final body weight (g)	462 ± 39	472 ± 25	459 ± 27	432 ± 39 (↓7%)	292 ± 32	321 ± 74	317 ± 35	292 ± 21
Liver weight, absolute (g)	12.5 ± 1.4	12.6 ± 0.7	13.4 ± 1.6	13.8 ± 2.0 (↑10%)	8.0 ± 0.7	9.0 ± 2.2	8.8 ± 0.9	8.3 ± 0.7
Liver weight relative (%)	2.70 ± 0.17	2.68 ± 0.20	2.91 ± 0.24	**3.20 ± 0.38 (↑19%)	2.79 ± 0.66	2.81 ± 0.30	2.78 ± 0.35	2.85 ± 0.13
Kidney weight absolute (g) – total	2.76 ± 0.15	2.69 ± 0.11	2.82 ± 0.30	*3.03 ± 0.24 (↑10%)	1.82 ± 0.10	1.88 ± 0.23	1.87 ± 0.04	1.89 ± 0.10
Kidney weight relative (%) – total	0.601 ± 0.056	0.570 ± 0.025	0.614 ± 0.052	*0.709 ± 0.106 (↑18%)	0.636 ± 0.114	0.602 ± 0.096	0.596 ± 0.072	0.649 ± 0.039
Adrenal weight absolute (mg) – total	60.3 ± 10.4	54.7 ± 4.1	57.0 ± 6.6	73.9 ± 18.7 (↑23%)	61.3 ± 10.7	61.3 ± 16.4	65.7 ± 5.3	61.8 ± 3.4
Adrenal weight relative (%) – total	0.0131 ± 0.0018	0.0116 ± 0.0010	0.0124 ± 0.0015	0.0176 ± 0.0065 (↑34%)	0.0218 ± 0.0077	0.0190 ± 0.0021	0.0210 ± 0.0034	0.0212 ± 0.0016
Epididymides wt absolute (mg) – total	480 ± 72	586 ± 188	503 ± 116	480 ± 72				
Epididymides wt relative (%) – total	0.105 ± 0.017	0.125 ± 0.042	0.110 ± 0.024	0.112 ± 0.016				
Uterus weight absolute (mg) – total					982 ± 395	788 ± 174	850 ± 144	1359 ± 678 (↑38%)
Uterus weight relative (%) – total					0.342 ± 0.142	0.257 ± 0.068	0.270 ± 0.044	0.465 ± 0.232 (↑36%)

	Males				Females)			
Dose (ppm)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Histopathological lesions								
Liver: bile duct hyperplasia (total) – all animals	50/50	30/31	31/31	50/50	25 / 50 (50.0%)	16 / 38 (42.1%)	15 / 44 (34.1%)	*35 / 50 (70.0%)
Testis: interstitial cell hyperplasia	12/50	2/50	1/48	4/50*				
Testis: interstitial cell tumour	40/50	46/50	45/48	40/50				
Mammary gland: duct dilation (total)					11 / 50 (22.0%)	3 / 25 (12.0%)	4 / 19 (21.1%)	10 / 50 (20.0%)
Mammary gland: diffuse hyperplasia					5/50	1/25	0/19	3/50
Mammary gland: adenoma					1/50	0/25	0/19	0/50
Mammary gland: adenocarcinoma					3/50	3/25	1/19	1/50
Uterus: endometrium hyperplasia					10/50	10/50	13/50	10/50
Uterus: stromal cell hyperplasia					1/50	0/50	1/50	1/50
Uterus: squamous epithelium metaplasia					1/50	0/50	0/50	0/50
Uterus: adenoma					2/50	1/50	2/50	3/50
Uterus: adenocarcinoma					4/50	1/50	2/50	10/50
Uterus: adenosquamous carcinoma					0/50	1/50	1/50	0/50

* $p < 0.05$ ** $p < 0.01$

Source: Yamashita, 2014

The NOAEL for chronic toxicity was 300 ppm (equal to 13 mg/kg bw per day) based on increased kidney, liver and adrenal weights in males and increased uterine weights, hyperplasia of the bile duct of the liver and increased incidence of uterine adenocarcinomas in females at the LOAEL of 1000 ppm (equal to 43 mg/kg bw per day). The NOAEL for carcinogenicity was 300 ppm (equal to 16 mg/kg bw per day) based on an increased incidence of uterine adenocarcinomas in females at the LOAEL of 1000 ppm (equal to 51 mg/kg bw per day) (Yamashita, 2014).

Study 4

In a two-year toxicity study, groups of 50 Fischer 344 rats of each sex received afidopyropen (purity 95.74%) in the diet at a concentration of 0, 1000 or 3000 ppm (equal to 0, 42 and 128 mg/kg bw per day in males, 0, 50 and 147 mg/kg bw per day in females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed at week 49. Body weight and body weight gain were measured weekly for the first 13 months of treatment and monthly thereafter. Feed consumption was measure once in the first week of treatment, twice weekly from weeks 2–13 and twice every month thereafter. Haematology parameters were analysed from samples taken from all animals at weeks 52, 78 and prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed from ten animals/sex per dose and tissues in control and high-dose groups examined histopathologically, as well as all tissues with gross lesions and the adrenals of males and uteruses of females in all dose groups.

Mortality was increased in 3000 ppm females. Clinical signs of toxicity were limited to unilateral eye opacity in females in both dose groups. Body weights were slightly decreased in males throughout the administration period at 3000 ppm. In females there were non-adverse decreases in body weight at 1000 ppm throughout the administration period and an adverse decrease at 3000 ppm. Body weight gains were decreased in females at 3000 ppm overall and between weeks 52 and 76. Feed consumption was decreased throughout the administration period in all treated groups; however, the most marked decreases were at the start of treatment and were not reflected in body weights.

Haematological changes were limited to non-statistically significant increases in white blood cells in both males and females at 3000 ppm. Spleen weights were increased in males and females at 3000 ppm.

In the liver, relative liver weights were increased in females in both dose groups and absolute and relative liver weights were increased in males and females at 3000 ppm. Hyperplasia of the bile ducts was noted in livers of females in both dose groups and there was an increase in the foci of altered cells in the liver in males at 3000 ppm.

In the reproductive organs, there was a non-adverse increase in epididymis weights at both doses and a reduced incidence of the number of animals with decreased number of sperm in the epididymides. The incidences of testicular interstitial cell tumours and hyperplasia were reduced at the top dose level. In females ovarian weights were decreased and there was an increase in adenocarcinomas at both doses and an increase in uterine weights at 3000 ppm (Table 19).

Kidney weights were increased in males and females at 3000 ppm and there was an increase in chronic progressive nephrosis in 3000 ppm females.

There was an increase in cysts in the pituitary in males and females and in the mandibular lymph node in males at 3000 ppm. Additionally, there was increased dilatation of the sinus in the mandibular lymph node in males at 3000 ppm.

Adrenal weights were decreased in 3000 ppm males.

There was a decrease in zymogen granules in the pancreatic acinar cells as seen in the one-year toxicity study; however, the change was only seen in the 3000 ppm in females.

Table 19. Effects in the two-year oral toxicity and carcinogenicity study in rats

	Males			Females		
Dose (ppm)	**0**	**1000**	**3000**	**0**	**100**	**300**
Organ weights						
Final body weight (g)	446 ± 47	447 ± 36	429 ± 39 (↓4%)	296 ± 26	288 ± 27 (↓3%)	**260 ± 17 (↓12%)
Liver weight, absolute (g)	12.1 ± 1.4	12.7 ± 1.6	**14.3 ± 1.9 (↑18%)	8.6 ± 1.4	9.0 ± 1.0 (↑5%)	**9.9 ± 1.2 (↑15%)
Liver weight relative (%)	2.73 ± 0.40	2.85 ± 0.32	**3.35 ± 0.39 (↑23%)	2.91 ± 0.34	*3.12 ± 0.27 (↑7%)	**3.85 ± 0.53 (↑32%)
Spleen weight, absolute (g)	1228 ± 1584	1168 ± 511	**1735 ± 2115 (↑41%)	776 ± 576	708 ± 316	*1013 ± 1205 (↑31%)
Spleen weight relative (%)	0.284 ± 0.421	0.263 ± 0.121	**0.426 ± 0.585 (↑50%)	0.261 ± 0.182	0.248 ± 0.113	**0.398 ± 0.488 (↑52%)
Kidney weight, absolute (g)	2.75 ± 0.20	2.86 ± 0.28	**2.94 ± 0.22 (↑7%)	1.98 ± 0.17	2.04 ± 0.16	**2.11 ± 0.23 (↑7%)
Kidney weight, relative (%)	0.628 ± 0.132	0.643 ± 0.064	**0.688 ± 0.051 (↑10%)	0.673 ± 0.077	0.714 ± 0.084	**0.813 ± 0.098 (↑21%)

	Males			Females		
Dose (ppm)	**0**	**1000**	**3000**	**0**	**100**	**300**
Adrenal weight, absolute (mg)	79.1 ± 88.9	77.2 ± 70.6	117.6 ± 354.7 (↑49%) §63.6 ± 15.6 (↓20%)	62.8 ± 6.6	78.3 ± 90.0	62.7 ± 8.1
Adrenal weight, relative (%)	0.0207 ± 0.0376	0.0179 ± 0.0192	0.0274 ± 0.0817 (↑32%) §0.0149 ± 0.004 (↓28%)	0.0214 ± 0.0030	0.0276 ± 0.0329	0.0243 ± 0.0038** (↑14%)
Epididymides weight absolute – total (mg)	513 ± 93	*595 ± 117 (↑16%)	**767 ± 152 (↑50%)	-	-	-
Epididymides weight relative – total (%)	0.116 ± 0.020	**0.133 ± 0.023 (↑15%)	**0.178 ± 0.028 (↑53%)	-	-	-
Uterus weight absolute (mg)	-	-	-	1083 ± 490	1081 ± 487	1945 ± 4680 (↑80%)
Uterus weight relative (%)	-	-	-	0.370 ± 0.179	0.381 ± 0.174	0.745 ± 1.768 (↑101%)
Ovaries weight absolute (mg)	-	-	-	124.7 ± 349.8	72.9 ± 50.4 (↓42%)	**57.5 ± 14.5 (↓54%)
Ovaries weight relative (%)	-	-	-	0.0423 ± 0.1190	0.0254 ± 0.0182 (↓40%)	0.0222 ± 0.0058 (↓48%)
Histopathological lesions						
Liver: bile duct hyperplasia – all animals						
slight	46/50	46/50	41/50	21/50	39/50**	43/50**
moderate	4/50	4/50	9/50	1/50	3/50	6/50
total	50/50	50/50	50/50	22/50	42/50**	49/50**
Testis: interstitial cell hyperplasia	41/50	34/50	11/50**	-	-	-
Testis: interstitial cell tumour	41/50	40/50	16/50**	-	-	-
Mammary gland:						
duct dilation (total)	-	-	-	23/50	17/50	11/50**
diffuse hyperplasia	-	-	-	6/50	9/50	4/50
adenoma	-	-	-	3/50	1/50	0/50
adenocarcinoma				3/50	2/50	0/50
Uterus:						
endometrium hyperplasia				7/50	11/50	16/50*
stromal cell hyperplasia				2/50	0/50	0/50
adenoma				1/50	3/50	4/50
adenocarcinoma				0/50	5/50*	12/50**

* $p < 0.05$ ** $p < 0.01$ Source: Oshima, 2015b

The NOAEL was not established in this study. At the LOAEL for chronic toxicity of 1000 ppm (equal to 42 mg/kg bw per day), epididymis weights were increased in males, there was an increase in unilateral opacity of the lens of the eyes, increased relative liver weights, decreased absolute heart weights, decreased ovarian weights and increased hyperplasia of the bile ducts. At the LOAEL for carcinogenicity of 1000 ppm (equal to 50 mg/kg bw per day) there were increased incidences of adenocarcinomas in the uterus (Oshima, 2015b).

2.4 Genotoxicity

(a) In vitro studies

A range of in vitro studies was conducted to assess the genotoxicity of afidopyropen, to assess its potential for inducing chromosomal aberration and gene mutation at the median purity, and reverse mutation at a variety of purities (summarized in Table 20). There was no evidence of genotoxicity.

(b) In vivo studies

Micronucleus assays were conducted on three purities to assess the potential of afidopyropen to damage chromosomes in vivo (summarized in Table 20). There was no evidence of genotoxicity.

Table 20. Genotoxicity studies with afidopyropen

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	***Test 1*** (–S9) *S. typhimurium*: 2.3–556 µg/plate; *E. coli*: 20.6–5000 µg/plate; ***Test 1*** (+S9) All strains: 61.7–5000 µg/plate; ***Test 2*** (–S9) *S. typhimurium*: 9.8–313 µg/plate; *E. coli*: 156–5000 µg/plate; ***Test 2*** (+S9) All strains: 313–5000 µg/plate.	95.74	Negative	Matsumoto, 2009a
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0–5300 µg/plate (± S9)	94.5	Negative	Woitkowiak, 2015a
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0–5300 µg/plate (± S9)	97.3	Negative	Woitkowiak, 2015b
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0–5600 µg/plate (± S9)	90.00	Negative	Woitkowiak, 2015c
Chromosomal aberration	Chinese hamster lung (CHL/IU) cells	Inhibition test: 19.5–5000 µg/mL; Chromosome aberration test: 78.1–625 µg/mL (short-term treatment); 30.9–156 µg/mL (continuous treatment)	95.74	Negative	Matsumoto, 2009b
Mammalian forward mutation	Chinese hamster ovary cells (CHO/HGPRT)	***Experiment 1***: 0–300 µg/mL −/+ S9 in DMSO ***Experiment 2***: 0–250 µg/mL −/+ S9 in DMSO	94.54	Negative	Kapp & Landsiedel, 2015

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vivo (oral)					
Mouse micronucleus[a]	NMRI mouse	0, 350, 700 and 1400 or 2000 mg/kg bw	90.0	Negative	Dony, 2016
Mouse micronucleus[a]	[Crlj:CD1 (ICR)] mouse	0, 250, 500 or 1000 mg/kg bw	95.7	Negative	Dony, 2015
Mouse micronucleus[a]	[Crlj:CD1 (ICR)] mouse	0, 500, 1000 or 2000 mg/kg bw	95.74	Negative	Wada, 2009

DMSO Dimethyl sulfoxide

CHO/HGPRT Chinese hamster ovary hypoxanthine-guanine phosphoribosyltransferase

S9 9000 × *g* supernatant fraction from liver homogenate from phenobarbital-treated and 5,6-benzoflavone-treated rats

[a] Bone marrow exposure was not demonstrated in this study; however, ADME studies indicate that the compound reached the bone marrow (e.g.Table 5)

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Study 1

In a range-finding one-generation reproductive toxicity study, groups of eight Wistar rats of each sex received afidopyropen (purity 95.74%) at a dietary concentration of 0, 150, 1500, 3000 or 6000 ppm (equal to 0, 10, 101, 192 and 390 mg/kg bw per day in males, 0, 15, 132, 243 and 431 mg/kg bw per day in females). Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured weekly in males and weekly during the prebreeding phase and on gestation days (GDs) 0, 7, 14 and 20 and lactation days (LDs) 0, 1, 4, 7, 14 and 21 in females. Feed consumption was measured weekly during prebreeding but was not measured during breeding due to cohousing. Estrus cycle length was monitored daily for a minimum of three weeks prior to mating, until evidence of copulation and a vaginal smear was examined at necropsy. Breeding commenced 75 days after the start of treatment, litters were recorded as soon as possible following delivery and F0 females reared their pups until postnatal day (PND 21). Blood samples were taken from eight animals/sex per group shortly before study termination. F0 males were killed prior to weaning of the F1 pups and F0 females and F1 pups were killed shortly after.

There were no effects on mortality or clinical signs of toxicity in parental animals. At 1500 ppm and above, body weight, body weight gain and feed consumption were decreased in females. Male body weight, body weight gain and feed consumption were decreased at 3000 ppm.

Adrenal, thymus and liver weights were increased in females at 1500 ppm and in males at 6000 ppm. In males, absolute pituitary weights were decreased at 3000 ppm and relative pituitary weights were decreased at 6000 ppm. Thyroid weights were increased in females at 3000 ppm. There was an increase in dark brown discolouration of the liver at 3000 ppm and above in males.

Regarding reproductive parameters, there were decreases in the mean number of implantations and mean number of pups delivered at 1500 ppm and above. Estrous length, gestation length, delivery and viability indices were decreased at 6000 ppm. Parental ovarian weights and offspring uterine weights were decreased at 3000 ppm and absolute epididymis weights were decreased in parental animals at 6000 ppm.

Changes to the offspring consisted of decreased body weights at 150 ppm. At 3000 ppm, there was an increase in pup deaths and a decrease in the viability index. At 6000 ppm there was an increase in whole litter losses. Other changes consist of decreased spleen and thymus weights at 3000 ppm and decreased absolute brain weights at 6000 ppm (Fujii et al. 2009).

In a one-generation reproductive toxicity study to investigate the effects of impurities, groups of 25 Wistar [Crl:WI(Han)] rats of each sex received afidopyropen at dietary concentrations of 0, 1500 ppm (purity 99.9% ; high purity) or 1500 ppm (purity 95.74%; standard purity), equal to

0, 127 and 126 mg/kg bw per day in males, 0, 131 and 132 mg/kg bw per day in females, in the control, high purity and standard purity test groups. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured weekly in males: for females measurements were weekly during the premating phase and on GDs 0, 7, 14 and 20 and LDs 0, 1, 4, 7, 14 and 21. Feed consumption was measured weekly during premating but was not measured during breeding due to cohousing. Estrus cycle length was monitored daily for a minimum of three weeks prior to mating, until evidence of copulation and a vaginal smear was examined at necropsy. Blood samples were taken from 12 animals/sex per group shortly before study termination. F0 males were killed prior to weaning of the F1 pups and F0 females and F1 pups were killed shortly after.

There were no effects on parental mortality. In males, body weight gains were decreased in the first week of treatment and increased compared to controls in males in both purity groups at weeks 11–12. There were no effects on body weights or effects on overall body weight gain in males. In premating females, body weight gains were increased in the standard-purity group in the first two weeks of treatment and body weight gains were decreased in both purity groups at weeks 3–4 and 7–8. In the first week of gestation, both purity groups had decreased body weight gain, however the change was only of sufficient magnitude to be considered adverse in the high-purity group. Body weight gains were increased compared to controls at all time periods in both purity groups during lactation. There were no effects on body weights or overall body weight gains in females during premating or gestation. Overall body weight gain was increased in both purity groups in the lactation period. There were no effects on feed consumption in males or females during premating or gestation. In females in both groups, feed consumption was decreased throughout the lacation period.

Regarding haematology parameters, reticulocytes and WBCs were increased in males of both purity groups and in females in the standard-purity group. Neutrophils were increased in high-purity males and standard-purity females. Lymphocytes were increased in both groups of males and unaffected in females. Bilirubin was decreased in all treated animals. In males, spleen weights were increased in both dose groups, however the magnitude of the change was on the cusp of adversity. In females, spleen weights were at the high end of historical controls, however the magnitude of the change was only adverse as an absolute organ weight gain in the standard-purity group.

Among clinical chemistry parameters, triglycerides were increased in all treated groups. Cholesterol was increased in all treated groups, but only reached adverse levels in the standard-purity groups. Inorganic phosphate was increased in both groups of females, but only reached adverse levels in the standard-purity group. Liver weights were increased in all treated groups, however the magnitude of change was only considered adverse for relative liver weight in standard-purity males and absolute and relative liver weights in standard-purity females. Livers were enlarged in two standard-purity females. At histopathological examination, there were slight increases in extramedullary haematopoiesis in standard-purity males and in grade 2 periportal fatty change in high- and standard-purity females.

Absolute kidney weights were slightly elevated in standard-purity females.

Absolute adrenal weights were increased in standard-purity males and both groups of females.

Thyroid weights were increased in high-purity males and in both groups of females.

With sperm measurements, there was an equivocal but statistically significant decrease in sperm counts in the standard-purity males. Absolute prostate weights were decreased in males in both groups. Additionally, sex ratios were skewed towards males in the standard-purity group.

With respect to pups, high-purity dose groups exhibited decreased body weights from PND 14 to 21 and decreased body weight gains from PND 1–21. Standard-purity pups exhibited decreased body weights from PND 7–21 and decreased body weight gains from PND 1–2. Both treatment groups exhibited decreased spleen weights in male and female pups. An increase in the percentage of male pups was seen in the standard-purity group only (Schneider et al., 2016a).

Study 2

In a two-generation reproductive toxicity study, groups of 24 BrlHan:WIST rats of each sex received afidopyropen (purity 95.74%) at a dietary concentration of 0, 100, 300 or 1000 ppm (equal to 0, 7.7, 22 and 75 mg/kg bw per day in males, 0, 9.0, 27 and 85 mg/kg bw per day in females). Animals were monitored twice daily for mortality and moribundity. Body weight, body weight gain and feed consumption were measured weekly in males and weekly during the premating phase and on GDs 0, 7, 14 and 20 and LDs 0, 1, 4, 7, 14 and 21 in females. Both males and females were weighed on the day of necropsy. Food consumption was not determined during the mating period. Haematology and clinical chemistry parameters were not examined. Breeding commenced ten weeks after the start of treatment, litters were recorded as soon as possible following delivery and litters were culled on LD 4 to four/sex per litter. On PND 21 F1 litters were weaned. From the F1 weanlings 24 males and 24 females were selected from litters born during a five-day period including the largest number of parturitions; these became the F1 parental animals to produce the second (F2) generation. Weanlings were selected, as far as was possible, so as to yield comparable group mean body weights. Selected F1 animals were monitored daily for onset of puberty. Gross examinations were performed and organs weighed in all adults, and the uteri of all females were stained for examination. Histopathological examination was performed on all adults from the control and high-dose groups. All pups not selected at various stages were autopsied and brain, thymus, spleen and uterus weights measured in F1 and F2 weanlings.

In parental animals, there were no adverse effects on mortality, body weight, feed consumption, clinical signs of toxicity, gross pathology, histopathology or organ weights. Body weights were slightly increased and feed consumption was slightly decreased at 1000 ppm in both generations, however the magnitude was small and not considered adverse. Liver weights in males and females, and adrenal weights in females were increased at 1000 ppm, however there was no correlation with histopathological changes.

Amongst the reproductive parameters, there was an increase in the ratio of males to females and a decrease in the prostate weights in F1 parental animals at 1000 ppm (Table 21). A change in sex ratios towards males at 100 and 300 mg/kg bw per day in the F1 generation was not repeated in the F2 generation or in another study on a closely related strain.

Table 21. Effects in the reproductive toxicity study in rats

Dose (ppm)	0	100	300	1000
Parental animals – organ weights				
Absolute prostate (mg)				
F0	541.3 ± 120.9	513.0 ± 116.9	549.8 ± 116.5	516.7 ± 106.3 (↓5%)
F1	572.1 ± 88.1	546.5 ± 86.7	565.4 ± 106.6	522.3 ± 95.8 (↓9%)
Relative prostate (10^{-3} %)				
F0	124.8 ± 31.7	116.1 ± 24.8	121.7 ± 24.8	115.0 ± 25.1 (↓8%)
F1	129.6 ± 17.1	120.7 ± 21.2	123.3 ± 24.7	112.0 ± 19.1* (↓14%)

(***Table 21 continued on the next page***)

Dose (ppm)	0	100	300	1000
Offspring				
Pups delivered [n]				
F1	297	272	291	288
F2	279	274	272	276
Litters				
F1	24	23	23	23
F2	23	24	24	24
Offspring per dam [mean *n*]				
F1	12.4 ± 1.8	11.3 ± 3.3	12.7 ± 2.1	12.5 ± 1.3
F2	12.1 ± 2.2	11.4 ± 2.2	11.3 ± 3.5	11.5 ± 2.8
Offspring liveborn [*n*]				
F1	296	271	291	287
F2	278	269	270	274
Litters not surviving to day 21, complete litter loss [*n*]				
F1	0	0	0	0
F2	0	0	1	1
Viability index [mean %]				
LD 0				
F1	99.58 ± 2.04	99.67 ± 1.61	100.0 ± 0.0	99.60 ± 1.90
F2	99.60 ± 1.90	98.63 ± 4.70	99.40 ± 2.92	99.29 ± 2.45
LD 4				
F1	96.13 ± 8.09	94.63 ± 14.34	87.80 ± 23.88	95.59 ± 11.48
F2	96.19 ± 7.76	92.86 ± 18.82	95.07 ± 8.71	91.21 ± 12.06
Pup mortality				
Total found dead PND 0–26				
F1	15 (9)	22 (8)	17 (9)	29 (13)
F2	13 (7)	24 (8)	30 (9)	18 (11)
Day 0				
F1	1 (1)	1 (1)	0	1 (1)
F2	1 (1)	5 (2)	2 (1)	2 (2)
Days 1 to 4				
F1	11 (6)	19 (6)	16 (9)	25 (11)
F2	11 (6)	16 (7)	26 (9)	12 (7)
% of pups dying PND 0				
F1	0.34	0.37	0.0	0.35
F2	0.36	1.86	0.74	0.73
% of pups dying PNDs 1–4				
F1	3.72	7.01	5.50	8.71
F2	3.96	5.95	9.63	4.38

Dose (ppm)		0	100	300	1000
% of pups dying PNDs 0–4					
	F1	4.05	7.38	5.50	9.06
	F2	4.3	7.7	10.3	4.3
Sex ratio [% live males]					
	F1	45.1	54.8*	54.0*	56.3**
	F2	46.2	47.8	51.1	52.5
Pup body weights					
Males					
LD 0					
	F1	5.93 ± 0.39	6.00 ± 0.49	5.83 ± 0.58	5.77 ± 0.35
	F2	5.81 ± 0.53	6.01 ± 0.41	5.74 ± 0.48	6.01 ± 0.65
LD 4					
	F1	9.26 ± 1.14	9.63 ± 1.84	9.24 ± 1.63	9.19 ± 1.10
	F2	9.31 ± 1.37	9.54 ± 1.75	9.29 ± 1.51	9.60 ± 2.14
LD 21					
	F1	50.84 ± 3.26	50.15 ± 4.24	50.32 ± 5.33	47.90 ± 2.52**
	F2	51.59 ± 4.54	50.51 ± 5.49	50.44 ± 5.11	47.37 ± 5.29*
Females					
LD 0					
	F1	5.60 ± 0.38	5.64 ± 0.44	5.55 ± 0.50	5.41 ± 0.33
	F2	5.49 ± 0.45	5.68 ± 0.40	5.55 ± 0.63	5.69 ± 0.58
LD 4					
	F1	8.78 ± 1.13	9.48 ± 1.64	89.15 ± 1.43	8.67 ± 1.24
	F2	8.93 ± 1.33	9.24 ± 1.47	8.86 ± 1.49	9.26 ± 1.72
LD 21					
	F1	48.78 ± 3.52	49.31 ± 3.65	48.63 ± 4.61	45.96 ± 2.22**
	F2	49.51 ± 4.97	49.12 ± 4.30	48.81 ± 4.58	46.20 ± 3.80*
Sexual maturity (males, F1 generation)					
Age at preputial separation (days)		42.1 ± 1.8	42.2 ± 1.4	43.0 ± 1.7	43.9 ± 1.8**
Body weight on the day of preputial separation (g)		182.5 ± 13.6	185.8 ± 13.5	190.6 ± 13.5	190.7 ± 16.9

* Significantly different from control, $p < 0.05$ ** Significantly different from control, $p < 0.01$

Source: Fujii, 2013a

In the offspring in both generations, there were decreased body weights at PND 21 in males and females at 1000 ppm. In the F1 generation at 1000 ppm, spleen weights were decreased in males and females. Sexual maturity was delayed in F1 male pups at the high dose in a manner that was not related to decreased body weight gain.

There was a slight and non-statistically significant increase in pup deaths between PNDs 0 and 4 at the highest dose tested in the F1 generation. While unclear in the study report, the Sponsor determined that further study of the finding would be required.

The NOAEL for parental toxicity was 1000 ppm (equal to 75 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 300 ppm (equal to 22 mg/kg bw per day), based on altered sex ratios at the LOAEL of 1000 ppm (75 mg/kg bw per day).

The NOAEL for offspring toxicity was 300 ppm (equal to 27 mg/kg bw per day), based on increased pup deaths and decreased body weights at PND 21 in F1 and F2 offspring, decreased spleen weight in F1 males and females, and delayed sexual maturation and decreased prostate weights in F1 males at the LOAEL of 1000 ppm (equal to 85 mg/kg bw per day) (Fujii, 2013d).

Study 3

In a two-generation reproductive toxicity study, groups of 25 Crl:WI(Han) rats of each sex received afidopyropen (purity 97.2%) at a dietary concentration of 0, 100, 500 or 2000 ppm (equal to 0, 7.8, 39 and 150 mg/kg bw per day in males, 0, 8.4, 41 and 155 mg/kg bw per day in females). Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight, body weight gain and feed consumption were measured weekly in males and weekly during the prebreeding phase and on GDs 0, 7, 14 and 20 and LDs 0, 1, 4, 7, 14 and 21 in females. Both males and females were weighed on the day of necropsy. Food consumption was not determined during the mating period. Breeding commenced 75 days after the start of treatment. Litters were recorded as soon as possible following delivery and litters were culled on LD 4. On PND 21 F1 litters were weaned. To form the F1 parental animals to produce the second (F2) generation, 24 males and 24 females were selected from the F1 weanlings from litters born during a five-day period including the largest number of parturitions. As far as was possible, weanlings were selected as to yield comparable group mean body weights. Selected F1 animals were monitored daily for onset of puberty. Haematological and clinical chemistry parameters were examined prior to terminal kill. Gross examinations were performed and organs weighed on all adults, and the uteri of all females were stained for examination. Histopathological examination was performed on all adults from the control and high-dose groups and adrenal glands were examined at the mid-dose in the female parental animals. All pups subject to terminal kill, stillborn or those that died prior to weaning, were examined externally and necropsied. Notable findings or abnormalities were further evaluated on a case-by-case basis, depending on the type of finding noted.

In parental animals, body weight gains were sporadically decreased in F0 generation males and females at 2000 ppm. In the F1 generation body weight and body weight gain were decreased in males and females at 2000 ppm. Feed consumption was decreased at 2000 ppm in both sexes and generations. The exception was an increase in body weight gain during lactation in both generations in the presence of decreased feed consumption.

There was a decrease in monocytes in the F0 generation males and treatment-related and adverse increases in reticuloctyes in F0 and F1 females at 2000 ppm. Lymphocytes were increased in F1 females at 500 ppm and above, and in the F0 females at 2000 ppm. Glucose was decreased (> 10%) in females at 500 ppm and above in F0 females and at 2000 ppm in F0 and F1 males and F1 females. Total bilirubin was decreased in 2000 ppm males and females of both generations and triglycerides were decreased in 2000 ppm F0 and F1 males and F0 females. Cholesterol was increased (> 10%) in 500 ppm and above F0 and 2000 ppm F1 females.

Adrenal weights were increased in F0 and F1 females at 500 ppm and above, however histopathological change was only noted at 2000 ppm, with an increase in adrenal vacuolation. Adrenal weights were increased in males at 2000 ppm in F0 and F1 generations in the absence of histopathological change (Table 22a on the following page).

Table 22a. Effects of afidopyropen on parental rats in two-generation reproductive toxicity study

Generation	F0 generation				F1 generation			
Dose (ppm)	**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**2000**
Males								
Organ weights								
Necropsy body weight (g)	402.3 ± 24.4	405.3 ± 39.8	412.7 ± 26.2	377.0 ± 43.1	422.4 ± 40.107	406.6 ± 30.253	417.8 ± 41.49	379.4 ± 34.3**
Absolute adrenal weight (g)	61.28 ± 7.46	61.08 ± 8.05	63.80 ± 6.89	62.60 ± 8.09	61.32 ± 6.762	59.46 ± 7.003	64.76 ± 11.016	65.60 ± 8.986
Relative liver weight (%)	0.015 ± 0.002	0.015 ± 0.002	0.015 ± 0.001	0.017 ± 0.002*	0.015 ± 0.002	0.015 ± 0.002	0.016 ± 0.002	0.017 ± 0.002**
Histopathology								
Prostate infiltration, lymphoid	6	-	1	1	4	-	0	8
Females								
Organ weights								
Necropsy body weight (g)	240.4 ± 17.3	237.5 ± 14.2	252.4 ± 19.9	245.8 ± 16.1	237.4 ± 20.68	233.7 ± 15.74	243.9 ± 16.74	240.3 ± 19.85
Absolute adrenal weight (g)	78.16 ± 9.38	81.12 ± 12.3	89.40 ± 10.8**	94.12 ± 12.5**	78.4 ± 7.878	75.1 ± 11.341	85.7 ± 8.473**	85.3 ± 11.866*
Relative liver weight (%)	0.033 ± 0.004	0.034 ± 0.004	0.036 ± 0.005*	0.038 ± 0.004**	0.033 ± 0.004	0.032 ± 0.004	0.035 ± 0.004*	0.036 ± 0.005
Histopathology								
Adrenal cortex								
Vacuolation increased	3	0	0	7	1	1	0	10
Grade 1	3	–	–	7	1	1	–	8
Grade 2	–	–	–	–	–	–	–	2

* Significantly different from control, $p < 0.05$ ** Significantly different from control, $p < 0.01$

Source: Schneider et al., 2016b

In the reproductive parameters, there was an increase in "improper nursing of offspring" in three dams in the F0 generation and six dams in the F1 generation at 2000 ppm. Sex ratios were skewed towards males at 2000 ppm in both generations in addition to decreased prostate and ovarian weights. In the 2000 ppm F0 generation, sperm counts in the testes were decreased. In the 2000 ppm F1 generation, there was a decrease in implantation sites, a decrease in the mean number of pups per dam and a decrease in the mean litter size. Additionally, there was an increase in lymphoid infiltration of the prostate glands and a decrease in uterus weights.

Table 22b. Reproductive performance of F0 and F1 generations in two-generation reproductive toxicity study

Parental generation	**F0**				**F1**			
Dose (ppm)	**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**2000**
Animals /sex / dose	25	25	25	25	24	24♂ 25♀	25	25
Male fertility								
mating index (%)	100	100	100	100	100	100	92	100
males with pregnant females (*n*)	25	24	22	22	24	24	22	24
fertility index (%)	100	96	88	88	100	100	88	96
Sperm parameters								
motile sperm (%)	86 ± 7	87 ± 8	88 ± 7	88 ± 6	87 ± 7	88 ± 7	84 ± 19	89 ± 6
TS/gT [a] (Mio/g)	132 ± 19	–	–	120 ± 25*	109 ± 18	–	–	116 ± 25
TS/gC[b] (Mio/g)	528 ± 76	–	–	551 ± 86	627 ± 189	–	–	660 ± 159
Abnormal sperm (%; cut off 6%)	6.2 ± 0.8	–	–	6.0 ± 0	6.2 ± 0.6	–	–	6.0 ± 0
Female fertility								
Placed with males (*n*)	25	25	25	25	24	25	25	25
Mated (*n*)	25	25	25	25	24	25	25	25
Mating index (%)	100	100	100	100	100	100	92	100
Pregnant (*n*)	25	24	22	22	24	25	22	24
Fertility index (%)	100	96	88	88	100	100	95.7	96
Implantation sites, total (*n*)	299	295	283	255	333	315	256	240
Implantation sites, mean	12 ± 2.6	12.3 ± 2.0	12.9 ± 1.2	11.6 ± 2.3	13.9 ± 4.8	12.6 ± 1.6	11.6 ± 2.8	10.0 ± 2.0**
Gestation index (%)	100	95.8	100	100	100	100	95.5	95.8
Dams with stillborn pups (*n*)	1	1	0	2	4	2	0	4
Dams with all stillborn pups (*n*)	0	0	0	0	0	0	0	0
Pups delivered (*n*)	282	267	271	239	289	303	242	225
per dam (mean %)	11.3 ± 2.6	11.6 ± 2.1	12.3 ± 1.2	10.9 ± 1.8	12 ± 2.1	12.1 ± 1.6	11.5 ± 2.1	9.8 ± 1.9**
liveborn (*n*)	281	266	271	237	285	301	242	217
stillborn (*n*)	1	1	0	2	4	2	0	8
Live birth index (mean %)	99.6	99.6	100	99.2	98.6	99.3	100	96.4
Sex ratio (% live males)								
Day 0	45.6	44.4	48.0	55.3	49.5	49.5	48.3	56.7
HC (2009–2014)[c]	42.6–53.8				44.0–50.1			

[a] TS/gT = total spermatids/gram testis

[b] TS/gC = total sperms/gram cauda epididymis

[c] Historical controls via correspondence with sponsor

Source: Schneider et al., 2016b

In the offspring, body weight and body weight gain were decreased in pups of both generations at 2000 ppm. There were slight decreases in body weight gain in males and body weight at PND 21 and body weight gain in females of both generations at 500 ppm. Preputial separation was delayed 1.5 days at 500 ppm and 5.4 days at 2000 ppm in the F1 pups and vaginal opening was delayed 3.6 days at 2000 ppm, but body weights at attainment of maturation were unaffected in males (Table 22). Thymus weights were decreased in F1 females and F2 males at 500 ppm and F1 males and F2 females at 2000 ppm. Spleen weights were decreased at 2000 ppm in F1 males and females, and F2 males and females at 500 ppm. Lactation indices and litter size at PND 4 were decreased in both generations at 2000 ppm and the viability was decreased in the 2000 ppm group in the F2 generation. Pup death and the number of pups with reduced nutritional condition were increased at 2000 ppm in both generations.

Table 22c. Offspring parameters of F0 and F1 generations in in two-generation reproductive toxicity study

Parental generation		**F0**				**F1**			
Dose [ppm]		**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**2000**
Mean litter size	Day 0	11.2 ± 2.6	11.6 ± 2.0	12.3 ± 1.2	10.8 ± 1.9	11.9 ± 2.1	12.0 ± 1.6	11.5 ± 2.1	9.4 ± 2.1**
	Day 4	11.2 ± 2.6	11.5 ± 2.0	12.3 ± 1.2	10.5 ± 1.7	11.9 ± 2.1	12.0 ± 1.6	11.5 ± 2.0	8.6 ± 2.7**
Viability index	(Mean%)	99.3 ± 2.5	99.7 ± 1.4	99.6 ± 1.9	97.6 ± 7.3	100 ± 0.0	100 ± 0.0	99.7 ± 1.6	83.9 ± 30.1*
Pups surviving days 4 to 21	(*n*)	194	183	174	160	191	200	166	151
Lactation index	(Mean%)	100 ± 0.0	100 ± 0.0	98.9 ± 5.3	92 ± 24.6	100 ± 0.0	100 ± 0.0	100 ± 0.0	90.2 ± 29.4
				MALE					
Animals examined		128	119	130	133	144	150	117	127
Reduced nutritional condition,	Total	0	0	0	8	0	0	0	32
	Severe	–	–	–	1	–	–	–	1
	Moderate	–	–	–	3	–	–	–	31
	Slight	–	–	–	4	–	–	–	0
Pup body weight									
Body weight at PND 1		7.0 ± 0.9	7.0 ± 0.8	7.0 ± 0.5	6.5 ± 0.8* (↓7%)	6.8 ± 0.7	6.7 ± 0.5	6.7 ± 0.7	6.4 ± 0.8 (↓6%)
Body weight at PND 21		54.9 ± 4.5	54.5 ± 3.7	52.9 ± 3.9	38.3 ± 3.3** (↓30%)	52.8 ± 3.0	52.7 ± 3.4	49.7 ± 3.4* (↓6%)	36.8 ± 5.2** (↓30%)
Body weight gain: Days 14 to 21		20.3 ± 2.4	19.7 ± 2.2	18.6 ± 1.9* (↓8%)	12.7 ± 1.2** (↓37%)	19.1 ± 1.4	19.1 ± 1.6	17.5 ± 1.6** (↓8%)	12.6 ± 1.9** (↓34%)
Overall body weight gain: Days 1 to 21		47.8 ± 3.9	47.5 ± 3.3	45.9 ± 3.5 (↓4%)	31.8 ± 2.9** (↓33%)	46.0 ± 2.7	46.0 ± 3.1	43.0 ± 3.0* (↓7%)	30.4 ± 4.6** (↓34%)

Parental generation	F0				F1			
Dose [ppm]	**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**2000**
Preputial separation								
Days (mean ± SD)	41.0 ± 1.7	41.3 ± 1.5	42.5 ± 2.6*	46.4 ± 2.3**				
N	25	24	25	25				
Body weight at day of preputial separation: mean ± SD (g)	180.1 ± 14.4	177.2 ± 11.4	183.8 ± 10.7	177.3 ± 16.1				
N	25	24	25	25				
Historical control values − Age at criterion (days)								
Mean	41.5							
Range of means	39.7–42.5							
Number of litters	210							
Historical control values − Body weight at criterion (g)								
Mean	168.7							
Range of means	156.5–181.0							
Number of litters	210							
FEMALE								
Animals examined	154	148	141	106	145	153	125	98
Reduced nutritional condition, total	0	0	0	5	0	0	0	27
Severe	–	–	–	1	–	–	–	1
Moderate	–	–	–	0	–	–	–	26
Slight	–	–	–	4	–	–	–	0
TOTAL								
Animals examined	282	267	271	239	289	303	242	225
Reduced nutritional condition: Total	0	0	0	13 [2]	0	0	0	2 [1]
Severe [PND 7–death or PND 7–21]	–	–	–	2 [1]	–	–	–	
Moderate [PND 7–death or PND 7–21]	–	–	–	3 [1]	–	–	–	57 [6]
Slight [PND 5–7]	–	–	–	8 [1]	–	–	–	2 [1]
Pup body weight								
Body weight at PND 1	6.7 ± 0.7	6.6 ± 0.7	6.6 ± 0.6	6.2 ± 0.7* (↓7%)	6.4 ± 0.7	6.3 ± 0.5	6.4 ± 0.7	6.0 ± 0.6 (↓6%)
Body weight at PND 21	52.6 ± 3.4	52.3 ± 3.4	51.0 ± 3.7	37.9 ± 2.7** (↓28%)	50.8 ± 2.9	50.1 ± 3.5	48.1 ± 3.7* (↓5%)	35.0 ± 4.4** (↓31%)
Body weight gain: Day 14 to 21	18.8 ± 1.5	18.5 ± 1.6	17.6 ± 2.0* (↓6%)	12.4 ± 1.6** (↓34%)	18.3 ± 1.4	17.8 ± 1.5	16.8 ± 1.7** (↓8%)	11.8 ± 1.3** (↓36%)
Overall body weight gain: Day 1 to 21	45.9 ± 2.9	45.6 ± 2.9	44.3 ± 3.2	31.6 ± 2.4** (↓31%)	44.4 ± 2.5	43.8 ± 3.1	41.7 ± 3.2* (↓6%)	28.9 ± 4.0** (↓35%)

Parental generation	F0				F1			
Dose [ppm]	**0**	**100**	**500**	**2000**	**0**	**100**	**500**	**2000**
Vaginal opening								
Days (mean ± SD)	29.8 ± 1.6	30.4 ± 1.4	30.5 ± 2.0	33.4 ± 2.7**				
N	25	25	25	25				
Body weight at day of vaginal opening Mean ± sd	92.2 ± 8.1	94.1 ± 8.0	96.8 ± 11.3	97.9 ± 13.6				
N	25	25	25	25				
Historical control values − Age at criterion (days)								
Mean	31.3							
Range of means	30.0–32.1							
Number of litters	213							
Historical control values − Body weight at criterion (g)								
Mean	93.7							
Range of means	86.4–99.6							
Number of litters	213							

* Significantly different from control, $p < 0.05$ ** Significantly different from control, $p < 0.01$

Source: Schneider et al., 2016b

The NOAEL for parental toxicity was 100 ppm (equal to 8.4 mg/kg bw per day), based on increased lymphocytes in F1 females, decreased glucose in F0 females and increased cholesterol in F0 females at the LOAEL of 500 ppm (equal to 41 mg/kg bw per day).

The NOAEL for reproductive toxicity was 500 ppm (equal to 39 mg/kg bw per day), based on improper nursing, altered sex ratios, decreased prostate weights and decreased ovarian weights in F0 and F1 generations, decreased sperm counts in F0 males and decreased implantation sites, mean number of pups per dams, mean litter size at PND 0, increased infiltration of the prostate gland and decreased uterus weights in the F1 generation adults at the LOAEL of 2000 ppm (equal to 150 mg/kg bw per day).

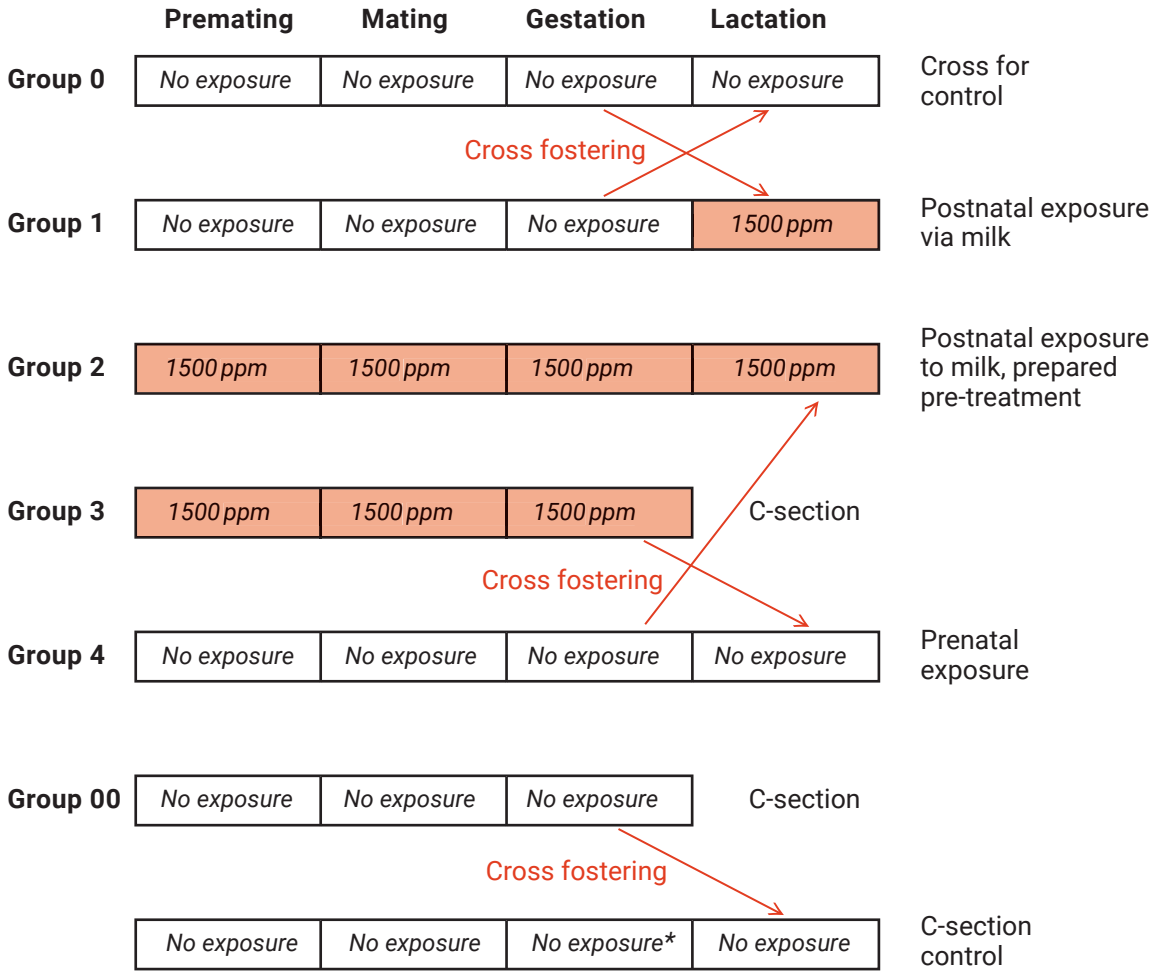
The NOAEL for offspring toxicity was 100 ppm (equal to 8.4 mg/kg bw per day) based on decreased thymus weights in F1 females and F2 males and decreased spleen weights in F2 males and females, and delayed preputial separation at the LOAEL of 500 ppm (equal to 41 mg/kg bw per day) (Schneider et al., 2016b).

Study 4

In a non-guideline, one-generation cross-fostering study, groups of 20 Crl:WI(Han) rats of each sex received afidopyropen (purity 94.54–95.74%) at a dietary concentration of 0 or 1500 ppm (equal to 0 and 131 mg/kg bw per day in males, 0 and 132-133 mg/kg bw per day in females). Test group 0 (TG0) did not receive afidopyropen at any point and dams fostered pups from Test group 1 (TG1). TG1 did not receive afidopyropen during premating or gestation, but received afidopyropen during lactation while fostering pups from TG0. TG2 received afidopyropen during premating and gestation, but not lactation while fostering pups from TG4. Pups from TG2 dams were culled following parturition. TG3 animals received afidopyropen during premating and gestation. Pups were delivered via caesarian section. TG4 did not receive afidopyropen at any point and fostered pups from TG3. A supplementary control group of 50 female Crl:WI(Han) rats (TG00) was added during the study where parental animals received no afidopyropen and pups were delivered via caesarian section. Dosing and fostering are described in Fig. 5. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight, body weight gain and feed consumption were measured weekly in males and weekly during the prebreeding phase and on GDs 0, 7, 14 and 20 and LDs 0, 1, 4, 7, 14 and 21 in females. Both males and females were weighed on the day of necropsy. Food consumption was not determined during the mating period. Breeding commenced 76 days after the start of treatment; litters were recorded

as soon as possible following delivery and litters were cross-fostered immediately as whole litters. Haematology and clinical chemistry parameters were measures in TG0, TG1 and TG2. Litters were weaned on PND 21. Gross examinations were performed on all the adults from TG0, TG1 and TG2. Animals from TG00, TG3 and TG4 were not examined. With the necropsied animals, organs from all adults were weighed and histopathological examination performed on the reproductive organs, adrenal glands, heart, liver, pituitary, reproductive organs and gross lesions from all animals of TG1, TG2 and TG3. Stillborn pups, or those that died prior to weaning, were examined externally and necropsied. At study termination one pup/sex per litter was necropsied and the brain, heart, spleen and thymus were weighed. Notable findings or abnormalities were further evaluated on a case-by-case basis, depending on the type of finding noted.

Figure 5. Cross-fostering scheme



* All pups from these dams were culled after parturition

Redrawn from Schneider et al., 2016c

In the parental animals, body weight gain was increased during lactation in TG1, TG2 and TG4; however, there was no effect on the final body weights. Haematological changes consisted of decreased neutrophils in TG2 males and TG1 and TG2 females, increased lymphocytes in TG1 and TG2 females, increased reticulocytes and decreased monocytes in TG2 males and increased platelets in TG1 females. In the clinical chemistry parameters, bilirubin and triglycerides were decreased in TG2 males and TG1 and TG2 females, glucose was decreased in TG2 males, inorganic phosphorus was decreased and cholesterol and GGTP were increased in TG1 and TG2 females and blood urea was increased in TG1 females. Organ weight changes in the TG2 males consisted of increased liver, kidney and spleen weights. In the TG1 females, liver weights were increased along with enlarged livers and an increase in diffuse hepatocellular hypertrophy. In TG2 females liver weights were increased.

Considering the reproductive parameters, there was an increase in pup death at PND 0 in TG4 animals. To determine whether the increased death was related to the caesarian section rather than treatment with afidopyropen, an additional dose group, TG00, was created, in which untreated animals were subject to caesarian section. While there was an increase in pup death in TG00 compared to TG0, the number of pup deaths was lower than TG4. The increased incidence of pup deaths in the TG4 group was determined to be related to in utero exposure (Table 23).

Table 23. Litter parameters for cross-fostering reproductive toxicity study (Study 4)

Observation	Test group				
	TG0	TG1	TG2	TG4	TG00 (*n*=50)
	F1 generation				
Exposure of foster dams	0 ppm premating, mating, gestation and lactation.	0 ppm premating, mating and gestation. 1500 ppm lactation.	1500 ppm premating, mating, gestation and lactation.	0 ppm premating, mating, gestation and lactation.	Arrived GD 0 from breeder. 0 ppm premating, mating, gestation and lactation.
Exposure of litters	0 ppm prenatal and postnatal.	0 ppm prenatal. 1500 ppm postnatal.	0 ppm prenatal. 1500 ppm postnatal.	Delivered via C-section 1500 ppm prenatal. 0 ppm postnatal.	Delivered via C-section 0 ppm prenatal and postnatal
Total number of litters	17	17	15	15 [b]	
Litters with liveborn pups (*n*)	17	17	15	15	23
Total number of pups delivered (*n*)	212	220	175	173	221
Mean # of pups delivered (%)	12.5 ± 2.48	12.9 ± 1.56	11.7 ± 2.87	11.5 ± 2.87	9.6 ± 1.34
Number born live (*n*)	210	216	173	173	221
Number stillborn [litters]	2 [2]	4 [3]	2 [2]	0	0
Number of deaths Day 0 (*n*)	0	2 [1]	0	17** [6]	10 [1]
Days 1–4 (*n*)	2 [1]	2 [2]	2 [2]	3 [3]	6 [5]
Day 5 (*n*)	0	0	1 [1]	0	0
Total number of pup deaths PND 0–21 (*n*)	2 [1]	4 [3]	3 [2]	20 [7]	16 [6]
% dead pup [% litters] PND 0–21	0.9 [5.8]	1.8 [18]	1.7 [13]	12 [47]	7 [26]
Mean litter size: Day 1	12.2 ± 2.54	12.5 ± 1.59	11.5 ± 3.09	10.3 ± 3.08	9.0 ± 2.43
Day 4	12.2 ± 2.54	12.5 ± 1.59	11.4 ± 3.02	10.2 ± 3.21	8.9 ± 2.41
Day 7	12.2 ± 2.54	12.5 ± 1.59	11.3 ± 2.97	10.2 ± 3.21	8.9 ± 2.41
Day 14	12.2 ± 2.54	12.5 ± 1.59	11.3 ± 2.97	10.2 ±3.21	8.9 ± 2.41
Day 21	12.2 ± 2.54	12.5 ± 1.59	11.3 ± 2.97	10.2 ± 3.21	8.9 ± 2.41
Live birth index (%)	99	98	99	100	100
Pups surviving days 0 to 4	208	212	171	153**	205
Viability index (%)	99	98	99	88	93
Lactation index (%)	100	100	99	100	100
Sex ratio Day 0 (%)	45.7	53.2	48.6	53.8	52.5

* Significantly different from control, $p < 0.05$ ** Significantly different from control, $p < 0.01$

Source: Schneider et al., 2016c

The TG1 and TG2 offspring (0 ppm prenatal exposure; 1500 ppm postnatal exposure) exhibited decreased body weight and body weight gain. TG4 offspring (1500 ppm prenatal exposure; 0 ppm postnatal exposure) had decreased body weights until PND 4, but were unchanged from treatment thereafter. Pup death was increased in TG4 from PND 0–4. Spleen, heart and thymus weights were decreased in groups TG1 and TG2 (Schneider et al., 2016c).

(b) Developmental toxicity

Rat

Study 1

In a preliminary developmental toxicity study, groups of eight premated female BrlHan:WIST Jcl(GALAS) rats were given afidopyropen (purity 95.74%) in 1% sodium carboxymethyl cellulose by gavage at dose levels of 0, 20, 100, 500 or 1000 mg/kg bw/day from GDs 6–19. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured on GDs 0, 3 and daily thereafter. Feed consumption was determined over GDs 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–20. All surviving animals were subjected to gross necropsy, uterine staining and histopathological examination of the heart and liver. Fetuses were examined for external malformations.

In the dams at 500 and 1000 mg/kg bw per day, there were increases in maternal mortality, vaginal haemorrhage, bradypnea, hypothermia and soiled fur. There was an increase in soiling of the fur at 500 mg/kg bw per day and above. Additionally, body weight, body weight gains and feed consumption were decreased at 500 mg/kg bw per day and above, as were gravid uterine weights.

There were no effects on the pregnancy rate, corpora lutea, implantations or implantation indices. The number of live fetuses and litters was decreased at 500 mg/kg bw per day and above and the number of resorptions and the post-implantation loss was increased.

At necropsy the thymus and spleen were atrophied, there was discolouration (multifocal dark red/black patches of the jejunum and glandular stomach mucosa and at 500 mg/kg bw per day and above there was hypertrophy of the adrenal gland apparent at gross examination. At 1000 mg/kg bw per day, there was discolouration of the adrenal gland, decreased heart weights and increased liver weights. Histopathologically, there were single incidences of myocardial degeneration at 20 and 100 mg/kg bw per day and multiple incidences at 500 and 1000 mg/kg bw per day. In the liver, there were increases in hepatocellular hypertrophy and periportal fatty change at 500 mg/kg bw per day and above.

In the offspring at 500 mg/kg bw per day and above, the number of live fetuses, litters and the viability indices were decreased, and resorptions and post-implantation losses increased. Due to the limited examinations, no NOAEL was derived (Fujii, 2013a).

Study 2

In a developmental toxicity study, groups of 24 premated female BrlHan:WIST@Jcl(GALAS) rats were given afidopyropen (purity 95.74%) in 1% sodium carboxymethyl cellulose by gavage at dose levels of 0, 10, 30 or 100 mg/kg bw per day from GDs 6–19. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured on GDs 0, 3 and daily thereafter. Feed consumption was determined over GDs 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–20. All surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex and external malformations. Half of each group was examined for visceral malformations, and the remaining half stained and examined for skeletal malformations.

In dams, there were no effects on mortality, body weight or feed consumption or clinical signs of toxicity. Reduced body weight gain on the first day of dosing appears to be related to reduced food consumption. Adrenal weights were increased at 100 mg/kg bw per day.

At 100 mg/kg bw per day, there was an increase in fetuses with skeletal variations and an increase in lumbar (supernumerary) ribs, increased metatarsal ossification and a change in sex ratios towards male offspring (Table 24).

Table 24. Body weight and sex ratio in developmental toxicity study in rats (Study 2)

Observation		Dose (mg/kg bw per day)			
		0	10	30	100
Maternal weight gain (g)	Days 6–7	4.1	4.3	3.5	2.6*
Maternal food consumption (g/day)	Days 6–9	21	21	21	19
Number of of litters examined		24	23	23	22
Implantations		13.3	12.0	12.0	12.5
Litter size		12.7	11.1	11.1	11.5
Sex ratio: overall		0.502	0.510	0.520	0.567
Sex ratio: mean		0.496 ± 0.147	0.530 ± 0.177	0.519 ± 0.111	0.572 ± 0.145
Number of litters with ratio of males ≥ 0.6		6	7	6	10
Percentage of litters with ≥ 0.6 males		25%	30%	26%	45%

* Significantly different from control, $p < 0.05$

Source: Fujii, 2013b

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on increased adrenal weights at the LOAEL of 100 mg/kg bw per day.

The NOAEL for embryo/fetal (and change in other studies) toxicity was 30 mg/kg bw per day, based on increased variations and altered sex ratios at the LOAEL of 100 mg/kg bw per day (Fujii, 2013b).

Study 3

In a developmental toxicity study, groups of 24 premated female BrlHan:WIST@Jcl(GALAS) rats were given afidopyropen (purity 95.74%) in 1% sodium carboxymethyl cellulose by gavage at dose levels of 0, 50, 100 or 200 mg/kg bw per day from GDs 6–19. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and body weight gain were measured on GDs 0, 3 and daily thereafter. Feed consumption was determined over GDs 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–20. All surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex and external malformations. Half of each group was examined for visceral malformations, and the remaining half was stained and examined for skeletal malformations.

In dams, there was one mortality at GD 19 at 200 mg/kg bw per day. At the same dose, body weight gain was decreased from GDs 6–9 with an associated reduction in food consumption (Table 24); however, there was no effect on absolute body weights. Feed consumption was likewise decreased from GDs 6–12 and overall from GDs 6–20.

In fetuses there was an increase in skeletal variations and supernumerary ribs at 50 mg/kg bw per day and above. At 200 mg/kg bw per day there was an increase in malformations, with increased fetal and litter incidences of the zygomatic bone fused with the maxilla, and two fetuses from the same litter with cleft palates (Table 25). The fetuses with cleft palate weighed 1.9 and 2.05 g; much lower than the "normal" fetuses in the litter (2.2–3.6 g) and the high-dose group mean of 3.6 g. This is possibly due to a delay in palate closure rather than any direct teratogenic effect.

Table 25. Offspring findings in developmental toxicity study in rats (Study 3)

Observation		Dose (mg/kg bw per day)			
		0	50	100	200
Maternal weight gain (g)	Days 6–9	9	9	8	2*
Maternal food consumption (g/day)	Days 6–9	19	18	16*	14*
Number of litters examined		24	24	24	21
Fetuses examined for skeletal variations		153	150	160	128
Skeletal variations, fetuses [litters] as a percentage, fetuses [litters]		95 [23] 62% [96%]	112* [24] 80% [100%]	132* [24] 83% [100%]	120* [21] 94% [100%]
Supernumerary rib, fetuses as a percentage, fetuses [litters]		79 52% [92%]	101* 62% [100%]	119* 74% [96%]	114* 89% [100%]
Sex ratio: overall		0.519	0.497	0.505	0.529
Sex ratio: mean		0.516 ± 0.117	0.495 ± 0.158	0.499 ± 0.148	0.525 ± 0.124
Number of litters with ratio of males ≥ 0.6 Percentage of litters with ≥ 0.6 males		6 25%	6 25%	7 29%	5 24%
Number of fetuses [litters] examined		318 [24]	312 [24]	329 [24]	263 [21]
Number of fetuses [litters] with external malformations		1 [1]	0	1 [1]	2 [1]
Omphalocele, fetuses [litters]		1 [1]	0	0	0
Cleft palate, fetuses [litters]		0	0	0	2 [1]

* Significantly different from control, $p < 0.05$

Source: Sato, 2014

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on decreased body weight gain and feed consumption at the LOAEL of 200 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was undetermined, based on increased skeletal variations and supernumerary ribs at the lowest dose tested of 50 mg/kg bw per day (Sato, 2014).

Rabbit

Study 1

In a preliminary developmental toxicity study, groups of eight female premated Japanese White rabbits were given afidopyropen (purity 95.74%) at a concentration of 0, 10, 30, 100 or 300 mg/kg bw per day in 1% sodium CMC from GDs 6–27. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and feed consumption were measured daily during treatment (GDs 7–28), and statistical analyses were conducted on GDs 0, 7, 10, 13, 16, 20, 24 and 28. All surviving animals were subjected to gross necropsy and uterine staining. Fetuses were counted, weighed and external examinations carried out.

Among the maternal animals at 100 mg/kg bw per day, five of eight animals aborted and there was one premature delivery. At 300 mg/kg bw per day six of eight does died or were killed in extremis between GDs 10 and 13. The one surviving doe from each group had a completely absorbed litter. Clinical signs of toxicity at 100 mg/kg bw per day consisted of soiled fur, while clinical signs at 300 mg/kg bw per day consisted of prone or lateral position. Body weight and body weight gain were decreased at 100 mg/kg bw per day and above. There was a significant decrease in body weight gain in all treated groups, however there was a lack of dose–response at the two lowest doses and the treatment-related nature of the change was unknown. Likewise, feed consumption was decreased in all treated groups from GD 12–24 at 10 mg/kg bw per day, GD 12–27 at 30 mg/kg bw per day, and throughout treatment at 100 mg/kg bw per day and above. The change was considered treatment-related and adverse at 100 mg/kg bw per day and above due to the effect on body weight. Postimplantation loss was increased at 30 mg/kg bw per day.

In the fetuses, there was a slight increase in deaths on a fetal basis and an increase in postimplantation loss at 30 mg/kg bw per day. At 100 mg/kg bw per day only one litter was available for examination with the remaining lost to abortions, premature delivery and total fetal resorption. In the remaining litter, fetal body weights were decreased. There were no live litters produced in the 300 mg/kg bw per day group. Due to the limited examinations, no NOAEL was derived (Rojo, 2009).

Study 2

In a developmental toxicity study, groups of 25 female premated Japanese White rabbits were given afidopyropen (purity 95.74%) at a concentration of 0, 8, 16 or 32 mg/kg bw per day in 1% sodium CMC from GDs 6–27. Animals were monitored twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and feed consumption were measured daily during treatment (GDs 7–28), and statistical analyses were conducted on GDs 0, 7, 10, 13, 16, 20, 24 and 28. All surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex, and external and visceral malformations. Half of each group was stained and examined for craniofacial malformations, and all fetuses were stained and examined for skeletal malformations.

In the maternal animals there were no effects on food consumption or body weight. There was an alteration of the sex ratio towards males at 16 mg/kg bw per day and above. At 32 mg/kg bw per day, there was an increase in early resorptions, total litter resorptions and postimplantation loss (Table 26). There were no treatment-related increases in the incidences of malformations or variations.

Table 26. Caesarian findings in developmental toxicity study in rabbits

Observation	Dose (mg/kg bw per day)				Historical control data: Mean (range)
	0	8	16	32	
Total number of resorptions[a] [including litters with total resorptions]	18 [21]	14 [16]	7 [13]	16 [34]	
Early [including litters with total resorptions]	14 [17]	11 [13]	3 [9]	10 [28]	
Late	4	3	4	6	
Litters with total resorptions[a]	1	1	1	3g	
Overall postimplantation loss (%) [including litters with total resorptions]	9.2 [10.4]	8.0 [8.8]	6.5 [9.0]	14.2 [21.6]	
Number of litters examined	24	23	23	22	–
Litter size	8.3	9.0	8.7	7.4	
Sex ratio; overall	0.475	0.488	0.542	0.546	0.489 (0.444–0.545)
Sex ratio; mean[b]	0.482 ± 0.187	0.493 ± 0.190	0.550 ± 0.123	0.584 ± 0.198	0.489 (0.444–0.545)
Number of litters with ratio of males ≥ 0.6 Percentage of litters with ≥ 0.6 males)	4 17%	6 26%	10 43%	12 55%	–

[a] The study report indicated that no caesarean data were available for litters with total resorptions since "the animals had 10% ammonium sulphide-stained implantations sites but had no grossly observable conceptus."

[b] Proportion of males on a per litter basis. Historical controls also calculated on a per litter basis, however, change in calculation did not result in a change in values.

Source: Fujii, 2013b

In the fetuses there was an alteration of the sex ratio towards males at 16 mg/kg bw per day and above. In addition to the increased resorptions and postimplantation loss noted in the does, there was a decrease in the number of live fetuses and an increase in the number of dead fetuses at 32 mg/kg bw per day.

The maternal NOAEL was 16 mg/kg bw per day, based on increased early and late resorptions and postimplantation losses at the LOAEL of 32 mg/kg bw per day.

The embryo/fetal NOAEL was 8 mg/kg bw per day, based on altered sex ratios at the LOAEL of 16 mg/kg bw per day (Hojo, 2011).

2.6 Special studies

(a) Neurotoxicity

Study 1

In an acute neurotoxicity study, groups of ten Wistar [Crl:WI(Han)] rats were given afidopyropen (purity 94.54%) as a gavage dose at a concentration of 0 (1% CMC), 200, 700 or 2000 mg/kg bw. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weights measurements; FOB and locomotor evaluations were conducted on days −7, 0, 7 and 14. At study termination, the first five animals per sex from each group were killed and subjected to gross and histopathological examinations of the brain and peripheral nervous system.

There were no effects on mortality, gross necropsy or the histopathological examination. There were various clinical signs of toxicity (altered gait, piloerection) seen on day 0 in individual animals at 700 mg/kg bw per day, however as there was a lack of consistency, the treatment-related nature of the change was unknown. At 2000 mg/kg bw per day there was a significant decrease in motor activity in males and females and slight tremors and hypothermia in females, all at day 0.

The NOAEL was 700 mg/kg bw based on decreased motor activity in males and females and slight tremors and hypothermia in females at the LOAEL of 2000 mg/kw bw. These effects were considered secondary to generalized systemic toxicity and the NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested (Buesen et al., 2012).

Study 2

In a 90-day neurotoxicity study, groups of ten Crl:WI(Han) rats were given afidopyropen (purity 94.54%) in the diet at concentrations of 0, 300, 1000 or 4000 ppm (equal to 0, 20, 73 and 396 mg/kg bw per day in males, 0, 24, 92 and 438 mg/kg bw per day in females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Body weight and feed consumption were measured prior to treatment, on day 0 and weekly thereafter. FOB and locomotor evaluations were performed on days −7, 0, 22, 50 and 85. At study termination the first five animals per sex from each group were killed and subjected to gross and histopathological examinations of the brain and peripheral nervous system.

There were no effects on mortality, FOB parameters, clinical signs of toxicity, gross or histopathology. Body weight and body weight gain were decreased in males at 4000 ppm. Feed consumption values were compromised due to excessive food spillage at 4000 ppm in males and females. As such, compound consumption is likely overestimated at the high dose.

The NOAEL was 1000 ppm (equal to 73 mg/kg bw per day) based on decreased body weight and body weight gain in males at the LOAEL of 4000 ppm (equal to 396 mg/kg bw per day). The NOAEL for subchronic neurotoxicity was 4000 ppm (equal to 396 mg/kg bw per day), the highest dose tested (Flick et al. 2016d).

(b) Immunotoxicity

In a 28-day immunotoxicity study, groups of ten female Wistar [Crl:WI(Han)] rats were given afidopyropen (purity 94.54%) in the diet at concentrations of 0, 300, 1000 or 4000 ppm (equivalent to 0, 25, 69 and 278 mg/kg bw per day). Compound intake was estimated for the 1000 and 4000 ppm dose groups due to excessive food spillage. An additional group of ten female Wistar rats was administered cyclophosphamide monohydrate in water via daily oral gavage at a dose level of 4.5 mg/kg bw/day for 28 days as a positive control. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. A detailed clinical examination was performed prior to study

initiation and weekly thereafter. Body weight and feed consumption were measured prior to treatment, on day 0 and weekly thereafter. Six days prior to euthanasia (day 23), all rats were immunized with 0.5 mL of sheep red blood cells (sRBCs; 4×10^8 cells/mL) via intraperitonial (i.p.) injection. On day 29 blood samples were obtained from all animals to measure a primary T-cell antibody response using an anti-sRBC IgM enzyme-linked immunosorbent assay (ELISA) for evaluation of immunotoxicity. The rats were then weighed, euthanized, necropsied, and the liver, spleen, and thymus dissected and weighed. Histopathological examinations were not performed.

There were no effects on mortality or clinical signs of toxicity. Overall body weight gain was decreased at 4000 ppm, however there was no effect on body weight. Calculations of feed consumption were compromised due to food spillage indicating a palability issue at 1000 ppm and above. Relative liver and thymus weights were increased at 4000 ppm. The changes were considered treatment-related in the absence of histopathological examination. There were no effects noted in the immunotoxicity assay; however, animals were immunized with SRBCs six days prior to study termination rather than at the guideline timepoint of four days.

The NOAEL for systemic toxicity was 1000 ppm (equal to 69 mg/kg bw per day) based on decreased body weight gain and increased liver and thymus weights at the LOAEL of 4000 ppm (equal to 278 mg/kg bw per day).

The NOAEL for immunotoxicity was 4000 ppm (equal to 278 mg/kg bw per day), the highest dose tested (Flick et al., 2016e).

(c) Studies on metabolites

The acute toxicity and genotoxicity of the metabolite M440I007 (the dimer, found in plants) and the acute toxicity of cyclopropanecarboxylic acid (CPCA, a major rat metabolite – present in plants) are summarized in Tables 27 and 28.

Table 27. Acute toxicity study on metabolites of afidopyropen

Test substance	Species	Strain	Sex	Route	Purity (%)	Result	Reference
M440I007	Rat	Wistar	Female	Oral	98.52	$LD_{50} > 2000$ mg/kg bw	Fukuyama (2012)
CPCA	Rat	Wistar	Female	Oral	99.2	$300 < LD_{50} < 500$ mg/kg bw	Hoeger (2017)

Table 28. Genotoxicity study on a metabolite of afidopyropen

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro						
M440I007	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0–5000 µg/plate (± S9)	98.52	Negative	Matsumoto, 2012
M440I007	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0–5200 µg/plate (± S9)	97.3	Negative	Woitkowiak, 2014
M440I007	In vitro forward mutation assay	Mouse lymphoma L5178Y cells	0–300 µg/mL	97.3	Negative	Schulz & Landsiedel, 2015
M440I007	In vitro micronucleus test	Human lymphocytes	4.0–2055 µg/mL ± S9 4.0–2055 µg/mL − S9 8.0–257 µg/mL + S9	97.3	Negative	Bohnenberger, 2015
M440I007	In vivo micronucleus assay	Mouse (NMRI)	0, 500, 1000 and 2000 mg/kg bw	97.3	Negative	Schulz, Mellert & Grauert, 2015

S9 9000 × *g* supernatant fraction from rat liver homogenate

Repeat dose toxicity: M440I007

In a 90-day toxicity study, groups of ten Crl:WI(Han) rats received M440I007 (purity 97.3%) in the diet at a concentration of 0, 600, 4000 or 10 000 ppm (equal to 0, 42, 277 and 708 mg/kg bw per day for males, 0, 47, 317 and 797 mg/kg bw per day for females). Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. Detailed clinical observations were performed weekly. An FOB was performed prior to study termination. Body weight, body weight gain and feed consumption were measured weekly. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues from control and high-dose groups examined histopathologically, as well as all tissues with gross lesions.

There were no effects on mortality, clinical signs of toxicity, FOB parameters, body weight, feed consumption or ophthalmoscopic examination. There were no effects on haematological or urinalysis parameters and no changes noted at gross necropsy.

Among the clinical chemistry parameters, bilirubin values were increased at 10 000 ppm in females along with an increase in haematopoiesis of the spleen.

Thymus weights were increased in males and females at 10 000 ppm.

There was an increase in necrosis/fibrosis of the heart in males at 10 000 ppm. As this was consistent with short and long-term studies on afidopyropen, the study was considered supplemental as no histopathological examination was performed at 600 and 4000 ppm (Flick et al., 2016f). The available data indicate M440I007 is likely to be of similar toxicity to afidopyropen.

Repeat dose toxicity: cyclopropane carboxylic acid (CPCA)

In a 90-day toxicity study, groups of 10 Crl:CD(SD) Sprague Dawley rats received CPCA (purity 99.7%) by gavage at a concentration of 0, 2, 10, 30 or 60 mg/kg bw. Animals were inspected twice daily for mortality and moribundity, and daily for clinical signs of toxicity. FOB and motor activity assessments were performed prior to study initiation and prior to study termination. Ophthalmoscopic examinations were performed before the start of treatment and at the end of the study. Haematology and clinical chemistry parameters were analysed and urinalysis was performed from samples taken prior to terminal kill. Gross examinations were performed on all animals, including those found dead, killed moribund or killed at the end of the study. Organs were weighed and tissues examined histopathologically in control and high-dose groups, as well as in all tissues with gross lesions and suspected target organs (heart, liver, thymus and pancreas) and female reproductive organs (ovaries, uterus, cervix and vagina) in all dose groups.

There were no treatment-related effects on mortality, clinical signs of toxicity, ophthalmoscopic evaluation or urinalysis. Body weights were slightly increased in females at 60 mg/kg bw per day, however the change was not considered to be adverse.

In the haematopoietic and circulatory systems, monocytes were increased in males and females and neutrophils were increased in males at 60 mg/kg bw per day. At 30 mg/kg bw per day and above in males and females there was an increase in the incidence and severity of cardiomyopathy, characterized by one or more areas of myocyte degeneration/necrosis with a mononuclear inflammatory cell infiltrate. Myocardial vacuolation was increased at the mid dose and above in females and at the high dose in males.

In the liver, globulin was decreased in males at 30 mg/kg bw per day and above. In females, total bile acids, BUN and inorganic phosphorus were increased, and cholesterol was decreased at the mid dose. Total bile acids and triglycerides were increased at the high dose in males. Liver weights were increased at the mid dose in females and at the high dose in males. At gross necropsy, there was an increase in discoloured livers at the mid dose and above in females and at the high dose in males. Histopathologically, periportal fatty change in the liver was increased at the mid dose and high dose in females and males, respectively. Mononuclear cell infiltrate of the liver was noted in females at the mid dose and above.

In the pancreas, there was a decrease in zymogen within the pancreatic acinar cells in males and females at the mid dose and above.

Kidney weights were increased in females at 30 mg/kg bw per day and above, and at 60 mg/kg bw per day in males.

In the thymus, thymphoid necrosis was increased in females at 30 mg/kg bw per day and above, and at 60 mg/kg bw per day in males.

The NOAEL was 10 mg/kg bw per day based on increased effects on the myocardium and decreased zymogen within acinar cells of the pancreas in males and females, decreased globulin in males, and increased bile acids, BUN and inorganic phosphorus along with decreased cholesterol, increased liver and kidney weights, increased discolouration of the liver and increased myocardial vacuolation, periportal fatty change and mononuclear cell infiltrate in the liver and lymphoid necrosis of the thymus in females at the LOAEL of 30 mg/kg bw per day (Carpenter, 2012).

(d) Mode of action/investigative studies

A range of studies with afidopyropen were performed to investigate a potential mode of action (MOA) for the uterine tumours seen in female rats.

In a 14-day mode of action (MOA) study, groups of five female Fischer F344 rats received afidopyropen (purity 94.54%) in diet at a concentration of 0 or 3000 ppm (equal to 0 and 197 mg/kg bw per day). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Body weights and feed consumption were measured on days 1, 5, 8 and 16. Following study termination, livers and uteri were excised, weighed and prepared for CYP1A1 and CYP1B1 gene expression analysis. Microsomes were isolated from a second liver sample then prepared for enzyme activity assays and protein determinations. Blood samples were taken for possible future use.

There were no clinical signs of toxicity reported and no mortality observed during the treatment period. There was an initial body weight loss until day 5 and body weight gain was decreased overall. Overall feed consumption was decreased.

Liver weights were increased and uterine weights decreased. Hepatic ethoxyresorufin-*O*-deethylase (EROD) activity and estradiol-2-hydroxylation were both increased in the treated group. Estradiol-4-hydroxylation levels were below the level of detection (Augello, 2017 ammendment to the report).

There was a statistically significant increase in hepatic CYP1A1 and a non-statistically significant increase in hepatic CYP1B1. While uterine CYP1A1 was increased, uterine CYP1B1 was slightly and non-statistically significantly decreased. (Elcombe, 2015)

In a 28-day MOA study, groups of 20 female Fischer F344 rats received afidopyropen (purity 93.23%) in the diet at a concentration of 0, 300, 1000 or 3000 ppm (equal to 0, 18, 81 and 368 mg/kg bw per day). Additionally, 20 females serving as positive controls were administered the dopamine agonist bromocriptine (10 mg/kg bw per day) via gavage. To stimulate the release of endogenous prolactin from the pituitary, all animals received metoclopramide (500 µg/kg bw) via i.p. injection on day 28, including a separate group of five females given untreated diet and sampled at 0.5, 1 and 2 h to determine the time point of maximum plasma concentration of prolactin after i.p. injection of metoclopramide. Animals were inspected daily for clinical signs of toxicity and twice daily for mortality. Body weight and feed consumption were measured weekly including day 0. The estrus cycle was evaluated on study days −8 and −7 and daily from study day 0 onwards. Blood was sampled for prolactin measurements based on estrus cycle, and also on days 24 and 28, . Following study termination all animals were subject to gross pathological examination and liver, ovaries, uterus, brain, pituitary and adrenal glands weighed. Histopathological examination was performed on the liver, ovaries, uterus, cervix, oviducts, vagina, brain, pituitary, adrenal and mammary glands, and on all gross lesions and masses of the negative control and afidopyropen-treated groups. The positive control group was not subject to histopathological examination.

There were no clinical signs of toxicity and no gross lesions at necropsy. Body weight gains and feed consumption were increased at doses at, and above, 1000 ppm.

At 3000 ppm, the number of estrus cycles was decreased and the length of the cycle was increased. In the positive control animals the number of cycles was increased and the length of the cycle decreased. However, the negative control animals also exhibited a longer than standard cycle, indicating a possible reaction to isoflurane anaethesia.

On day 24, prior to stimulation, the majority of animals in all groups were in metestrus. Prolactin values were decreased to a nonstatistically significant extent at 1000 ppm and above in animals in diestrus and at 1000 ppm and above in animals in metestrus. In the positive control animals prolactin values were decreased in the animals in estrus ($n = 2$) and metestrus ($n = 18$). In samples specifically taken from animals in proestrus, there were nonstatistically significant decreases in prolactin values at the mid-dose and above during days 0–4 and in the high-dose animals during days 16–22. In the positive control group, prolactin values were decreased over both time periods. Prolactin values in animals in estrus were only available in the control, 300 ppm and positive control groups. Decreases only occurred in the positive control group.

It was only possible to compare the difference between stimulated and basal prolactin values of animals in estrus (0, 300, 1000 ppm and positive controls) and metestrus (all groups) due to the lack of animals in diestrus and proetrus. For animals in estrus, the positive control group had decreased prolactin values. In animals in metestrus, prolactin values were decreased at 1000 ppm and above and in the positive control group.

In the positive control group, ovarian and uterine weights were increased and pituitary weights were decreased (10%). At 3000 ppm, terminal body and liver weights were increased slightly and adrenal gland weights were decreased. As in the positive control, pituitary weights were decreased (26%), however, ovarian and uterine weights were decreased (44 and 60% respectively). Changes at 1000 ppm were limited to decreased relative pituitary weights (8%).

Gross necropsy changes in the 3000 ppm group consisted of atrophy of the ovaries, uterus, cervix and vagina and increased mucification of the vagina in all animals. Changes in the 1000 ppm group were only slightly increased compared to controls and were of equivocal significance.

Under the conditions tested, exposure to afidopyropen at ≥ 81 mg/kg bw/day (1000 ppm) appeared to cause less of an increase in prolactin levels following stimulation with metoclopramide in animals that are in metestrus, when compared to the untreated control (Flick et al. 2016g).

In an estrogen receptor transcriptional activation assay, stably transfected human estrogen cell receptor(hERα)-HeLa-9903 cells were exposed to afidopyropen (purity 94.54%) at seven log serial concentrations between 10^{-12} M and 10^{-5} M in DMSO (final concentration of 0.1% v/v; insoluble concentration) for 20 to 24 h. The experiments were performed using 96-well plates and all concentrations were tested in replicates of six/plate. Cells were exposed to the test agent to induce reporter (luciferase) gene products. Luciferase expression in response to activation of the estrogen receptor by afidopyropen was measured upon addition of a luciferase substrate and detection with a luminometer with acceptable sensitivity.

The maximum soluble concentrations of afidopyropen induced a maximum level of response (RPC_{max}) of less than 10% of the positive control, therefore afidopyropen was considered negative for estrogen receptor transcriptional activation by the study's author. (Willoughby, 2015a)

In an estrogen receptor binding assay, uterine cytosol from Sprague Dawley rats was exposed to concentrations of afidopyropen (purity 94.54%) and two of its metabolites (M440I002; a plant, animal, soil and water metabolite and a urine, bile and faecal metabolite in rats; purity 92.5%) and M440I001 (a plant, animal, soil and water metabolite and a urine, bile and faecal metabolite in rats; purity 93.9%) ranging from 10^{-10} M to 10^{-3} M in 4% DMSO, in serial dilutions. A saturation binding experiment was conducted to confirm that the cytosol was appropriate for the main test. A competitive binding experiment was conducted to measure the binding of a single concentration of [^{3}H]-17β-estradiol (1 MnM) in the presence of increasing concentrations of test substance. The assay included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17-β-estradiol as the natural ligand reference material.

The saturation binding experiment demonstrated that the estrogen receptor was present in reasonable concentrations in the rat uterine cytosol and was functioning with appropriate affinity for the reference ligand 17β-estradiol.

The main assay was performed as three independent runs. Afidopyropen was equivocal in two runs and non-interacting in one run. M440I002 and M440I001 were non-interacting in all three runs. The positive controls validated the test system (Willoughby, 2015b).

In a series of assays, afidopyropen (purity 94.56%) and metabolites, M440I001 (purity 93.9%), M440I002 (purity 92.5%), M440I003 (a plant, animal, soil and water metabolite and and a urine, bile and faecal metabolite in rats; purity 98.6%) and M440II017 (a plant and animal metabolite and and a urine and bile metabolite in rats; purity 93.7%), were subject to various in vitro assays to investigate effects on the human dopamine transporter (human recombinant CHO cells), dopamine D_1(h) agonist and antagonist activity (human recombinant CHO cells), dopamine uptake (rat striatum synaptosomes), and tissue bioassays with the D_1 receptor agonist and antagonist activity (human recombinant CHO cells rabbit splenic artery) and the D_2 receptor agonist and antagonist activity (field-stimulated rabbit ear artery).

In a binding assay, there was no indication that any of the test compounds inhibited the dopamine transporter.

In cellular and nuclear receptor functional assays, none of the five compounds had an agonistic or antagonistic effect on the dopamine D_1 receptor.

In an enzyme uptake assay, the test substances did not inhibit dopamine uptake.

In the rabbit splenic artery tissue bioassay, the five test compounds did showed neither an agonist nor antagonist effect on the dopamine D_1 receptor.

In the rabbit ear artery tissue bioassays, afidopyropen and M440I002 induced a concentration-dependent decrease in the twitch contraction amplitude that was not blocked with sulpiride (a dopamine antagonist). These responses were higher than 50%, suggesting that afidopyropen and M440I002 may have an agonist-like effect on the D_2 receptor in this tissue. Twitch contraction amplitude was also decreased with M440I017; however, the responses did not reach 50% of the control response and it was not considered indicative of an agonist-like effect.

Afidopyropen and M440I002 were considered possible agonists of the D_2 receptor (Jolas, 2015a).

In a subsequent tissue bioassay, afidopyropen (purity 94.56%) and M440I002 (purity 92.5%) were assessed for the dopamine D_2 receptor inhibition. The compounds were tested a concentration of 10^{-5}–10^{-7} M. The assay was performed using rabbit ear arteries to assess inhibition of twitch response, and the test compounds were used in the presence and absence of sulpiride (dopamine antagonist; 3 μM). Quinpirole (dopamine agonist; 0.3 μM) was used to verify responsiveness and to obtain a control response.

After a 20-minute pretreatment with solvent, afidopyropen and M440I002 induced a concentration-dependent decrease in the twitch contraction amplitude that was not blocked with further addition of sulpiride. After a 20-minute pretreatment with sulpiride, afidopyropen and M440I002 induced a concentration-dependent decrease in the twitch contraction amplitude that was right-shifted when compared to their effect after solvent pretreatment. These results indicate that afidopyropen and M440I002 may have agonist-like effects on the dopamine D_2 receptor. (Jolas, 2015b).

In assays with the short and long form of the dopamine receptor, $D2_S$(h) and D_{2L}(h), afidopyropen (purity 94.56%) and M440I002 (purity 92.5%) were tested to investigate radioligand binding. Compound binding was calculated as a % of inhibition of the binding of a radioactively labelled ligand specific for each target. Test compound concentrations were between 10^{-4} and 10^{-11} M.

Afidopyropen inhibited the D_{2S} receptor between −2.0 and −20.2%, and the D_{2L} receptor between −0.6 and −11.0%. Metabolite M440I002 was found to inhibit the D_{2S} receptor between 0.7 and −17.0%, and the D_{2L} receptor between 12.8 and −10.5%. Since these numbers were all below 50%, the test compounds were not determined to show inhibition of the dopamine D_{2S} and the D_{2L} receptors (Jolas, 2015c).

The molecular structures of afidopyropen and its metabolites M440I001, M440I002, M440I003 and M440I017 were submitted to in silico activity prediction models (QSAR) of the human dopamine D_2 receptor and dopamine transporter. The QSAR models predicted the strength of inhibition based on the structural similarity of the input molecules to those with a reported measured inhibition value (Van Cott & Fabien, 2018).

High inhibition values were predicted regarding the dopamine receptor for these metabolites (except M440I017) and afidopyropen, although these predictions were labelled as borderline due to the high level of uncertainty. Predictions for M440I017 were out-of-domain for the dopamine D_2 receptor since this metabolite was less structurally similar to the other test compounds. Inhibition of the dopamine transporter seemed unlikely given the low activity (pIC_{50} predictions) for this target.

It is worth noting that the QSAR models refer to the in vitro activity at the target only, not taking into account toxicokinetic effects like distribution or metabolism of the compounds in vivo. Additionally, no information was provided in regards to the input molecules (Wolf, 2016).

Overall, afidopyropen, M440I002 and M440I017 exhibit some evidence of acting as weak agonists on the dopamine D_2 receptor.

Studies on prolactin levels in rats (described in the repeat-dose toxicity section) identified a nonlinear response to afidopyropen at tumourigenic doses.

Overall, the MOA data do not adequately address all aspects of the production of uterine tumours in rats, and human relevance cannot be wholly excluded.

The Meeting concluded that the existing database on afidopyropen was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Comments

Biochemical aspects

In ADME studies conducted in rats using afidopyropen labelled with ^{14}C at the nicotinic acid, ^{13}C at the pyranone-4 and pyranone-6 positions, and ^{14}C at the pyranone-6 position, the absorption of afidopyropen was rapid (T_{max} < 1–4 h), approximately 70% based on urine and bile and radioactivity was widely distributed to tissues. Area under the concentration–time curve for the interval 0–168 h ($AUC_{0–168}$) values indicated a saturation of the elimination pathways with high-dose (300 mg/kg bw) values more than 300 times those of the low-dose (3 mg/kg bw) groups (McClanahan, 2015). Elimination half-life ($T_{½}$) was rapid at 2–5 h at low doses (3 mg/kg bw) but 6–12 hours at high doses (300 mg/kg bw) (Fabian & Landsiedel, 2015).

Radioactivity was readily excreted within 96–120 h with the majority of radioactivity (up to 86% of administered dose) excreted via the faeces and lower amounts (up to 21% of administered dose) excreted via the urine (Glaessgen & Thiaener, 2011). Biliary excretion is the predominant route of elimination for afidopyropen, accounting for approximately half of the radioactivity in faeces based on the findings from studies with bile duct-cannulated rats (Ohyama, 2015; Glaessgen & Thiaener, 2011). Generally, levels of radioactivity in urine increased with dose, whereas those in bile and faeces decreased with dose, indicating saturation of excretion. Bioavailability was not significantly different between the sexes and was not impacted by the dose level administered (Ohyama, 2015; Fabian & Landsiedel, 2015; Glaessgen & Thiaener, 2011).

There were no changes following repeat-dose treatment in absorption, distribution and excretion studies (Capello, 2016).

Highest levels of radioactivity were observed in the GI tract, liver, adrenal glands, ovaries and kidney, at one hour and four hours following low- and high-dose administration, respectively. Very low levels of radioactivity were observed in the ovaries, testes and brain 36 h following administration of high-doses. No sex differences were noted in the above parameters (Fabian & Landsiedel, 2015).

Afidopyropen was extensively metabolized in the rat with no significant sex differences identified. Most metabolites were structurally similar to the parent compound, with changes in one or two functional groups, and in some cases, loss of one or two cyclopropane carboxylic acid (CPCA) ester moieties. Following single or repeat dosing with low or high levels of ^{14}C-radiolabelled test material a range of metabolites were identified in the urine, faeces and bile, including M440I001 (urine, 2–11%; faeces, 3–23%; bile 2–4%) and M440I002 (urine, 0.4–5%; faeces, 2–10%) and bile (0.1–0.4%). Significant levels of unchanged afidopyropen were detected only in the faeces; 21–37% of the AD in single low-dose assays and 5–21% of AD in single and repeat high-dose assays. The proposed metabolic pathway involves hydrolytic loss of one or both CPCA moieties, *N*-oxidation at the pyridine ring, hydroxylation of one of the methyl groups, and conjugation of hydroxyl groups of the metabolites (Glaessgen & Thiaener, 2011).

Toxicological data

In rats, afidopyropen had an acute oral LD_{50} greater than 2000 mg/kg bw, an acute dermal LD_{50} of greater than 2000 mg/kg bw and an acute inhalation median LC_{50} greater than 5.48 mg/L (Fukuyama, 2009a, b, 2010). Afidopyropen was non-irritating to the skin and transiently irritating to the eyes of rabbits (Ueda, 2009a, b). It was not a dermal sensitizer in guinea pigs in a maximization test (Ueda, 2009c).

The main toxic effects of afidopyropen observed in short- and long-term studies were changes to the liver in mice, liver, heart and female reproductive system in rats, and the brain and kidney in dogs.

In a 90-day study, mice received a dietary concentration of afidopyropen of 0, 150, 500, 2000 or 6000 ppm (equal to 0, 21, 69, 285 and 819 mg/kg bw per day for males, 0, 25, 83, 327 and 919 mg/kg bw per day for females). The NOAEL was 500 ppm (equal to 69 mg/kg bw per day), based on increased blood bilirubin in males and females and increased blood triglycerides and spleen weights in females at the LOAEL of 2000 ppm (equal to 285 mg/kg bw per day) (Takahashi, 2010).

In a 90-day toxicity study, rats received a dietary concentration of afidopyropen of 0, 150, 300, 1000 or 3000 ppm (equal to 0, 8.9, 18, 61 and 182 mg/kg bw per day for males, 0, 10.2, 20, 68 and 197 mg/kg bw per day for females). The NOAEL was 300 ppm (equal to 18 mg/kg bw per day), based on increased relative liver weights in females, increased urobilinogen in males and increased blood urea, nitrogen and potassium, and increased vacuolar change (fatty change) of the liver and myocardium in females at the LOAEL of 1000 ppm (equal to 61 mg/kg bw per day) (Yamashita, 2010).

In a 90-day toxicity study, dogs received afidopyropen in capsules at a dose of 0, 15, 30 or 90→60 mg/kg bw per day. The high dose was reduced from 90 to 60 mg/kg bw per day and suspended at various points due to excessive toxicity. At 90→60 mg/kg bw per day, there was an increase in vomiting within the first week of treatment. At 30 mg/kg bw per day, increased vomiting occurred at week 3. The NOAEL was 15 mg/kg bw per day based on increased vomiting and hyaline droplet deposition in the hepatocytes of males and females and increased haematuria in males at the LOAEL of 30 mg/kg bw per day (Yoshida, 2010).

In a one-year toxicity study, dogs received afidopyropen in capsules at a dose of 0, 8, 20 or 50→40 mg/kg bw per day. The high dose was reduced from 50 to 40 mg/kg bw per day and suspended at various points due to excessive toxicity. The NOAEL was 8 mg/kg bw per day, based on decreased neutrophils and hyaline droplet deposition of the hepatocytes in males and females, vacuolation of the white matter and neuropil of the cerebrum in males at the LOAEL of 20 mg/kg bw per day (Yoshida, 2011).

In an 18-month carcinogenicity study, mice received a dietary concentration of afidopyropen of 0, 120, 700 or 4000 ppm for males (equal to 0, 13, 79 and 445 mg/kg bw per day) and 0, 120, 700 or 4000→3000→2000 mg ppm for females (equal to 0, 13, 76 and 333 mg/kg bw per day for females). In females, the dose was changed to 3000 ppm at week 24 and then to 2000 ppm at week 44 due to death or moribundity. The NOAEL was 700 ppm (equal to 76 mg/kg bw per day), based on clinical signs of toxicity culminating in mortality, decreased body weight, decreased feed consumption, increased WBC counts, increased spleen and ovarian weights, increased pale coloured livers, decreased haematopoieisis in the bone marrow, atrophy of the spleen, myocardial fibrosis, apoptosis of lymphocytes of the thymus and lymph nodes and vacuolation of various tissues at the LOAEL of 2000 ppm (equal to 333 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 333 mg/kg bw per day) in females and 4000 ppm (equal to 445 mg/kg bw per day) in males, the highest doses tested (Takahashi, 2012).

Two 1-year chronic toxicity studies and two 2-year oncogenicity studies were submitted on rats.

In a one-year toxicity study, rats received a dietary concentration of afidopyropen of 0, 75, 150, 300 or 1000 ppm (equal to 0, 3.7, 7.3, 15 and 48 mg/kg bw per day in males, 0, 4.4, 8.9, 18 and 56 mg/kg bw per day in females). The NOAEL was 300 ppm (equal to 15 mg/kg bw per day) based on increased platelets and decreased triglycerides in males and females and increased vacuolar change in the liver and myocardium of females at the LOAEL of 1000 ppm (equal to 48 mg/kg bw per day) (Yamashita, 2011).

A second study was performed to investigate the effects at higher doses. The findings at 3000 ppm were consistent with those of the high dose in the main study.

In a two-year toxicity study, rats received a dietary concentration of afidopyropen of 0, 100, 300 or 1000 ppm (equal to 0, 4.4, 13, and 43 mg/kg bw per day in males, 0, 5.3, 16 and 51 mg/kg bw per day in females). The NOAEL for chronic toxicity was 300 ppm (equal to 13 mg/kg bw per day) based on increased kidney, liver and adrenal weights in males and increased uterine weights and hyperplasia of the bile duct of the liver at the LOAEL of 1000 ppm (equal to 43 mg/kg bw per day). The NOAEL for carcinogenicity was 300 ppm (equal to 16 mg/kg bw per day) based on an increased incidence of uterine adenocarcinomas in females at the LOAEL of 1000 ppm (equal to 51 mg/kg bw per day) (Yamashita, 2014).

A second study was performed to investigate the effects at higher doses. The findings at 3000 ppm were consistent with those at the high dose of the main study.

A number of studies were performed in order to investigate a proposed dopamine agonist MOA for uterine tumours in rats. Inadequacies in the measurement of endpoints meant the MOA study failed to give support to all of the key events described.

The Meeting concluded that afidopyropen is carcinogenic in female rats, but not in mice or male rats. Based on the weight of evidence, the Meeting was unable to establish an MOA for the tumours and, therefore, human relevance could not be ruled out.

Afidopyropen was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that afidopyropen is unlikely to be genotoxic.

As afidopyropen is unlikely to be genotoxic and the uterine tumours in rats occurred by a mechanism that will exhibit a threshold, the Meeting concluded that afidopyropen is unlikely to pose a carcinogenic risk to humans via the diet.

Multiple reproductive toxicity studies were submitted. Following a range-finding one-generation study, a two-generation main study was performed. A subsequent one-generation study was performed to compare toxicity resulting from different purity levels, and a cross-fostering study performed to determine whether pup mortality in the first two-generation study was related to maternal care. A final two-generation study was performed with slightly higher dose levels and a higher purity of the active ingredient.

In a two-generation reproductive toxicity study, rats received a dietary concentration of afidopyropen (purity 95.74%) of 0, 100, 300 or 1000 ppm (equal to 0, 7.7, 22 and 75 mg/kg bw per day in males, 0, 9.0, 27 and 85 mg/kg bw per day in females). The NOAEL for parental toxicity was 1000 ppm (equal to 75 mg/kg bw per day), the highest dose tested. The NOAEL for reproductive toxicity was 300 ppm (equal to 22 mg/kg bw per day), based on altered sex ratios at the LOAEL of 1000 ppm (75 mg/kg bw per day). The NOAEL for offspring toxicity was 300 ppm (equal to 27 mg/kg bw per day), based on increased pup deaths and decreased body weights at PND 21 in F1 and F2 offspring, decreased spleen weight in F1 males and females and delayed sexual maturation and decreased prostate weights in F1 males at the LOAEL of 1000 ppm (equal to 85 mg/kg bw per day) (Fujii, 2013c).

There were no effects on parental toxicity when the lower (94.54%) and higher (99.0%) purity batches of afidopyropen were compared. Prostate weights were decreased in both treatment groups, however, an equivocal decrease in sperm counts in the testes and skewing of the sex ratios towards males was noted in the lower purity batch only. In the offspring, body weight, body weight gain and spleen weights were decreased in both treatment groups. Pup deaths were increased only in the lower purity group.

In the cross-fostering study, pup death was determined to be related to in utero exposure, while reduced body weights were directly related to exposure whether in utero or through lactation.

In a two-generation reproductive toxicity study rats received a dietary concentration of afidopyropen (purity 97.2%) of 0, 100, 500 or 2000 ppm (equal to 0, 7.8, 39 and 150 mg/kg bw per day in males, 0, 8.4, 41 and 155 mg/kg bw per day in females). The NOAEL for parental toxicity was 100 ppm (equal to 8.4 mg/kg bw per day), decreased glucose in F0 females and increased cholesterol in F0 females at the LOAEL of 500 ppm (equal to 41 mg/kg bw per day). The NOAEL for reproductive toxicity was 500 ppm (equal to 39 mg/kg bw per day), based on improper nursing, altered sex ratios, decreased prostate weights and decreased ovarian weights in F0 and F1 generations, decreased sperm counts in F0 males and decreased implantation sites, mean number of pups per dam, mean litter size at PND 0, increased infiltration of the prostate gland and decreased uterus weights in the F1 generation adults at the LOAEL of 2000 ppm (equal to 150 mg/kg bw per day). The NOAEL for offspring toxicity was 100 ppm (equal to 8.4 mg/kg bw per day) based on decreased thymus weights in F1 females and F2 males and decreased spleen weights in F2 males and females, and delayed preputial separation at the LOAEL of 500 ppm (equal to 41 mg/kg bw per day) (Schneider et al., 2016b).

In a developmental toxicity study female rats received a gavage dose of afidopyropen at dose levels of 0, 10, 30 or 100 mg/kg bw per day from days six through 19 of gestation. The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on increased adrenal weights at the LOAEL of 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day, based on increased lumbar rib variations and altered sex ratios at the LOAEL of 100 mg/kg bw per day (Fujii, 2013b).

In a developmental toxicity study, female rats received a gavage dose of afidopyropen at dose levels of 0, 50, 100 or 200 mg/kg bw per day from days six through 19 of gestation. The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on decreased body weight gain and feed consumption at the LOAEL of 200 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was not identified, based on increased skeletal variations and supernumerary ribs at the lowest dose tested of 50 mg/kg bw per day (Sato, 2014).

In a developmental toxicity study, female rabbits received a gavage dose of afidopyropen at 0, 8, 16 or 32 mg/kg bw per day from GD 6–27. The maternal NOAEL was 16 mg/kg bw per day based on increased early and late resorptions and post-implantation losses at the LOAEL of 32 mg/kg bw per day. The embryo/fetal NOAEL was 8 mg/kg bw per day based on altered sex ratios at the LOAEL of 16 mg/kg bw per day (Hojo, 2011).

The Meeting concluded that afidopyropen was not teratogenic.

In an acute neurotoxicity study, rats were given gavage doses of afidopyropen at a concentration of 0, 200, 700 or 2000 mg/kg bw. The NOAEL was 700 mg/kg bw based on decreased motor activity in males and females and slight tremors and hypothermia in females at the LOAEL of 2000 mg/kw bw. These effects were considered secondary to generalized systemic toxicity and the NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested (Buesen et al., 2012).

In a subchronic neurotoxicity study rats received a dietary concentration of afidopyropen of 0, 300, 1000 or 4000 ppm (equal to 0, 20, 73 and 396 mg/kg bw per day in males, 0, 24, 92 and 438 mg/kg bw per day in females). The NOAEL was 1000 ppm (equal to 73 mg/kg bw per day) based on decreased body weight and body weight gain in males at the LOAEL of 4000 ppm (equal to 396 mg/kg bw per day). The NOAEL for subchronic neurotoxicity was 4000 ppm (equal to 396 mg/kg bw per day), the highest dose tested (Flick et al., 2016d).

The Meeting concluded that afidopyropen was not neurotoxic in rats, but produced brain vacuolation in dogs.

In an immunotoxicity study, rats were given a dietary concentration of afidopyropen at 0, 300, 1000 or 4000 ppm (equivalent to 0, 25, 69 and 278 mg/kg bw per day). The NOAEL for systemic toxicity was 1000 ppm (equal to 69 mg/kg bw per day) based on decreased body weight gain and increased liver and thymus weights at the LOAEL of 4000 ppm (equal to 278 mg/kg bw per day). No specific indications of immunotoxicity were noted, therefore, the NOAEL for immunotoxicity was 4000 ppm (equal to 278 mg/kg bw per day), the highest dose tested (Flick et al., 2016e).

The Meeting concluded that afidopyropen was not immunotoxic.

Toxicological data on metabolites and/or degradates

The acute oral LD_{50} of M440I007, a plant metabolite, was > 2000 mg/kg bw (Fukuyama, 2012) and it tested negative for in vitro reverse mutation (Matsumoto, 2012; Woitkowiak, 2014), forward mutation (Schulz & Landsiedel, 2015) and micronucleus assays (Bohnenberger, 2015) and in vivo micronucleus assay (Schulz, Mellert & Grauert, 2015). A 90-day oral toxicity study was performed with dietary concentrations of M440I007 of 0, 600, 4000 or 10 000 ppm (equal to 0, 42, 277 and 708 mg/kg bw per day for males, 0, 47, 317 and 797 mg/kg bw per day for females). Effects were seen in the myocardium at the high dose that were consistent with the short- and long-term studies of the parent compound, however, histopathological investigations at lower doses were not adequate to identify a NOAEL/LOAEL. The data indicate M440I007 is likely to be of similar toxicity to its parent.

The acute oral LD_{50} of CPCA, a rat and plant metabolite, is between 300 and 500 mg/kg bw. A 90-day oral toxicity study in the rat was performed with gavage doses of CPCA of 0, 2, 10, 30 or 60 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day based on increased effects on the myocardium and decreased zymogen within acinar cells of the pancreas in males and females, decreased globulin in males and increased bile acids, blood urea nitrogen and inorganic phosphorus and decreased cholesterol, increased liver and kidney weights, increased discolouration of the liver and increased myocardial vacuolation, periportal fatty change and mononuclear cell infiltrate in the liver and lymphoid necrosis of the thymus in females at the LOAEL of 30 mg/kg bw per day (Carpenter, 2012). Although CPCA was not detected in rat metabolism studies due to the position of the radiolabel, it is likely to be present at more than 10% of the administered dose, based on the metabolic pathway. Therefore CPCA, is considered of similar toxicity to the parent compound.

Microbiological data

No data for antimicrobial activity or impact of afidopyropen on the human gut microbiome are available.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted (BASF, 2018).

The Meeting concluded that the existing database on afidopyropen was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI for afidopyropen of 0–0.08 mg/kg bw, on the one-year study on dogs and the rabbit developmental study, both having NOAELs of 8 mg/kg bw per day. Findings at the LOAEL consisted of vacuolation of the white matter of the cerebrum at 16 mg/kg bw per day in dogs and altered sex ratio (more males) and increased resorptions at 16 mg/kg bw per day in rabbits. A safety factor of 100 was applied. There was a margin of 540 to the uterine tumours in rats.

The Meeting established, for afidopyropen, an ARfD for women of childbearing age of 0.2 mg/kg bw, on the basis of the NOAEL of 16 mg/kg bw per day from the rabbit developmental toxicity study for increased early resorptions at 32 mg/kg bw per day. A safety factor of 100 was applied.

The Meeting established an ARfD for afidopyropen, for the general population, of 0.3 mg/kg bw, on the basis of increased vomiting in the first days of treatment at 60 mg/kg bw per day in the 90-day dog toxicity study. A safety factor of 100 was applied.

Levels relevant to risk assessment of afidopyropen

Species	Study	Effect	NOAEL	LOAEL
Mouse	90-day oral toxicity study[a]	Toxicity	500 ppm, equal to 69 mg/kg bw per day	2000 ppm, equal to 285 mg/kg bw per day
	Eighteen-month study of carcinogenicity[a]	Toxicity	700 ppm, equal to 76 mg/kg bw per day	2000 ppm, equal to 333 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 333 mg/kg bw per day[e]	-
Rat	90-day oral toxicity study[a]	Toxicity	300 ppm equal to 18 mg/kg bw per day	1000 ppm equal to 61 mg/kg bw
	One-year study of toxicity[a]	Toxicity	300 ppm, equal to 15 mg/kg bw per day	1000 ppm, equal to 48 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	300 ppm, equal to 13 mg/kg bw per day	1000 ppm, equal to 43 mg/kg bw per day
		Carcinogenicity	300 ppm, equal to 13 mg/kg bw per day	1000 ppm, equal to 43 mg/kg bw per day
	Two-generation study of reproductive toxicity[a, b]	Reproductive toxicity	300 ppm, equal to 22 mg/kg bw per day	1000 ppm, equal to 75 mg/kg bw per day
		Parental toxicity	100 ppm, equal to 8.4 mg/kg bw per day	500 ppm, equal to 41 mg/kg bw per day
		Offspring toxicity	100 ppm, equal to 8.4 mg/kg bw per day[c]	500 ppm, equal to 41 mg/kg bw per day
	Developmental toxicity study[b c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	-	50 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	16 mg/kg bw per day	32 mg/kg bw per day
		Embryo and fetal toxicity	8 mg/kg bw per day	16 mg/kg bw per day
Dog	90-day study of toxicity[d]	Toxicity	15 mg/kg bw per day	30 mg/kg bw per day
	One-year study of toxicity[d]	Toxicity	8 mg/kg bw per day	20 mg/kg bw per day
Metabolite M440I007				
Rat	13-week study of toxicity[a]	Toxicity	-	10 000 ppm equal to 708 mg/kg bw[f]
Metabolite CPCA				
Rat	13-week study of toxicity[a]	Toxicity	10 mg/kg bw per day	30 mg/kg bw per day

a Dietary administration. b Two studies combined c Gavage administration d Capsule administration
e Highest dose tested. f Limited examination at lower dose levels.

Acceptable daily intake (ADI), applies to afidopyropen, M440I001, M440I007, M440I017 and CPCA, expressed as afidopyropen

0–0.08 mg/kg bw

Acute reference dose for women of childbearing age (ARfD), applies to afidopyropen, M440I001, M440I007, M440I017 and CPCA, expressed as afidopyropen

0.2 mg/kg bw

Acute reference dose for the general population (ARfD), applies to afidopyropen, M440I001, M440I007, M440I017 and CPCA, expressed as afidopyropen

0.3 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to afidopyropen

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} 1–4 h depending on dose (3–300 mg/kg bw); and oral absorption of 70% based on urine and bile (rats)
Dermal absorption	1–6%, depending on concentration (rat, 50 g/L product)
Distribution	Extensive; highest concentrations in liver, adrenals and ovaries
Potential for accumulation	Low
Rate and extent of excretion	Relatively rapid (80% within 48 h in rats, mainly in faeces)
Metabolism in animals	Major metabolic reactions: cleavage of cyclopropylcarboxylic acid, *N*-oxidation and hydroxylation (rat)
Toxicologically significant compounds in animals and plants	Afidopyropen, M440I001, M440I007, M440I017, CPCA
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.5 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Transiently irritating (resolved within 24 h)
Guinea pig, dermal sensitization	Not sensitizing (maximization)
Short-term studies of toxicity	
Target/critical effect	Vacuolation, fibrosis, necrosis of myocardium; decreased ovary and uterus weight (rat) vacuolation of the brain white matter (dog)
Lowest relevant oral NOAEL	8 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw, the highest dose tested.
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Uterine adenocarcinoma, increased liver, kidney, adrenal weight (rat)
Lowest relevant NOAEL	13 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in female rats, not carcinogenic in mice or male rats[a]
Genotoxicity	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Altered sex ratio (more males)
Lowest relevant parental NOAEL	8.4 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	8.4 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	22 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Altered sex ratio (more males) (rats and rabbits) increased resorptions (rabbit)
Lowest relevant maternal NOAEL	16 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	8 mg/kg bw per day (rabbit)

Neurotoxicity	
Target/critical effect	Not neurotoxic (rats); brain vacuolation (dog)
Lowest relevant oral NOAEL	8 mg/kg bw (1-year dog)
Developmental neurotoxicity	No data
Immunotoxicity	Not immunotoxic (rat)
Lowest relevant oral NOAEL	> 278 mg/kg bw (rat)
Human data	No data

Studies on toxicologically relevant metabolites

Acute toxicity

M440I007, oral	LD_{50} > 2000 mg/kg bw (rat)
CPCA, oral	300 < LD_{50} < 500 mg/kg bw (rat)

Short-term toxicity

M440I007	
Target/critical effect	Necrosis / fibrosis of myocardium
Lowest relevant oral NOAEL	< 708 mg/kg bw per day (rat; limited examination)
CPCA	
Target/critical effect	Cardiomyopathy/myocardial vacuolation, decreased zymogen in pancreas, liver and kidney effects
Lowest relevant oral NOAEL	10 mg/kg bw per day (rat)

Genotoxicity

M440I007	Unlikely to be genotoxic[a]

a Unlikely to pose a carcinogenic threat to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.08 mg/kg bw[c]	One-year toxicity (dog) and developmental toxicity study (rabbit)	100
ARfD[a]	0.2 mg/kg bw[c]	Developmental toxicity study (rabbit)	100
ARfD[b]	0.3 mg/kg bw[c]	90-day toxicity study (dog)	100

a Women of childbearing age

b General population

c Applies to afidopyropen, M440I001, M440I007, M440I017 and CPCA, expressed as afidopyropen

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Annex 1: Mode of Action

Carcinogenicity of Afidopyropen

In a 2-year carcinogenicity study with rats, afidopyropen was administered to groups of Fischer rats for 24 months, at dietary concentrations of 0, 100, 300 or 1000 ppm. Adenocarcinoma of the uterus in females increased significantly in the 1000 ppm group.

Table I. Carcinogenicity Study of Afidopyropen in Rats; Incidence of Uterine Adenomas and Adenocarcinomas

		Dose			
Organ	ppm	0	100	300	1000
	mg/kg bw	0	5.3	15.5	50.8
Uterus	No. of animals	50	50	50	50
	Adenoma	2	1	2	3
	Adenocarcinoma	4	1	2	10[V]

* p<0.05 (Fisher's exact probability test)
** p<0.01 (Fisher's exact probability test)
[V] Incidence of this lesion in all animals examined (N=50) was not statistically significant. In animals killed by design (N=40), the 10/40 incidence was statistically significant at p≤0.05

Source: Van Cott, Frericks & Honarvar (2016)

The Dose Adequacy Review Team (DART) of the Health Effects Division of the US Environmental Protection Agency reviewed the dose levels in the previously described 2-year rat cancer study (2014/8000287) and concluded that an additional dose of 3000 ppm was required to meet the maximum tolerated dose (MTD) requirements. The agency also suggested that the 1000 ppm dose be repeated to bridge the results to the rat cancer study that was ongoing at dose levels of 100, 300 and 1000 ppm.

In a second rat cancer study afidopyropen was given in the feed to F344/DuCrlCrlj rats (50/sex/dose) at doses of 0, 1000 or 3000 ppm (equal to 0, 41.6 and 128.2 mg/kg bw/d for males and 0, 50.4 and 146.9 mg/kg bw/d for females) for 104 weeks. Adenocarcinoma of the uterus in females increased significantly in both the 1000 and 3000 ppm group. (Oshima, 2015b)

Table II. Carcinogenicity Study of Afidopyropen in Rats Administration via the Diet. Incidence of Uterine Adenomas and Adenocarcinomas

Organ		Dose (ppm)		
	ppm	0	1000	3000
	mg/kg bw	0	50.4	146.9
Uterus	No. of animals	50	50	50
	Adenoma	1	3	4
	Adenocarcinoma	0	5*	12**

* p<0.05 (Fisher's exact probability test)
** p<0.01 (Fisher's exact probability test)

Source: Van Cott, Frericks & Honarvar (2016)

In an 18-month carcinogenicity study (52/sex/dose), ICR [Crlj:CD1(ICR)] mice were given 0,120,700, 4000 (males) or 4000/3000/2000 females of afidopyropen in the diet. There were no increases of rare types of tumors, earlier occurrence of spontaneous neoplasm, or other sign indicating carcinogenicity. (Takahashi, 2012a)

Other than the observed uterine adenocarcinomas in the female rat, there were no other afidopyropen treatment related increases in neoplastic lesions.

Table III: Summary of carcinogenicity studies with afidopyropen

Study Dose levels (Batch / purity)	Incidence of Neoplastic Lesion in the Uterus All Animals Examined		Reference
	Adenoma	Adenocarcinoma	
Rat Fischer strain (F344/DuCrlCrlj) of SPF 2-year cancer study, dietary administration at 0, 100, 300 or 1000 ppm (080722, 95.74%)	0 ppm: 2/50 100 ppm: 1/50 300 ppm: 2/50 1000 ppm: 3/50	0 ppm: 4/50 100 ppm: 1/50 300 ppm: 2/50 1000 ppm: 10/50[1]	Yamashita, R. 2014 Reg. Doc. # 2014/8000287
Rat Fischer strain (F344/DuCrlCrlj) 2-year cancer study, dietary administration at 0, 1000 or 3000 ppm (080722, 95.74%)	0 ppm: 1/50 1000 ppm: 3/50 3000 ppm: 4/50	0 ppm: 0/50 1000 ppm: 5/50* 3000 ppm: 12/50**	Oshima, A 2015 Reg Doc # 2014/1215781
Mouse 18 mo cancer study, dietary administration 0,120,700, 4000 (males) or 4000/3000/2000 females (080722, 95.74%)	None[2]	0 ppm: 0/52 120 ppm: 0/12 700 ppm: 1/16 4000 ppm: 0/52	Naofumi, T. 2012 Reg Doc # 2012/8000283

*, P≤0.05; **, P≤0.01 Fisher's exact probability test
[1] Incidence of this lesion in all animals examined (N=50) was not statistically significant. In animals killed by design (N=40), the 10/40 incidence was statistically significant at p≤0.05
[2] In the mouse, Adenomas in the uterine cervix were not observed at any dose. Benign leiomyoma incidence in the uterine cervix was 0/52. 0/12, 0/16 and 2/52 for the respective doses of 0, 120, 700 and 4000 ppm

Source: Van Cott, Frericks & Honarvar (2016)

The Sponsor has submitted a Mode of Action (MoA) document that postulates that the tumor profile exhibited in these cancer studies is typical for administration of a compound that acts to decrease prolactin concentration in rats.

Sponsor's Postulated MoA

According to the Sponsor, "pharmacokinetic studies indicate that the doses where uterine adenocarcinomas were observed clearly exceeded a kinetically derived maximum tolerated dose (KMD or MTD). Doses exceeding the KMD have limited value to establishing actual risk due to the onset of high-dose restricted modes of action.[(Conolly, Beck & Goodman, 1999; Van & Fabien, 2016a)] Had PK data identifying clear dose-dependent metabolic saturation of afidopyropen been available prior to the design of the initial bioassay conducted at dietary doses up to 1000 ppm, current OECD guidance for dose selection in chronic bioassays would have recommended that the top dose in this bioassay should have been no higher than 50 mg/kg/day (1000 ppm), and potentially even lower (approaching 300 ppm or 15 mg/kg/day). Had such a guideline-acceptable dose selection strategy been employed, afidopyropen would not have (justifiably) been identified as a rat carcinogen, and no further mode of action studies to evaluate high-dose-specific tumors would have been needed.

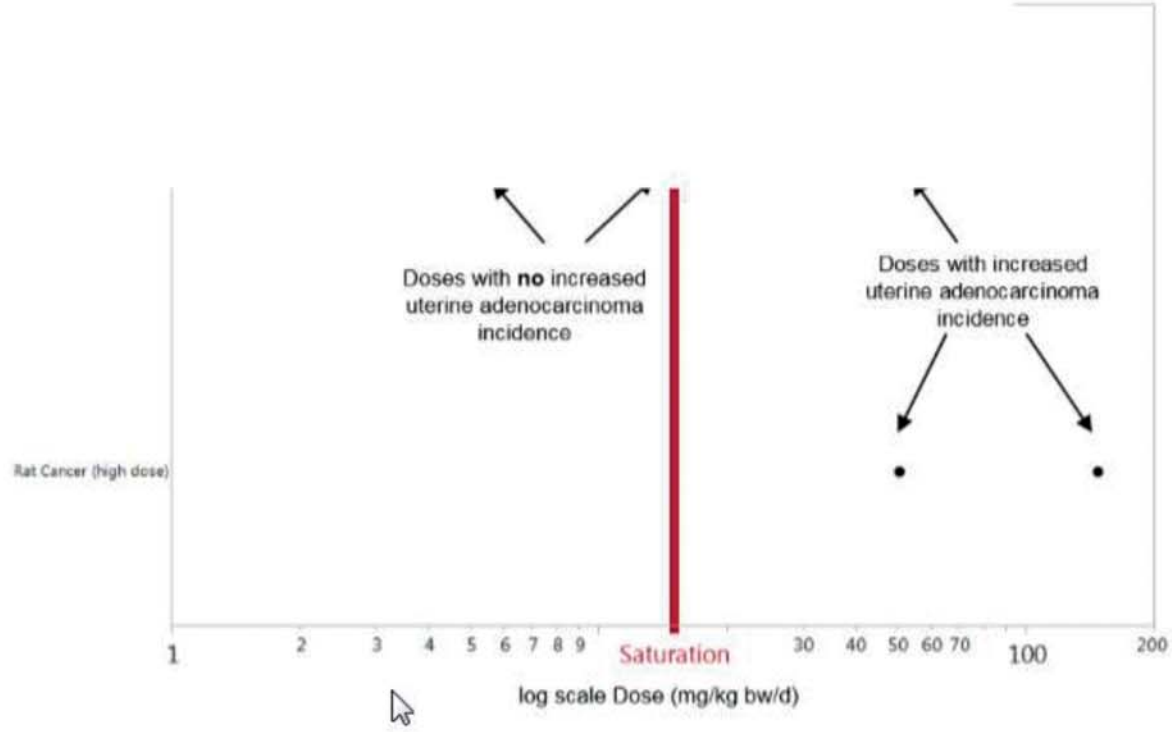
In the rat cancer studies, rat uterine adenocarcinomas were observed at 50.4 and 146.9 mg/kg in the F344/DuCrlCrlj rat. Both of these dose levels are doses where non-linear saturation kinetics occur." (Van Cott, Frericks & Honarvar, 2016)

Table IV. Pharmacokinetics in Female F344/DuCrlCrlj rats. Repeated dose (14D dietary + D15 gavage) at 3, 15 or 50 mg/kg bw. Study Capello, 2016a

External Dose		Plasma Afidopyropen			
mg/kg/bw/d	External Dose Difference	C_{max} ug-Eq/mL	C_{max} Difference	AUC hr*ug-Eq/mL	AUC Difference
3	1X	262	1X	800	1X
15	5X	2800	10.7X	10800	13.5X
50	16.7X	6980	26.7X	42300	53X

Source: Van Cott, Frericks & Honarvar (2016)

Figure I. F344/DuCrlCrlj rat cancer study doses; the red reference line indicates the dose level where saturation occurs following a bolus dose (15 mg/kg bw/d). Increased uterine adenocarcinoma incidence was only found at doses where saturation kinetics were clearly observed

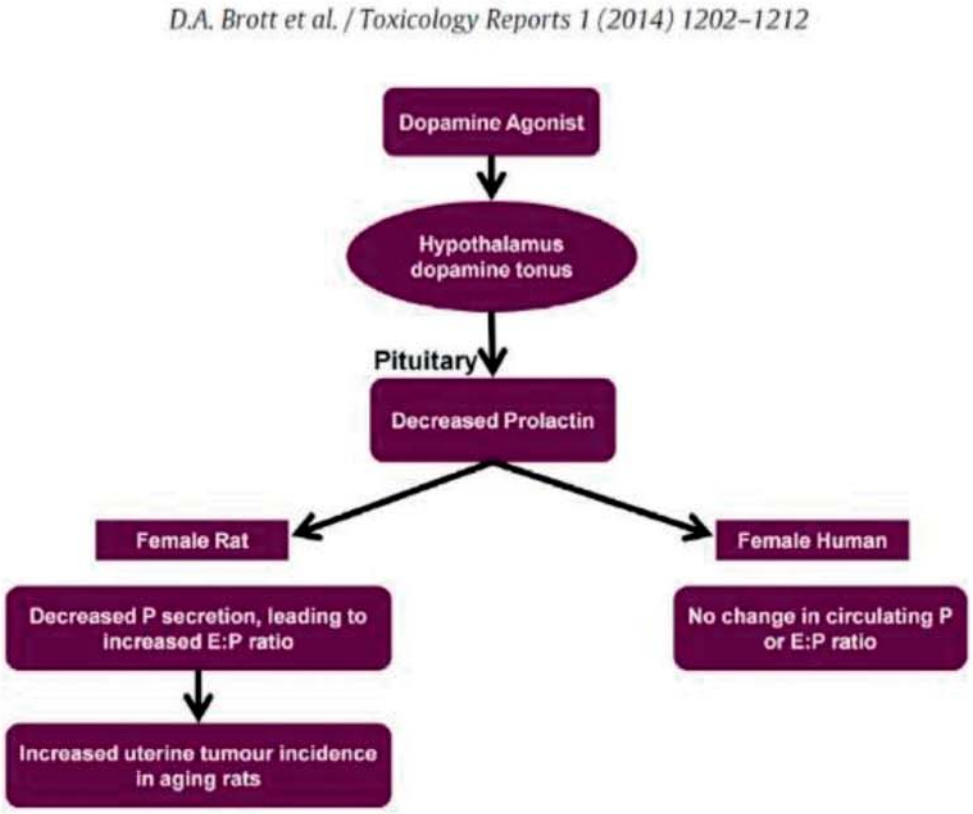


Source: Van Cott, Frericks & Honarvar (2016)

While it is possible that a KMD analysis proposed prior to the determination of the toxicological study design would have affected the doses chosen, the presence of tumours in the database requires them to be addressed. It is also noted that the kinetic data come from bolus dosing, not dietary exposure and a three-fold increase above linearity is not on the extreme end following short-term dosing.

While the Sponsor asserts that the tumours would not have been seen if a KMD-based dose selection strategy had been implemented, they have submitted an MoA based on a high-dose specific (>KMD) dopamine agonist-like effect in the rat. Subsequent to the dopamine agonist effect is an inhibition of prolactin (Prl) release from the pituitary gland, a key event leading to uterine adenocarcinomas in the Fischer rat. Because of the marked difference in hormone regulation between the rat and the human, rat uterine adenocarcinomas produced by this mode of action in the rat are considered to have no relevance to humans. Thus, the increased incidence of rat uterine adenocarcinomas is not relevant to humans based not only on quantitative rat PK and human exposure considerations, but also on qualitative differences in rat and human hormonal drivers of uterine cancer.

Figure II: Schematic for promotion of uterine adenocarcinomas in rat via a dopamine agonist

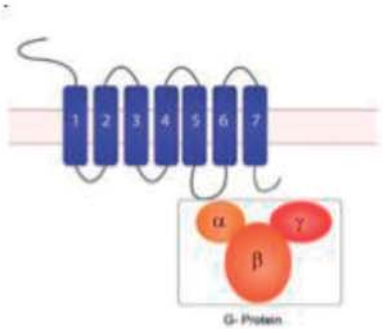


Source: Van Cott, Frericks & Honarvar (2016)

Key Events for the formation of uterine adenocarcinomas

Key event 1. Agonism of Dopamine receptor

A dopamine agonist is a compound that activates dopamine receptors in the absence of the receptor's physiological ligand, the neurotransmitter dopamine. Dopamine agonists can activate pathways through orthosteric binding (binding to the active site), allosteric interaction (binding to other regions of the protein causing conformational changes) or both orthosteric and allosteric interactions. There are few truly specific molecules for the dopamine receptor, primarily due to high conservation of the orthosteric binding site.



Source: Van Cott, Frericks & Honarvar (2016) (Figure from Panesar et al. D2 Receptors in Psychopharmacology)

Various dopamine receptors have been classified by receptor subtypes based upon their homology in their structure and their function (D1 – D5). The dopamine receptors are more broadly classified as either a D1-like (including D1 and D5) or D2-like (including D2-D4). The D2-like receptors are typically associated with inhibitory functions while the D1-like are associated with stimulatory functions. While dopamine binds all five receptors, dopamine agonists and antagonists may bind preferentially to D1-like or D2-like receptors.(Pivonello et al., 2007)

For this mode of action, the focus is on the dopamine receptors located in the pituitary gland of the rat. These receptors in the pituitary are important for regulating hormone secretion and synthesis. Of particular interest are those receptors that can affect prolactin concentration. In rat, the D2 receptor is mainly expressed in lactotropes (prolactin producing cells). The major role of the pituitary D2 receptor in the rat is the inhibitory control of prolactin synthesis and secretion, as well as the growth of the lactotrope cell.

Therefore, while assays were conducted to observe whether afidopyropen binds to various dopamine receptors or affects dopamine transport and uptake, the primary interest was the binding properties of afidopyropen and its metabolites to the D2 receptor. The D2 receptor activity in the rat has a direct impact on lactotrope cells in the rat pituitary and the subsequent concentration of serum prolactin. Ultimately, the D2 receptor in the rat pituitary has a downstream effect on levels of uterine proliferation.

Other dopamine related targets that could be involved with this key event include the dopamine transporter, a protein that spans the membrane and pumps the dopamine neurotransmitter out of the synapse into the cytosol. Reuptake of dopamine from the cytosol via the dopamine transporter clears the dopamine from the synapse. Blocking or agonizing the dopamine transporter has a direct effect on serum prolactin concentration. (Ben-Jonathan, N & Hnasko, R, 2001)

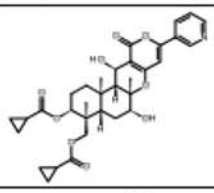
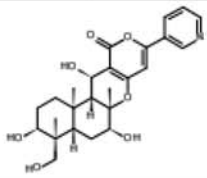
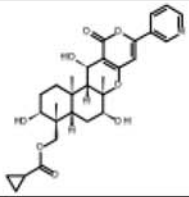
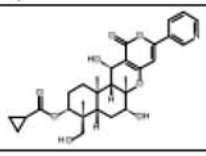
Data supporting key event #1

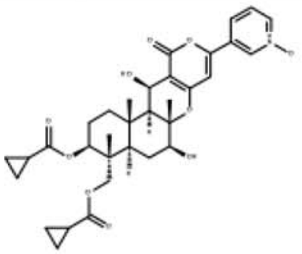
1. in silico

An in silico assay of activity at the D2 (hd2-) receptor and dopamine transporter (hdat-) was conducted using BASF in house in-silico activity prediction models (Wolf, 2016a). These in-house QSAR models predict the strength of inhibition of the above mentioned targets based on the structural similarity of the input molecules to those with a reported measured inhibition value (Anger et al. 2014).

The prediction output can be found in the below table for each input compound. In addition to afidopyropen, the model evaluated major rat metabolites of afidopyropen.

Table V: In-silico activity predictions for BAS 440 I (afidopyropen) and its main metabolites at the dopamine receptor (hd2-) and the dopamine transporter (hdat-). The activity is given as a plC50 value ($\log(IC_{50})$ in molar concentration) along with an error estimation.

Compound ID	Structure	Activity[11]	
		Dopamine Receptor (hd2-)	Dopamine Transporter (hdat-)
Afidopyropen (BAS 440 I)		7.23±0.99 **Borderline Activity**	5.55±0.7 **Inactive**
M440I001		7.09±0.93 **Borderline Activity**	5.6±0.78 **Inactive**
M440I002		7.31±0.95 **Borderline Activity**	5.57±0.72 **Inactive**
M440I003		7.33±0.95 **Borderline Activity**	5.56±0.72 **Inactive**

Compound ID	Structure	Activity[11]	
		Dopamine Receptor (hd2-)	Dopamine Transporter (hdat-)
M440I017		**Out of domain[12]**	5.54±0.71 **Inactive**

Source: Van Cott, Frericks & Honarvar (2016)

Overall, the predictions for BAS 440 I and its metabolites are similar. For BAS 440 I and all but one metabolite, high inhibition values are predicted for the D2 dopamine receptor in the range of 10^{-8} molar. The predictions are labeled as borderline because the high uncertainty of the predictions does not allow a clear classification above the cut-off (median of the distribution). However, the in-silico predictions suggest that afidopyropen and several of its rat metabolites are capable of modulating the dopamine receptor (D2) target.

An inhibition of the dopamine transporter (hdat-) by the investigated compounds was judged as unlikely, given the low plC50 predictions for this target.

2. In vitro binding assays (Jolas, 2015ab)

Afidopyropen and four rat metabolites (M440I001, M440I002, M440I003 and M440I017) were tested in a battery of screening assays measuring interaction with the dopamine related receptors and functions.

In radioligand binding assays neither afidopyropen nor any of the tested metabolites showed any agonist or antagonist activity on the D1 or $D2_{S\&L}$ human receptors. These results indicate afidopyropen and its metabolites probably do not interact orthosterically with the D1 or D2 receptors. Alternatively, the particular conditions of the binding assay (conformation etc.) may not be physiologically relevant for binding to occur.

Afidopyropen and the metabolites were also tested for interaction with the dopamine transporter complex (DAT) in a receptor binding assay. In conjunction with the transporter assay, the dopamine uptake assay, using synaptosomes from rat striatum, served as a functional bioassay of the DAT. In both of these assays, neither afidopyropen nor its metabolites showed any potential for interaction with the DAT on a molecular or a functional level.

Two types of tissue bioassays were conducted: One which measures D1 activation and another which measures D2 activation.

- In the rabbit splenic artery assay of the D1 receptor, there was neither an agonist nor antagonist effect observed.
- In the field stimulated rabbit ear artery assay of the D2 receptor, afidopyorpen and M440I002 indicated a strong D2 agonist-like activity. M440I017 also showed some D2 agonist activity. The observed activity was not reversed by the addition of a dopamine antagonist (-)sulpiride. Neither afidopyropen nor its metabolites showed antagonist activity in this assay.
- The field stimulated rabbit ear artery assay of the D2 receptor was repeated with afidopyorpen and M440I002, again showing the D2 agonist response that was not reversed by (-)sulpiride. Then a modified protocol of the assay was used (discussed later) that confirmed the afidopyropen and M440I002 D2 agonist activity.

Table VI: In-vitro Dopamine receptor interaction studies

Assay	Receptor	Tested Compounds	Results	Reference
Radioligand binding	dopamine receptor ($D1_h$) agonist effect	Afidopyropen M440I001 M440I002 M440I003 M440I017	No Activity	2015/1117539
	dopamine receptor ($D1_h$) antagonist effect		No Activity	
Radioligand binding	dopamine transporter(h)		No Activity	
Dopamine uptake	synaptosomes from rat striatum		No Activity	
Tissue bioassay	Rabbit splenic artery; D1 Agonist effect		No Activity	
	Rabbit splenic artery; D1 Antagonist effect		No Activity	
D2 Tissue bioassay I	Field stimulated rabbit ear artery; D2; agonist effect		Afidopyropen and M440I002 showed D2 agonist-like effects that were not reversed by (-)Sulpiride*	
	Field stimulated rabbit ear artery; D2; antagonist effect		No Activity	
Radioligand binding	dopamine receptor ($D2_h$) agonist effect	Afidopyropen M440I002	No Activity	2015/1204929
	dopamine receptor ($D2_h$) antagonist effect		No Activity	
D2 Tissue bioassay II	Field stimulated rabbit ear artery; D2; agonist effect; Standard protocol		Both molecules showed D2 agonist-like effects that were not reversed by (-)Sulpiride*	2015/1204930
	Field stimulated rabbit ear artery; D2; agonist effect; Modified protocol; pre-incubation with (-) Sulpiride		Concentration-dependent decrease in the twitch contraction amplitude, that was reduced by pre-incubation with (-)sulpiride; Confirmed D2 agonist effect	

*(-)Sulpiride is an antagonist of the D2 receptor

Source: Van Cott, Frericks & Honarvar (2016)

3. D2 tissue bioassay discussion

There are few specific molecules for binding to the dopamine receptor, primarily due to high conservation of the orthosteric binding site. 13 Therefore, compounds that are ineffective in competing for binding may still exert their functional activity via allosteric mechanisms. A molecule with this characteristic exhibits insignificant effects on radioligand binding to the receptor, yet may show maximal functional effects at similar or lower concentrations. These compounds are classified as allosteric agonists and negative allosteric modulators of the D2 dopamine receptor. Therefore, even though the radioligand results were clearly negative, an additional tissue bioassay was conducted.

The rabbit isolated perfused ear artery represents an excellent model for the quantitative study of the prejunctional dopamine receptor.(Steinsland & Hieble, 1978) The prejunctional dopamine receptor of the sympathetic nerve varicosities of the rabbit ear artery is typical of the D2 receptor subtype. The action of dopaminergic agonists is reflected by an inhibition of adrenergic neurotransmission, which is quantified by the inhibition of the constrictor response of the artery (decreased twitch activity). The receptor system is a simple, accurate and sensitive model for quantitative studies with dopamine receptor agonists and antagonists.(Hieble, J, Nelson, S & Steinsland, O, 1985)

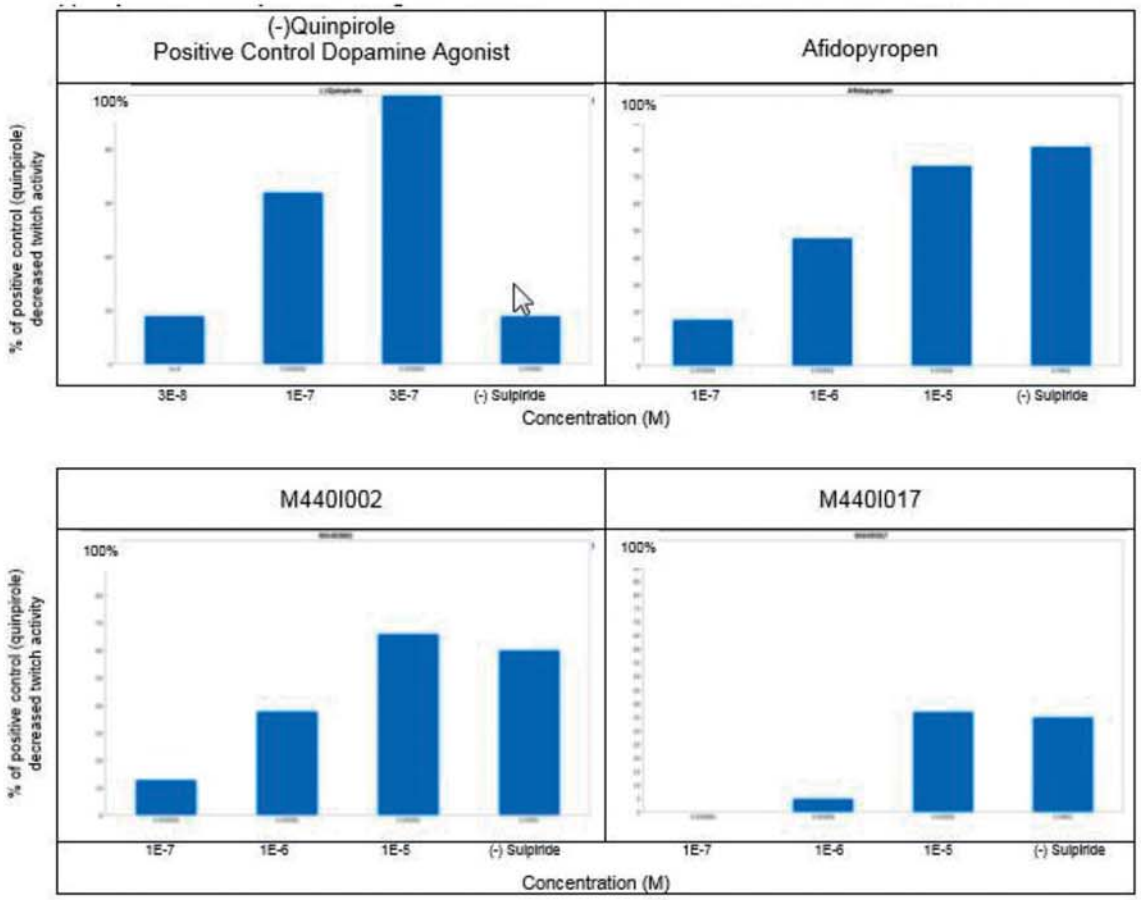
In the perfused ear artery, dopamine inhibits the constrictor response to field stimulation of noradrenergic nerve terminals. Molecules with actions on dopamine receptors such as quinpirole and

bromocriptine act as agonists on this receptor while metoclopramide and sulpiride are potent antagonists of the dopamine receptor.

D2 Tissue bioassay 1

The first field stimulated rabbit ear artery assay indicated apparent agonist activity with afidopyropen and metabolite M440I002 (Jolas, 2015a). However, because the decrease in twitch amplitude (constrictor response) observed with the afidopyropen and the M440I002 was not blocked with the later addition of the dopamine antagonist, (-)sulpiride, the initial assessment of the experiment was that these compounds did not interact as classical agonists of the D2 receptor. The results with (-)quinpirole, show the expected performance of the assay for a dopamine agonist. The dopamine antagonist (-)sulpiride reverses the agonist activity.This observed effect on the dopamine D2 receptor caused by afidopyropen and M440I002 was further explored in later experiments.

Figure III: First field stimulated rabbit ear artery assay results; D2 receptor (Jolas, 2015a). Results are shown as a percentage of the response of prototypical dopamine agonist (-)quinpirole. (-)sulpiride is a dopamine antagonist that was administered at a concentration of 3E-6 M.



Source: Van Cott, Frericks & Honarvar (2016)

D2 Tissue bioassay 2 (Jolas. 2015c)

The field stimulated rabbit ear artery assay was repeated

1. Using the same conditions as the prior assay: ((-)sulpiride addition after test compound treatment).

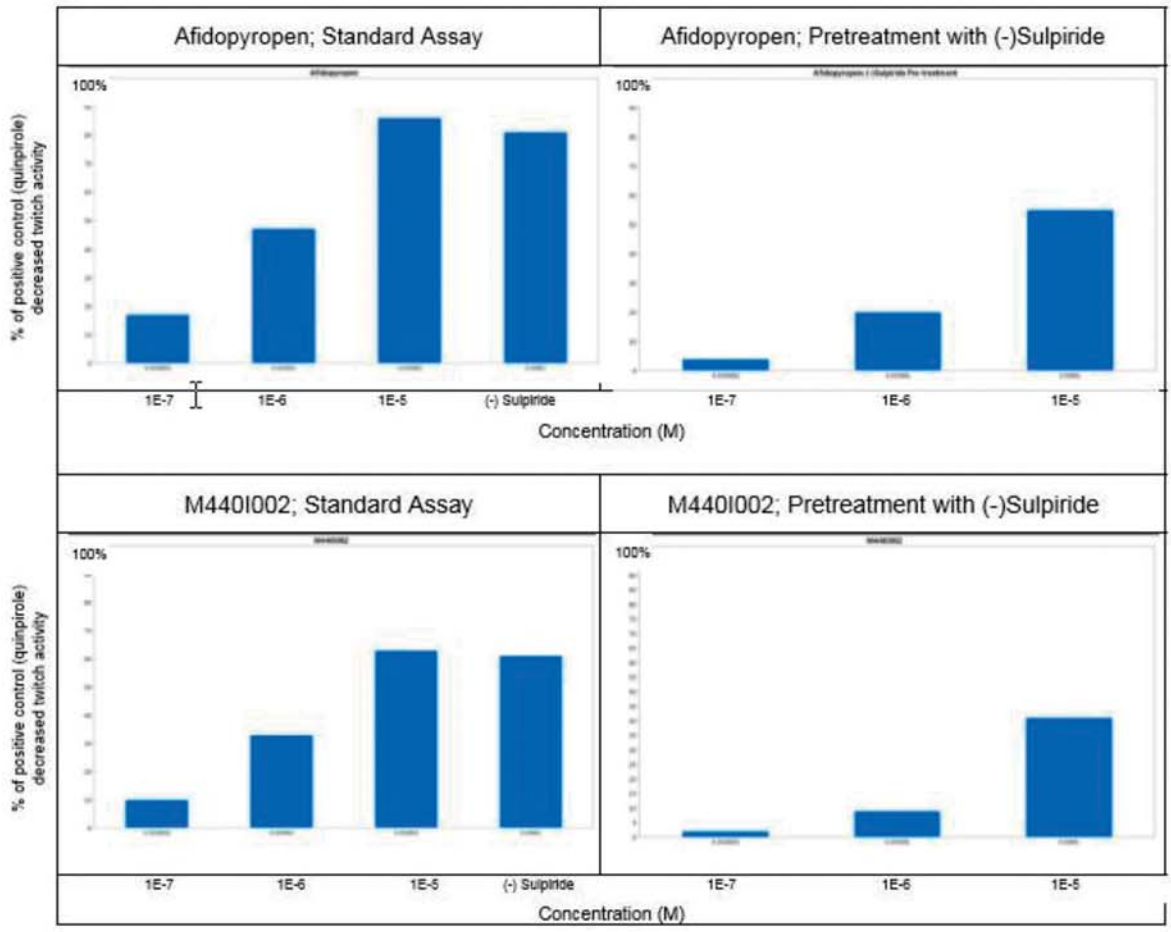
2. Using a modified protocol: Afidopyropen (09/0676-1) and M440I002 (15/0197-1) were tested at three concentrations in duplicate after a 20-minute pre-treatment with solvent or(-)sulpiride at 3.0E-06 M. The results are expressed as a percent of the control agonist (quinpirole) response.

Results of bioassay 2

1. Repeat of prior experiment: After a 20-minute pre-treatment with solvent, afidopyropen (09/0676-1) and M440I002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude, which was not blocked with further addition of (-)sulpiride at 3.0E-06 M. This is a repeat of the finding observed in the initial field-stimulated rabbit ear artery assay (Jolas, 2015a) – i.e. dopamine agonism that was not reversed after addition of the dopamine antagonist.

2. Modified protocol: after a 20-minute pre-treatment with (-)sulpiride at 3.0E-06 M, Afidopyropen (09/0676-1) and M440I002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude that was right shifted when compared their effect after solvent pre-treatment. This demonstrated a dopamine agonist response that was reduced by pre-treatment with a dopamine antagonist (see Figure IV)

Figure IV: Refined protocol; field stimulated rabbit ear artery assay results. D2 receptor (Jolas, 2015c). Results are shown as a percentage of the response of prototypical dopamine agonist (-)quinpirole. (-)sulpiride is a dopamine antagonist that was administered at a concentration of 3E-6 M.



Source: Van Cott, Frericks & Honarvar (2016)

These results indicate the importance of the (-)sulpiride addition protocol to reveal the specificity of the afidopyropen and M440I002 response. Addition of (-)sulpiride 20 minutes before treatment with afidopyropen and M440I002 decreases the dopamine agonist effect, while addition of (-)sulpiride after addition of the afidopyropen and M440I002 has little effect.

This effect is likely due to a high binding affinity of afidopyropen to the D2 receptor, with (-)sulpiride not being able to reverse the binding of afidopyropen. However, afidopyropen is able to partially displace the bound D2 antagonist (-)sulpiride.

In conclusion, both afidopyropen (09/0676-1) and M440I002 (15/0197-1) behave as functional agonists of the D2 receptor in the tissue bioassay.

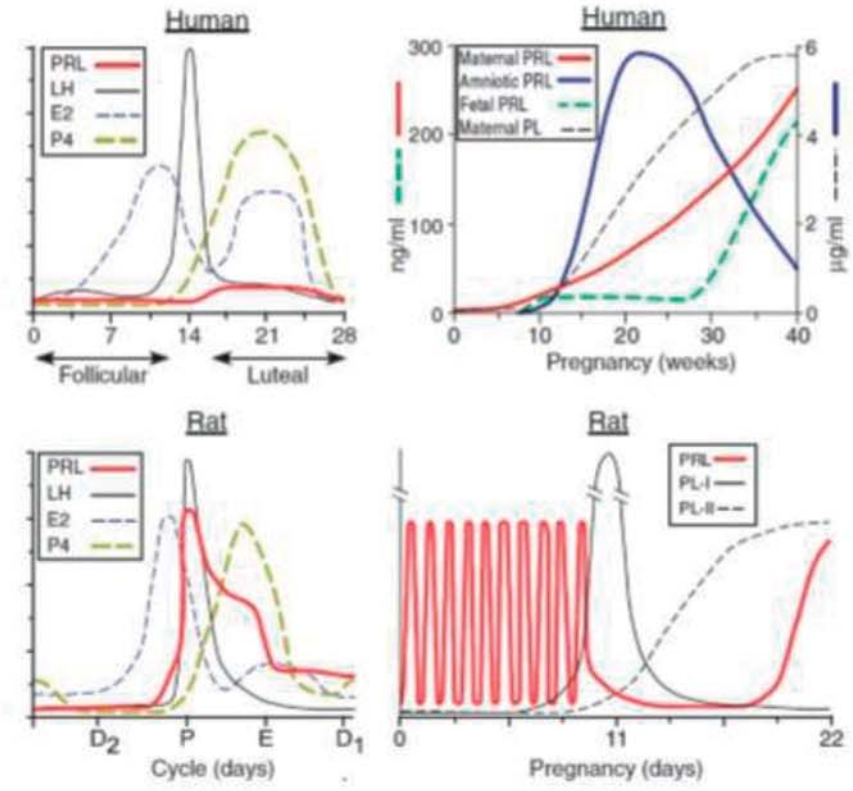
Key event 2. Decreased serum prolactin (Prl) levels

A direct response to dopamine release from the hypothalamus to the anterior pituitary gland is an inhibition of prolactin secretion to the systemic circulation.

In pituitary prolactin producing cells (lactotrophes), upon binding of dopamine and/or a dopamine agonist, there is an increase in potassium conductance and inactivation of voltage sensitive calcium channels. This results in membrane hyperpolarization, reduced intracellular calcium and an inhibition of prolactin release. Within minutes to hours, dopamine suppresses adenylyl cyclase and inositol phosphate metabolism, leading to down-regulation of the prolactin gene. Within days, dopamine inhibits lactotroph proliferation.(Ben-Jonathan, LaPensee & LaPensee, 2008)

It is critical to note the difficulty in measuring changes in prolactin in vivo. These difficulties in measuring prolactin are outlined in detail in a recent paper by Andersson et al. (2013) Prolactin is circadian-gated, altered by stress, and shows a distinct pattern of peaks during the ovulatory cycle; therefore, careful study design and a targeted sampling strategy are usually needed to detect treatment effects on prolactin concentration. Similar to gonadotropins, the stage of the cycle is important when measuring prolactin. Since prolactin concentration is lower during most stages of the estrous cycle in rodents, detecting treatment related decreases in prolactin is particularly challenging.

Figure V. Comparison of hormone profiles during the reproductive cycle (left panels) and pregnancy (right panels) in humans and rats. Note the variable levels in Prl in the rat over the estrus cycle.



Source: Van Cott, Frericks & Honarvar (2016) from Ben-Jonathan, LaPensee & LaPensee (2008)

Data supporting key event #2

The mechanistic studies conducted demonstrate afidopyropen decreases prolactin concentration in vivo. The in vivo decreases in Prl were observed at both doses where carcinogenic effects were observed in the rat (1000 and 3000 ppm). No changes in serum Prl concentration was observed at the lowest in vivo dose (300 ppm), indicating a clear threshold for this key event.

1. Decreased Prolactin in subchronic rat studies

Prolactin concentration was measured at the conclusion of two mechanistic 90D rat studies. Since these studies' primary objective was not to measure hormone levels, many of the parameters that ordinarily would be tightly controlled in a hormone study (such as increasing the number of animals, measurement of estrus cycle) were not controlled. Nevertheless, decreased serum concentration of Prl was detected in both studies at the high dose (4000 ppm). However, prolactin levels were increased reproducibly at 1000 ppm (Table VII and VIII), a finding inconsistent with the proposed MOA.

Table VII: Prolactin (Prl) concentration at the end of a 90D oral study in the F344 rat (Flick et al. 2016c)

Dose (ppm)	Prolactin (ug/L)			
	0	**300**	**1000**	**4000**
Mean	43.27v	168.19	152.78	**10.88***
SD	45.78	243.31	245.97	5.26
N	20	10	10	10
Median	28.73	26.29	58.80	10.86

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics; v=KRUSKAL-WALLIS-WILCOX

Source: Van Cott, Frericks & Honarvar (2016)

Table VIII: Prolactin (Prl) concentration at the end of a 90D oral study in the F344/DuCrlCrlj rat (Flick et al., 2016a)

Dose (ppm)	Prolactin (ug/L)			
	0	300	1000	4000
Mean	70.07	237.53	146.44	27.50
SD	174.51	326.77	181.97	29.83
N	20	10	10	10
Median	26.43	55.52	88.79	11.05

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics; v=KRUSKAL-WALLIS-WILCOX

Source: Van Cott, Frericks & Honarvar (2016)

2. Decreased prolactin in vivo in a hormone study with F344/DuCrlCrlj rats:

An afidopyropen dietary repeated dose 28-day toxicity study was conducted in Fischer F344/DuCrlCrlj rats to determine treatment-related effects on prolactin. (Flick et al., 2016d)

Because of the difficulty in measuring decreases in prolactin in vivo, there were multiple controls placed on the study in an effort to increase the likelihood of detecting changes in prolactin.

Study outline

1. Rat strain: F344/DuCrlCrlj from Charles River Laboratories Japan, Inc., Yokohama, Japan. This F344 strain is not the typical rat strain used to monitor hormone levels, but it was the same rat strain used in the rat cancer studies.
2. Dose groups; Females; N=20 per dose group
   a. 300 ppm afidopyropen: This dose is close to a NOAEL in the rat guideline studies. This dose is approximately the same level as the kinetically-derived maximum tolerated dose.
   b. 1000 ppm afidopyropen: This dose had a slightly increased incidence of uterine adenocarcinomas in both cancer studies. This dose exceeds a kinetically derived maximum tolerated dose.
   c. 3000 ppm afidopyropen: This dose had an increased incidence of uterine adenocarcinomas in the high dose cancer study. This dose also exceeds a kinetically derived maximum tolerated dose.
   d. Bromocriptine at 10 mg/kg bw/d. Bromocriptine is a known dopamine agonist that has been shown to reduce serum Prl concentration.

3. A long acclimatization/adjustment period (28D) was employed to reduce potential stress-mediated effects on prolactin concentration. The rats were handled daily to accustom the rats to the future procedures

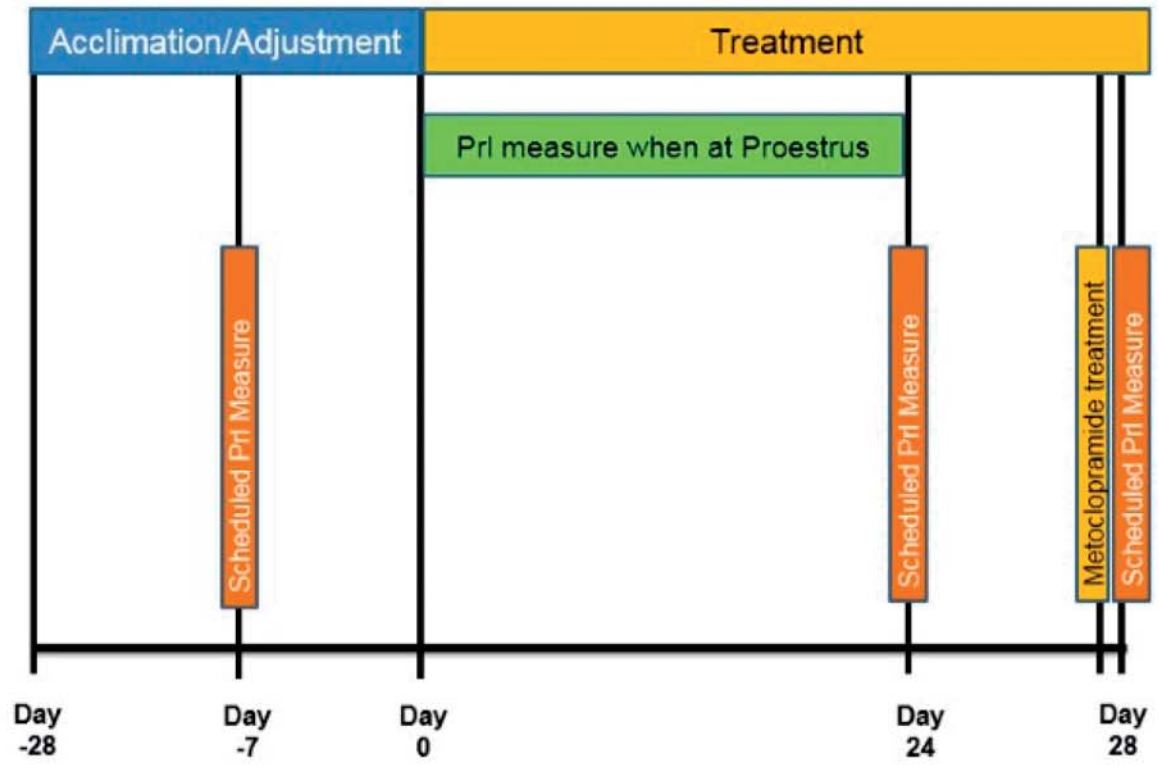
4. Estrus cycle state was measured daily in each animal.

5. There were several approaches employed to measure Prl concentration

   a. Measurements were taken at three fixed times in all animals regardless of the estrus cycle state of the rat: Study day -7, Study day 24 and study day 28.
   b. In addition to the samples taken at fixed times, from Day 0 to Day 24, Prl measurements were taken only when an animal was in proestrus or estrus.21

c. Immediately before sacrifice on Day 28, all animals were injected intraperitoneally with 500 ug/kg bw/d metoclopramide prior to prolactin measurement. 22

Figure VI. Study Timeline. Flick et al. (2016d)



Source: Van Cott, Frericks & Honarvar (2016)

Results

Bromocriptine mesylate (positive control dopamine agonist at 10 mg/kg bw/d).

The bromocriptine reduced serum Prl during treatment at multiple measures, showing that the study protocol was effective at detecting treatment-related decreases in Prl. The following tables show bromocriptine's effect on serum Prl concentration in this protocol. In almost all measurements there is a bromocriptine-induced statistically significant decrease in Prl.

Table IX. Mean Prolactin in Females. Comparison of study Days -7 (untreated) with study day 24 (bromocriptine at 10 mg/kg); Comparison of Control and study day 28 (bromocriptine at 10 mg/kg); (Flick et al., 2016d)

	Prolactin (ng/mL)			
	Day -7 compared to D24		Study D28	
Sex Cycle	Untreated Rats at D-7	D24 Bromocriptine 10 mg/kg bw/d	Control	Bromocriptine 10 mg/kg bw/d
Estrus	331.18	**5.00***	52.24	107.42
N	28	2	2	7
Metestrus	56.20	**5.72****	14.09	**96.31****
N	24	18	18	11

Wilcoxon test (two-sided), * $p \leq 0.05$, ** $p \leq 0.01$

Source: Van Cott, Frericks & Honarvar (2016)

Table X. Bromocriptine (10 mg/kg) effect on Prolactin concentration (ng/mL) in Females in proestrus or estrus (Flick et al., 2016d)

Sex Cycle	Proestrus		Proestrus		Estrus	
	Study days 0-3		Study days 16-22		Study days 18-22	
Dose	Control	10 mg/kg Bromocriptine	Control	10 mg/kg Bromocriptine	Control	10 mg/kg Bromocriptine
Mean	415.98	**8.75****	746.77	**8.49****	500.22	**5.71****
SD	370.48	6.88	420.77	4.12	491.57	1.07
N	9	11	6	13	9	6
Median	564.91	5.99	880.36	6.03	245.09	5.17

Wilcoxon test (two-sided), * $p \leq 0.05$, ** $p \leq 0.01$

Source: Van Cott, Frericks & Honarvar (2016)

Table XI. Bromocriptine (10 mg/kg) effect on Prolactin concentration (ng/mL); Study day 28; 30 minutes post prolactin metoclopramide stimulation (Flick et al., 2016d)

		Prolactin (ng/mL)	
Sex Cycle	Parameter	Control	Bromocriptine 10 mg/kg bw/d
Metestrus	Mean	1063.47	**326.71****
	S.D.	582.35	170.90
	N	18	11
	Median	919.94	373.56

Kruskal-Wallis test + Wilcoxon test (two-sided), * $p \leq 0.05$, ** $p \leq 0.01$

Source: Van Cott, Frericks & Honarvar (2016)

Afidopyropen treatment

At 3000 ppm there was a statistically significantly decreased prolactin in proestrus between study days 0 and 4. At 1000 ppm, there was also a decrease in in prolactin, but the decrease was not statistically significant.

Table XII: Prolactin (Prl) levels (ng/mL); Females in proestrus at study days 0-4. (Flick et al., 2016d)

Sex Cycle	Dose	BAS 440 I			
		Control	300 ppm	1000 ppm	3000 ppm
Proestrus	Mean	486.12	395.71	200.89	**167.67***
	S.D.	330.33	306.60	223.03	267.83
	N	10	10	9	9
	Median	568.23	438.12	128.24	27.84

Kruskal-Wallis test + Wilcoxon test (two-sided), * $p \leq 0.05$, ** $p \leq 0.01$

Source: Van Cott, Frericks & Honarvar (2016)

After stimulation with metoclopramide, there were statistically significant decreased prolactin values in both the 1000 ppm and 3000 ppm dose groups. The decrease in prolactin concentration observed with these afidopyropen treatment groups post-metoclopramide was similar to the effects observed with bromocriptine.

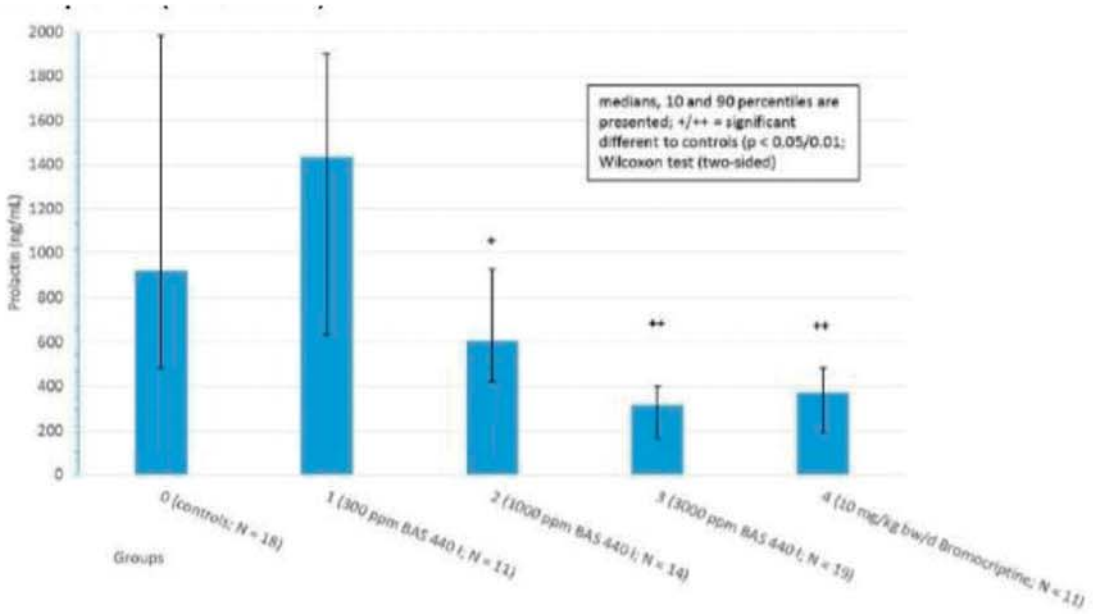
Table XIII: Prolactin (PrL) levels (ng/mL); Study day 28; 30 minutes post prolactin metoclopramide stimulation (Flick et al., 2016d)

Sex Cycle	Dose	BAS 440 I				Bromocriptine
		Control	300 ppm	1000 ppm	3000 ppm	10 mg/kg bw/d
Metestrus	Mean	1063.47	1298.10	**637.59***	**313.33****	**326.71****
	S.D.	582.35	731.16	190.91	107.76	170.90
	N	18	11	14	19	11
	Median	919.94	1432.90	606.48	314.43	373.56

Kruskal-Wallis test + Wilcoxon test (two-sided), * $p \leq 0.05$, ** $p \leq 0.01$

Source: Van Cott, Frericks & Honarvar (2016)

Figure VII. Prolactin concentration at study day 28, 30 minutes after stimulation with 500 µg/kg bw metoclopramide (Flick et al., 2016d)



Source: Van Cott, Frericks & Honarvar (2016)

At 300 ppm afidopyropen treatment, there was no change in prolactin concentration.

Conclusion

As expected, in vivo detection of prolactin concentration decreases was difficult without the metoclopramide treatment. Nevertheless, statistically significant decreases in Prl concentration were observed both with bromocriptine mesylate and with the 3000 ppm dose of afidopyropen without metoclopramide-stimulated Prl release. Decreases (though not statistically significant) were also observed with the 1000 ppm dose of afidopyropen.

After metoclopramide release of prolactin stores, statistically significant decreases of prolactin concentration were observed at both 1000 ppm and 3000 ppm afidopyropen treatments, as well as with the positive control Bromocriptine treatment. There were no effects on Prl concentration at the 300 ppm afidopyropen dose level.

This in vivo study provides clear evidence of decreased serum prolactin at the carcinogenic doses of 1000 and 3000 ppm in the F344/DuCrlCrlj rat.

Key event 3. Decreased Corpus Luteum Support in rats, resulting in decreased progesterone production and estrogen dominance.

The corpus luteum is a small, transient endocrine gland formed following ovulation from the secretory cells of the ovarian follicles. The main function of the rat corpus luteum is the production of progesterone. Prolactin inhibits the progesterone-catabolizing enzyme, 20α-hydroxysteroid dehydrogenase. (Ben-Jonathan, LaPensee & LaPensee, 2008) The consequence of a decrease in prolactin is a decrease in corpus luteum support - resulting in a decreased level of progesterone in the rat.

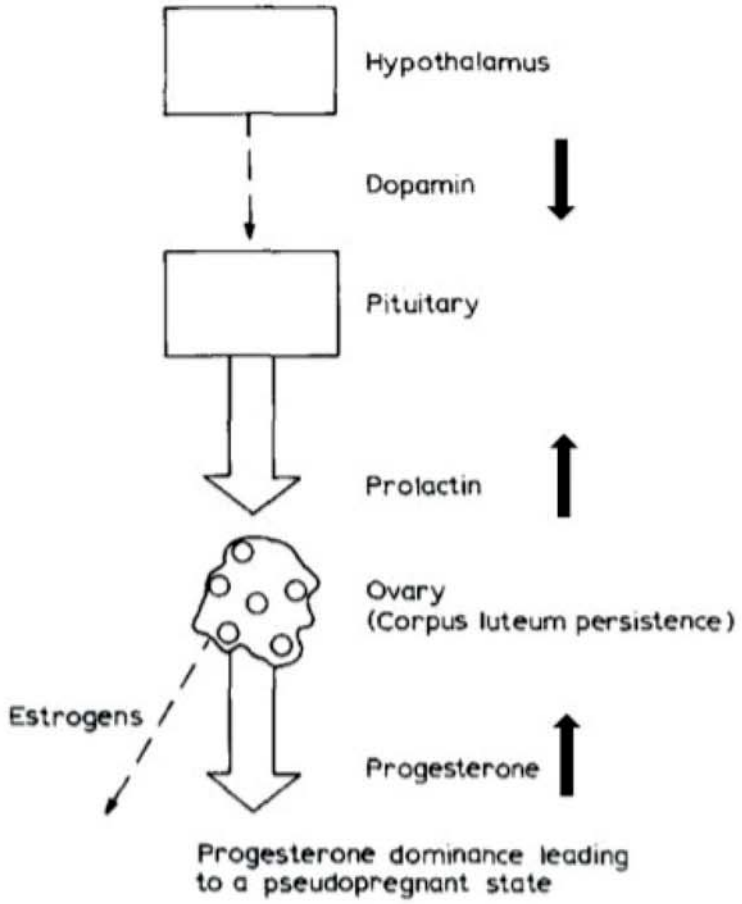
While this key event is not relevant to humans since prolactin is not luteotropic in humans (prolactin provides no stimulating action on development or function of the corpus luteum); no examination of the corpora lutea was performed in the reproductive toxicity studies or in the aged rat. (Harleman, et al., 2012; Jones & Lopez, 2006) Further, decreased uterine and ovarian weights were seen throughout the database, which would undermine a finding of estrogen dominance.

Key event 4. Altered reproductive senescence in aged rats

Normally aging female rat

In a normally aging female rat, menopause is not caused by a loss of ovarian responsiveness, as in women, but by aging processes in the hypothalamus that results in a deficit of dopamine. This dopamine deficit, leads to an increase of prolactin secretion in rodents, but not in primates where it is not a luteotrophic hormone. The result is corpus luteum persistence, and consequently, a decrease in the E2:P ratio (progesterone dominance)

Figure VIII. Normal hormonal situation in aged pseudo-pregnant menopausal rats. The normally increased level of prolactin leads to a decrease in the E2:P ratio (Progesterone dominance) and a pseudopregnant state (from Neumann, 1991)

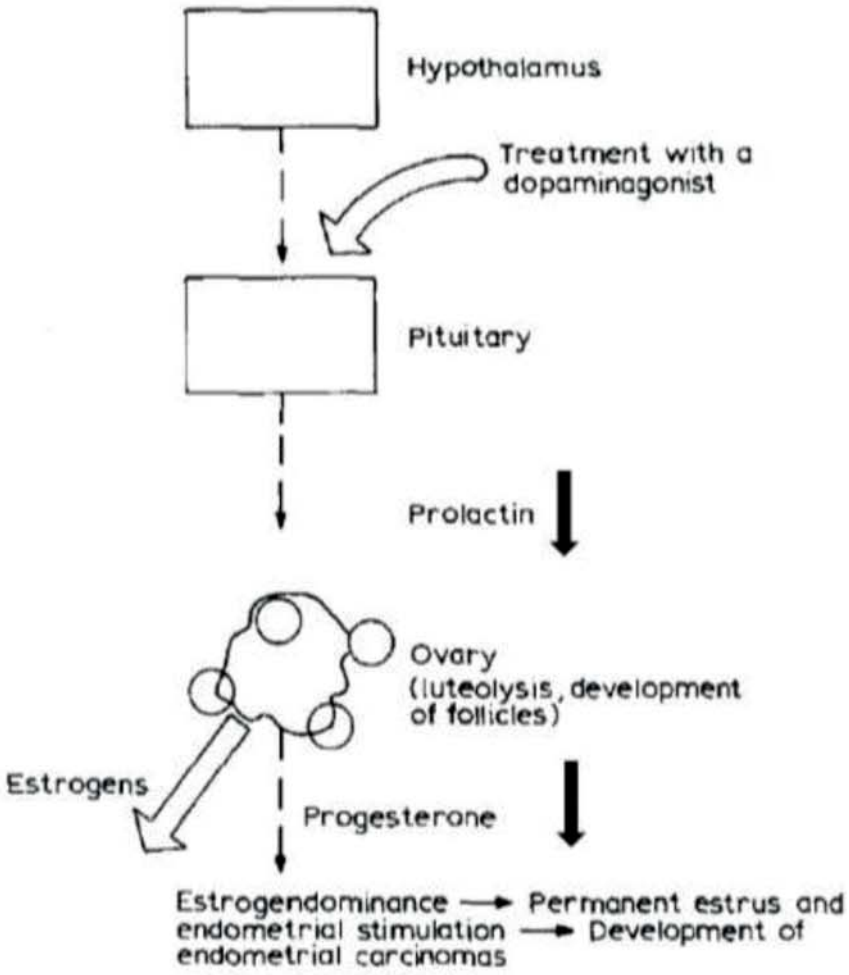


Source: Van Cott, Frericks & Honarvar (2016)

Aging rat - influenced by dopamine agonist

A dopamine agonist will decrease prolactin concentration in aging rats, which cause not only decreased stimulation of mammary glands, but also luteolysis (structural and functional degradation of the previously persistent corpora luteum). It will also decrease new follicle development, and the rat will continue to be exposed to recurrent estradiol. Thus in older female rats, decreased prolactin increases the estradiol:progesterone ratio over a series of cycles (relative estrogen dominance). This prolonged estrogen stimulation of the endometrium can lead to the observed endometrial adenocarcinoma seen with bromocriptine (in rat, not human) or other compounds that increase dopaminergic stimulation (Neumann, 1991)

Figure IX. Influence of dopamine agonists in old pseudo-pregnant menopausal rats. An decreased level of prolactin leads to an increase in the E2:P ratio (Estrogen dominance) and endometrial stimulation (from Neumann, 1991)



Source: Van Cott, Frericks & Honarvar (2016)

Data supporting key event #4

With afidopyropen there is a lack of precursor lesions to uterine adenocarcinoma (endometrial hyperproliferation) at early time points (up to and including the 1yr chronic rat study). If the mode of action were estrogen receptor agonism, proliferation of the mammary tissue and uterine tissue would be expected to be observed in early time points, subsequently progressing to uterine adenocarcinomas. However, in afidopyropen studies it was only in menopausal rats that altered proliferation of the uterus (increased) and mammary tissue (decreased) was observed. This E2:P imbalance only observed late in life indicates that the normal progesterone (P) dominance that occurs in senescence and leads to a pseudopregnant state did not occur.

The dilatation of the mammary gland duct is a normal consequence of reproductive senescence in the virgin female F344 rats as they become middle aged (from 8-14 months of age). As normal reproductive senescence ensues there is an increasing level of prolactin (PRL) secretion that contributes to the development of a number of spontaneous morphologic changes that include increased secretions, duct dilation, alveolar and tubular epithelial hyperplasia, and periductal fibrosis. Xenobiotics that increase pituitary PRL secretion like dopamine receptor antagonists can also cause these rat mammary gland changes. In contrast, dopamine agonists may reduce PRL and decrease the

incidence of the above histologic changes as well as the incidence of spontaneous mammary gland neoplasia. (Rudmann et al. 2012)

The Sponsor states that this is supported in the high dose 2-year rat cancer study. In that study, the non-neoplastic histopathology of the mammary gland reported a decreased incidence in the dilatation of the duct at both 1000 and 3000 ppm. The decreased incidence of this lesion was statistically significant at 3000 ppm ($p<0.01$; Table XIVa). However, when combined with the incidences from the low dose 2-year rat cancer study, there is a lack of dose response (Table XIVb).

Table XIV: Incidence of selected histopathological findings in rat administered afidopyropen for 2-years: All animals examined (Oshima, 2015b)

Organ /Lesion	Female (ppm)		
	0	1000	3000
Mammary gland N	50	50	50
Dilatation, duct +	22	15	11*
Dilatation, duct ++	0	1	0
Dilatation, duct +++	1	1	0
Total (all severities)Dilatation, duct	23	17	**11****

*, P≤0.05; **, P≤0.01 Fisher's exact probability test

+ slight ++moderate +++ severe

Source: Van Cott, Frericks & Honarvar (2016)

Table XIVb. Combined data from the first and second rat oncogenicity studies

ppm	0	100	300	1000	3000
Females					
Mammary gland: Dilation, duct, total – all animals	34/100 (34%)	3/25 (12%)	4/19 (21%)	27/100 (27%)	11/50 (22%)

Source: Yamashita (2014) and Oshima (2015b)

Overall, there was a lack of assessment of reproductive senescence in aged animals. The available evidence was considered insufficient/inadequate to support this key event.

Key event 5. Endometrial hyperproliferation

The association between estrogen exposure and endometrial hyperplasia has been demonstrated in aged Fischer rats in vitro and in vivo. The development of hyperplasia is prevented in the rat by the cyclic appearance of progesterone associated with pseudopregnancy at(?) 29 months of age, when hyperplasia and adenocarcinoma develop despite the presence of elevated progesterone, suggesting that the development of more advanced adenomatous hyperplasia and adenocarcinoma may be due to a decrease in sensitivity to progesterone.(Sponsor included citation, but it`s missing information)

Data supporting key event #5

Other modes of action for development of uterine adenocarcinomas (genotoxic, estrogen receptor agonists) are capable of inducing uterine hyperplasia that can be observed throughout the life stage of the rat. Late onset of endometrial hyperplasia is consistent with the dopamine agonist mechanism of action where a delayed entry into senescence is the trigger for endometrial proliferation.

There was no reported increase of hyperplasia in the uterus following afidopyropen exposure for 12 months.(Yamashita, 2011a; Oshima, 2015a) However at 3000 ppm in the high dose cancer study there was an increased incidence of endometrial proliferation. There was a slight increase in endometrial hyperplasia at 1000 ppm in the second rat cancer study (Oshima, 2015b) but no increased incidence in the first study at 1000 ppm (Yamashita, 2014a). When the two studies are combined, there was no clear dose-response observed at the tumourgenic dose of 1000 ppm.

Table XVa: Incidence of selected histopathological findings in rat administered afidopyropen for 2-years: All animals examined (Oshima, 2015b)

Organ /Lesion	Female (ppm)		
	0	1000	3000
Uterus N	50	50	50
Hyperplasia, endometrium+	7	10	13
Hyperplasia, endometrium++	0	0	1
Hyperplasia, endometrium+++	0	1	2
Hyperplasia, endometrium	7	11	**16***
Cyst, endometrium +	23	14*	20

*, P≤0.05; **, P≤0.01 Fisher's exact probability test
+ slight ++moderate +++ severe

Source: Van Cott, Frericks & Honarvar (2016)

Table XVb. Combined data from the first and second rat oncogenicity studies

ppm	0	100	300	1000	3000
Females					
Uterus - Hyperplasia, endometrium, total – all animals	17/100 (17%)	10/50 (20%)	13/50 (26%)	21/100 (21%)	16/50 (32%)

Source: Yamashita (2014) and Oshima (2015b)

Key event 6. Promotion of uterine adenocarcinoma

There was a treatment-related increase in the incidence of uterine adenocarcinoma and the combined incidence of uterine adenoma/adenocarcinoma in Fischer rats was observed at 1000 and 3000 ppm.

Data supporting key event #6

As the following tables indicate, there is evidence for an increase in uterine adenocarcinomas in the F344/DuCrlCrlj rat at 1000 and 3000 ppm.

Table XVI. Carcinogenicity Study of afidopyropen in Rats; Incidence of Uterine Adenomas and Adenocarcinomas (Yamashita, 2014a)

		Dose (ppm)			
Organ	ppm	0	100	300	1000
	mg/kg bw	0	5.3	15.5	50.8
Uterus	No. of animals	50	50	50	50
	Adenoma	2	1	2	3
	Adenocarcinoma	4	1	2	10*

* p<0.05 (Fisher's exact probability test)
** p<0.01 (Fisher's exact probability test)

Source: Van Cott, Frericks & Honarvar (2016)

Table XVII. Carcinogenicity Study of Afidopyropen in Rats Administration via the Diet; Study Oshima, 2015b. Incidence of Uterine Adenomas and Adenocarcinomas

		Dose (ppm)		
Organ	ppm	0	1000	3000
	mg/kg bw	0	50.4	146.9
Uterus	No. of animals	50	50	50
	Adenoma	1	3	4
	Adenocarcinoma	0	**5***	**12****

* p<0.05 (Fisher's exact probability test)
** p<0.01 (Fisher's exact probability test)

Source: Van Cott, Frericks & Honarvar (2016)

If it the increase in uterine adenocarcinomas is indeed linked to the proposed MOA, this final key event for development of uterine adenocarcinoma in rats would not be relevant for humans. Dopamine agonists do not increase the E2:P ratio in humans and women do not have pseudo-pregnancy. For

example, there is no epidemiological evidence of endometrial neoplasia in women receiving bromocriptine treatment (a potent dopamine agonist). (Neumann, 1991)

Dose-response relationship

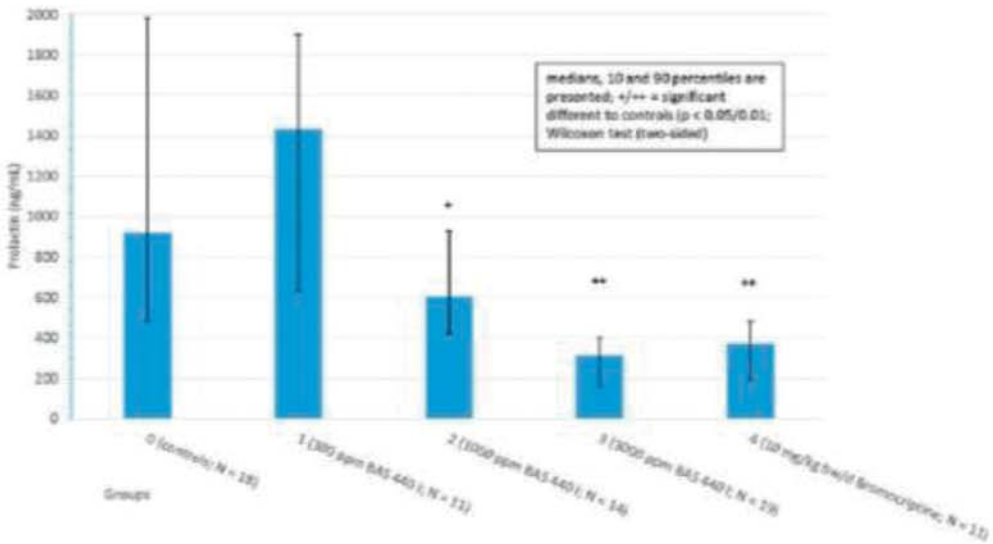
While the key events occur within the range the Sponsors considerd affected by saturation kinetics, the current dose selection of the database requires further investigation of the tumours.

The Dose-Response of each key event can be considered.

1) Agonism of dopamine receptor: there is no meaningful in-vivo endpoint to establish an in vivo threshold for this key event. The later key events serve as surrogates to indicate the doses where biologically relevant levels of dopamine agonist activity occurs.

2) Decreased serum prolactin (Prl) levels; the in vivo 28d feeding study with F344/DuCrlCrlj rats established a threshold for afidopyropen-decreased Prl concentration. No change in prolactin concentration was observed at 300 ppm, while there was dose-dependent decreases in prolactin concentration at 1000 ppm and 3000 ppm. However, the 28-day study is confounded by small sample sizes, large variability among animals and within animals, variations in blood collections times and a lack of a gavage control group. In the second set of studies, the prolactin values were measured at study termination. Again the study was confounded by mall sample sizes, increased variability in data and a lack of estrous stage assessments. Overall, there is some evidence of decreased prolactin, but there is a great deal of uncertainty.

Figure X. Prolactin concentration at study day 28, 30 minutes after stimulation with 500 µg/kg bw metoclopramide (Flick et al., 2016d)



Source: Van Cott, Frericks & Honarvar (2016)

3) Decreased corpus luteum support leading to decreased production of progesterone and estrogen dominance. There are not specific data indicating a threshold for this key event in that corpora lutea measurements were not taken. Further, the decreased ovary and uterus weights do not support the finding of estrogen dominance.

4) Altered reproductive senescence in aged rats.

a. Uterine hyperplasia did not occur until female rats were in senescence. This delay indicates that the normal progesterone dominance present in aged rats was altered. The measurements that indicate this alteration are in the key event 5.
b. The non-neoplastic histopathology of the mammary gland may be used as a surrogate endpoint that indicates the alteration or reproductive senescence in aged rats. The dilatation of the duct was reduced at 3000 ppm in the high dose rat cancer study; however, there was no dose-response when the two two-year studies were combined. Further, the increased tumour incidence at 1000 ppm is unsupported by this finding.

5) Endometrial hyperproliferation was only observed in the high dose rat cancer study (Oshima, 2015b). It was observed at 3000 ppm (statistically significant) with an indication of an increase (not statistically significant) at 1000 ppm. However, the incidence in the high-dose cancer study at 1000 ppm was comparable with the control incidence in the low-dose cancer study where there was no treatment related increase in endometrial hyperplasia at any dose.

Table XIX: Incidence of selected histopathological findings in rat administered afidopyropen for 2-years: All animals examined (Oshima, 2015b)

Organ /Lesion	Female (ppm)		
	0	**1000**	**3000**
Uterus N	50	50	50
Hyperplasia, endometrium+	7	10	13
Hyperplasia, endometrium++	0	0	1
Hyperplasia, endometrium+++	0	1	2
Hyperplasia, endometrium	7	11	**16***

*, P≤0.05; **, P≤0.01 Fisher's exact probability test

Source: Van Cott, Frericks & Honarvar (2016)

Table XX: Incidence of selected histopathological findings in rat administered afidopyropen for 2-years: All animals examined (Yamashita, 2014a)

Organ /Lesion	Female (ppm)			
	0	100	300	1000
Uterus N	50	50	50	50
Hyperplasia, endometrium+	7	8	12	9
Hyperplasia, endometrium++	2	2	1	0
Hyperplasia, endometrium+++	1	0	0	1
Hyperplasia, endometrium	10	10	13	10

*, P≤0.05; **, P≤0.01 Fisher's exact probability test

Source: Van Cott, Frericks & Honarvar (2016)

6) Promotion of uterine adenocarcinomas

The data from both cancer studies in the F344/DuCrlCrlj rat indicates a clear threshold for this key event at 300 ppm (15.5 mg/kg bw/d). There is a dose dependent increase in uterine adenocarcinomas at 1000 and 3000 ppm.

Temporal Relationship

There is a logical temporal relationship with the key events preceding the appearance of uterine adenocarcinomas.

Table XXI. Dose-Response Temporality Concordance Table

Temporal →

Dose ↓

Dose (ppm)	Key Event 1 Dopamine Receptor Agonism	Key Event 2 Decreased serum prolactin levels	Key Event 3 E2:P Imbalance due to decreased CL support	Key Event 4 Altered reproductive senescence	Key Event 5 Endometrial hyper-proliferation	Key Event 6 Promotion of Uterine Tumors
100			-	-	-	-
300		-	-	-	-	-
1000	+*	+	+*	+	+	+
3000	+*	+	+*	+	+	+

+ indicates effect present; - indicates effect absent; Blank cell indicates no data.
*Indirect data based on earlier and later key events
CL: Corpus Luteum
---The dark line indicates the inflection point for observed saturation kinetics (15 mg/kg bw, approximately 300 ppm). Doses below this line exceed a kinetically derived maximum tolerated dose

Source: Van Cott, Frericks & Honarvar (2016)

Dopamine agonism is expected to occur soon after afidopyropen exposure. Decreases in serum prolactin were observed within five days of the initiation of afidopyropen treatment. The lack of uterine proliferation at time points up to and including 1 year is consistent with this mode of action. Indications of delayed senescence are not observed until the end of the 2-year rat study, but this delay is consistent with this mechanism of action. It is not until the end of the two years of treatment that uterine hyperproliferation and adenocarcinoma are observed. This late development of hyperproliferation and adenocarcinoma is typical of the dopamine agonist mode of uterine adenocarcinoma formation.

There is a logical temporal response for the key events in the afidopyropen induced uterine adenocarcinomas in which all necessary key events precede tumor formation.

Strength, consistency and specificity of the association of the tumor response with the key events

The quantifiable precursor events leading to uterine adenocarcinomas are consistent with the formation of uterine adenocarcinomas. The decreased levels of prolactin is a specific finding that choreographs the occurrence and timings of the later key events. No uterine adenocarcinoma was observed at doses where there was not a decrease in prolactin. As cited before, by definition, every substance that reduces prolactin increases the incidence of endometrial carcinoma in old rats (Neumann, 1991).

Also adding to the weight of evidence for this mode of action and associated key events is the characteristic "lesion signature" of a chronic, compound-induced hypoprolactinemia. Three lesions characteristic (though not always observed) of dopamine agonists are:

1) a decreased incidence of pituitary neoplasia,

2) a decreased incidence of mammary gland neoplasia and

3) an increased incidence of uterine neoplasia.

Occurrence of this lesion signature can contribute to the weight of evidence that indicates a decrease in prolactin signaling (Hargreaves & Harleman, 2011). The incidence of uterine neoplasia has already been discussed.

In the high dose cancer study with afidopyropen, there was a statistically significant decrease of neoplastic lesions in the mammary gland. There was a decreasing trend of neoplastic lesions in the incidence of adenoma in the anterior lobe of the males, but no discernable change in the pituitary lesions in females.

Table XXII: Carcinogenicity Study of Afidopyropen in Rats Administration via the Diet; Study Oshima, 2015b. Incidence of mammary and pituitary neoplastic lesions.

		Dose (ppm)					
		Male			**Female**		
Organ	**ppm**	**0**	**1000**	**3000**	**0**	**1000**	**3000**
	mg/kg bw	**0**	**41.6**	**128.2**	**0**	**50.4**	**146.9**
Mammary Gland	No. of animals	-	-	-	50	50	50
	Fibroadenoma	-	-	-	9	8	4
	Adenoma	-	-	-	3	1	0
	Adenocarcinoma	-	-	-	3	2	0
	Combined Adenoma / Adenocarcinoma	-	-	-	6	3	**0***
	Combined Fibroadenoma / Adenoma / Adenocarcinoma	-	-	-	15	11	**4****
Pituitary	No. of animals	50	50	50	50	50	50
	Adenoma, anterior lobe	7	4	3	18	14	19
	Carcinoma, anterior lobe	1	0	1	5	4	6

* $p<0.05$ (Fisher's exact probability test, two-sided) ** $p<0.01$ (Fisher's exact probability test, two-sided)

Source: Van Cott, Frericks & Honarvar (2016)

Biological Plausibility and Coherence

The lactotroph cells in that rat that release prolactin are governed by the action of dopamine. When dopamine or an agonist binds to the D2 receptor, within seconds, dopamine increases potassium conductance and inactivates voltage sensitive calcium channels. This results in membrane hyperpolarization, reduced intracellular calcium, and inhibition of prolactin release. An elevated intracellular calcium accounts for high basal prolactin release in the absence of dopamine and its suppression upon exposure to dopamine. Within minutes to hours, dopamine suppresses adenylyl cyclase and inositol phosphate metabolism, leading to down-regulation of the prolactin gene. Within days, dopamine inhibits lactotroph proliferation.(Ben-Johnathan, LaPensee & LaPensee, 2008) Just as with dopamine, agonists of the D2 receptor, including such well-studied D2 agonists like bromocriptine and cabergoline are known to reduce prolactin release from lactotrophs (Eguchi et al., 1995).D2 agonists reduce prolactin concentration so effectively, they are used clinically to treat hyperprolactinemia. (Chanson et al., 2007).

The observed D2 receptor functional agonism induced by afidopyropen are attributed by the Sponsors as a causal key event to produce the observed decrease in serum prolactin. Secondary to the direct bioassay confirming dopamine agonism, is the indirect evidence provided by decreased levels of serum prolactin. Because inhibition of prolactin secretion is primarily dependent on dopamine signaling, a decrease in circulating serum prolactin is an appropriate indirect measure of neuronal dopamine enhancement and also a unique identifying feature of dopamine agonism/ enhancement (Chambers et al., 2014). Therefore, both the direct D2 receptor bioassay, as well as the indirect measure of serum prolactin concentration in rat are indicative that afidopyropen at high doses interacts with the D2 receptor. The effect on prolactin in the in vivo study confirms that the observed in vitro activation of the D2 receptor is not just a stand-alone finding only occurring in vitro, but that the same D2 activation occurs in the in vivo system and is biologically relevant, albeit at doses that are not human relevant.

The most compelling observation in discussing the biological plausibility of the remaining key events is the observed afidopyropen-induced decrease in serum prolactin at doses that caused an increased uterine adenocarcinomas in rats. The in vivo mechanistic study detected dose-dependent, statistically significant decreases in prolactin at both of the doses where uterine adenocarcinomas were observed However, prolactin levels in the 90-day supplementary studies were measured at a single set point and not at the most appropriate timepoint in the estrus cycle (proestrus). As such, the decreases at 4000 ppm are not supported by the increased prolactin values at 1000 ppm, a tumourgenic dose.

The Sponsor considers additional confidence that this [decreased PRL-uterine adenocarcinoma] causal relationship is present at high doses of afidopyropen is provided by the characteristic lesion signature induced by hypoprolactinemia (both the increased incidence of uterine adenocarcinomas and reduced incidence of mammary neoplasia; Harleman et al, 2012). Dopamine agonists do not always exhibit exactly the same lesion signature. However, even without establishing the initial key events for this MOA, afidopyropen would be suspected as a dopamine agonist, based only upon the profile of lesions observed in the cancer studies.

Biological Plausibility and Coherence Conclusion

While there is evidence that afidopyropen acts as a weak, partial dopamine agonist there is insufficient evidence that afidopyropen works along the same mode of action as bromocriptine. In addition to the inconsistency of prolactin measurements at 1000 and 3000 ppm, there were

inconsistencies with precursor lesions, target organ weights and non-existent measurements of the corpora lutea.

Based on the weight of evidence, the Meeting was unable to establish an MOA for the tumours and, therefore, human relevance could not be ruled out.

Other modes of action

1. Alternate mode of action: The lesions are not treatment related.

BASF considered the possibility that the observed uterine adenocarcinomas may not be treatment related. The low dose rat cancer study report came to this "not treatment related" conclusion based upon the increased background incidence of this lesion in this F344/DuCrlCrlj strain of rat. (2014/8000287)

Though there was no historical control available for this strain of rat at the facility conducting the study (Nisseiken Co Tokyo,Japan), a publication from Bozo Laboratories in Japan (2013) indicates a statistically significant rise in F344/DUCrlCrlj uterine adenocarcinomas in the past years, with a maximum incidence at 22% (Maronpot, 2013).

Table XXIII. Incidence of Uterine Adenocarcinomas in F344/DUCrlCrlj rats at Bozo Laboratories.

Years	1990-1999	2000-2004	2005-2009
No. Study	11	5	4
No. Rats	873	275	215
Mean (%)	3.3%	12.0%**	13.5%**
Range (%)	0-8%	9-16%	9-22%

** p<0.01 vs. 1990-1999 (Bonferroni t-test)

Source: Van Cott, Frericks & Honarvar (2016) from Maronpot (2013)

This increasing incidence of uterine tumors observed with the F344/DUCrlCrlj rats is in contrast to the incidence of uterine neoplasia observed in the F344/N strain used by the NTP, where incidence in a 2013 report was 0.29% for uterus adenoma and 0.29% for uterus carcinoma (NTP Historical Controls report. All routes and vehicles. F344/N rats. June 2013; 14 studies with start dates 2003-2005; Total N=700).

However, the circumstances surrounding the cancer studies indicate that these lesions are likely treatment related.

1. The two studies conducted by Meiji and BASF at Nisseiken Co (Tokyo,Japan) are the only cancer studies available with this F344/DUCrlCrlj strain of rat at that lab. The historical control study count is therefore only 2, and the incidences were outside of this historical control.

2. At 1000 ppm, incidences were statistically significant increased and slightly outside of concurrent controls. At 3000 ppm, incidences were statistically significantly increased and well outside concurrent controls.
3. Incidences were slightly outside of the historical controls of other Japanese labs using this strain of rat.
4. There is an apparent dose response for the tumor incidences.

While there are reasons to think the lesions might not be treatment related (especially at 1000 ppm), the available data suggests that the lesions are related to afidopyropen treatment.

2. Alternate mode of action: Genotoxicity.

Afidopyropen showed no evidence of causing genotoxicity.

Table XXIV: Genotoxicity studies with Afidopyropen

Table 24: Genotoxicity studies with Afidopyropen

Study type	Test system	Batch / Purity Information	Result	Reference
Bacterial assay for gene mutation	*Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) *Escherichia coli* (WP_2uvrA)	Batch 080722 95.74%	Without S9: Negative With S9: Negative	Matsumoto K ; 2009/8000061
		Batch COD-002002 94.5%		Woitkowiak C 2015/1096110
		Batch COD-001545 97.3%		Woitkowiak C 2015/1099097
		Batch COD-002107 90.0%		Woitkowiak C 2015/1238581
Clastogenicity in mammalian (CHL) cells	CHL cells established from the lung of Chinese hamster	Batch 080722 95.74%	Without S9: Negative	Matsumoto K. 2009/8000063
			With S9: Negative	
In vitro Gene mutation; HPRT locus assay	CHO (Chinese hamster ovary) established from the Chinese hamster	Batch 080722 94.54%	Without S9: Negative	Kapp 2015/1110623
			With S9: Negative	
Mouse bone marrow micronucleus assay	NMRI male mice 350, 700 and 1400 and 2000 mg/kg bw	Batch COD-002107 90%	Negative	Dony E. 2015/1001181
	ICR (Crj:CD-1) male mice 500, 1000 and 2000 mg/kg bw	Batch 080722 95.74%	Negative	Wado K. 2009/8000065
	NMRI male mice 250, 500 and 1000 mg/kg bw	Batch COD-002002 95.7%	Negative	Dony E 2014/1313074

Source: Van Cott, Frericks & Honarvar (2016)

3. Alternate Mode of Action: Endocrine (estrogen receptor agonist) mode of action

With uterine tumors, estrogen receptor agonism is an obvious potential mechanism for tumor formation. The estrogen to progesterone ration (E2:P) is the critical endpoint for uterus tissue proliferation. Any direct alteration in effective E2 concentration would upset this critical ratio. A primary suspect in the perturbation of this ratio is E2 receptor agonism.

Figure XI: Estradiol (E2) hormonal mediated formation of uterine adenocarcinomas

Source: Van Cott, Frericks & Honarvar (2016)

Key events for this hypothesized MOA are as follows

1. Increase in effective estradiol (E2) levels
2. Increased E2:P ratio
3. Uterus cell proliferation, hyperplasia
4. Uterine adenocarcinomas

The afidopyropen data surrounding the rat reproduction studies as well as the lack of other ED related endpoints do not indicate an estrogen receptor agonist. The rat 2-generation studies saw no consistent evidence of estrogenic activity.

Other evidence against this mode of action includes the pattern of uterus tissue proliferation consistently observed in subchronic studies at high doses with the F344/DuCrlCrlj rat (not with other strains of rat or species). A significant reduction in uterus weight occurred as early as 14 days into treatment. Although this endpoint indicated that the uterus was a target in this strain of rat, this particular finding is not consistent with what would be expected with an estrogen receptor agonist where proliferation and weight increases would be observed. The uterus proliferation that was observed with afidopyropen treatment was only observed late in life - also not typical of an estrogen receptor agonist.

Though BASF saw no indication of estrogen receptor interaction in the guideline toxicology studies, several assays from the Endocrine Disruption Screening Program (EDSP) were conducted.

1. Estrogen receptor binding assay (OPPTS 890.1250): Assay on Afidopyropen and two major rat metabolites (M440I001 and M440I002); Willoughby, 2015b

2. Transcriptional activation assay (OPPTS 890.1300): Afidopyropen; Willoughby, 2015a

Results from the three estrogen receptor binding assays showed M440I001 and M440I002 to be "non-interacting" with the estrogen receptor. Afidopyropen was classified of "equivocal." A result of "equivocal" is not a positive or a potential positive classification, but rather, it means the result is ambiguous, or cannot be properly identified due to limitations of the assay.

In two independent runs of the transcriptional activation assay, afidopyropen did not increase luciferase activity at any of the viable concentrations tested (RPCmax<10%). These data suggest afidopyropen is not an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system.

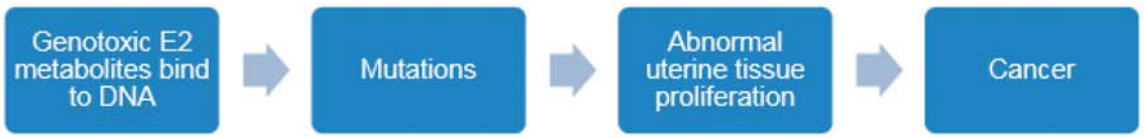
Conclusion

The data does not support the first key event in this estrogen receptor agonist hypothesized mode of action. Though there are shared key events with the dopamine agonist MOA, the timing of the key events observed in the toxicology studies is not consistent with an estrogen receptor agonist mode of action.

4. Alternate Mode of Action: Enzyme induction

Estradiol (E2) mediated carcinogenesis may also have a non-hormonal source.

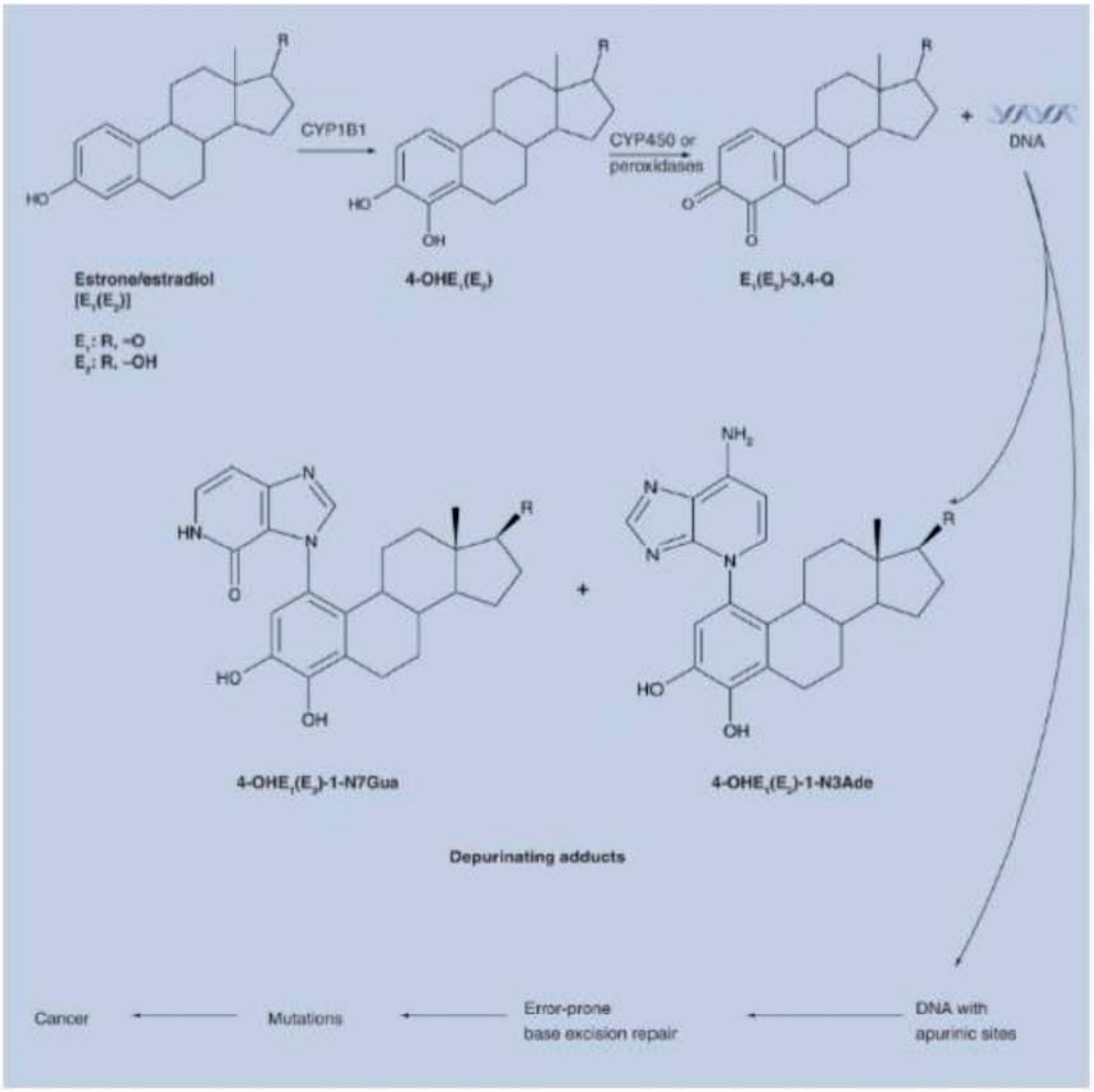
Figure XXII. Non-hormonal estradiol (E2) mediated carcinogenesis



Source: Van Cott, Frericks & Honarvar (2016)

One of the known modes of action for formation of uterine adenocarcinomas is enzyme induction. The metabolism of estradiol to 4-hydroxyestradiol - a suspected tumor initiator and/or promoter in estrogen responsive tissues such as the uterus is primarily driven by CYP1B1.

Figure XIII. 4-OHE2 initiation pathway



Source: Van Cott, Frericks & Honarvar (2016) from Cavalieri & Rogan (2014)

Enzyme induction through a receptor-mediated mechanism is generally accepted to be a threshold-based response.

Key events for this hypothesized MOA are as follows

1. Enzymes responsible for estradiol (E2) metabolism are induced (e.g. CYP1B1, CYP1A1)
2. Unbalanced metabolism of E2 via CYP1B1 leads to an increased production of genotoxic metabolite 4OH-E2.
3. Formation of 4OH-E2 DNA adducts
4. Error prone base excision / repair
5. Abnormal uterine cell proliferation
6. Uterine Adenocarcinomas

There was suggestive evidence for this hypothesized mode of action from the guideline toxicology studies. The evidence consisted of treatment-related liver effects. In both the 28 and the 90-day rat feeding studies, the absolute and relative liver weights of female rats were significantly increased,

compared to the controls, at dietary concentrations of 1000 and 3000 ppm. In addition, histopathology in the 90D study revealed vacuolar changes in 8/10 and 10/10 females at dietary concentrations of 1000 or 3000 ppm. These observations raised the possibility of enzyme induction, which formed the basis for further investigation of this endpoint.

To investigate the possibility of Afidopyropen enzyme induction, an in vivo enzyme induction assay was conducted (2015/1183794, MRID 49689008). The primary goal was to investigate the first hypothesized key event for this potential mode of action, the induction of CYP1B1.

Study Summary

Female Fischer 344 rats were treated for 14 days with 3000 ppm BAS 440 I (Afidopyropen). At termination, liver was processed for isolation of microsomes and the analysis of CYP1A1 and CYP1B1 enzymatic activity. Liver and uteri were processed for Taqman® analysis of CYP1A1 and CYP1B1 mRNA expression.

In the treated rats, there was a slight yet statistically significant 1.6-fold increase in hepatic microsomal ethoxyresorufin-O-deethylation (EROD) (used as a marker for CYP1A) at day 14 following administration of BAS 440 I (Afidopyropen) at 3000 ppm in the diet. Estradiol-2-hydroxylation was similarly increased 1.7-fold. The increases in hepatic microsomal enzyme activities were accompanied by a statistically significant 4-fold increase in hepatic CYP1A1 mRNA. No increases in hepatic microsomal estradiol-4-hydroxylation or CYP1B1 expression were observed. Uterine CYP1A1 mRNA was significantly increased 57-fold in BAS 440 I (Afidopyropen) - treated rats. However, there were no changes in the expression of uterine CYP1B1.

In conclusion, treatment of female F344/DuCrlCrlj rats with Afidopyropen via the diet at 3000 ppm did not induce an increase in the CYP1B1 activity and no significant increase in the level of 4-hydroxyestradiol was detected. The elevated CYP1A1 mRNA levels and as well the slightly increase EROD activity are not representative for a prototypical Aryl-Hydrocarbon Receptor (AHR)-inducer (EROD induction >100 fold (e.g. NTP reports 520, 521, 525, 529, 525, 530, 531) mRNA >1000 fold).

Conclusion on Plausibility of Non-hormonal estradiol (E2) mediated carcinogenesis

Because the crucial key event (enzyme induction) for this mode of action was not observed, this MOA was not considered plausible for the induction of uterine adenocarcinomas in rat.

Uncertainties

Based upon reported data from other laboratories in Japan, the F344/DUCrlCrlj strain of rat appears to be uniquely susceptible to uterine adenocarcinomas. The lack of historical control data in the laboratory conducting the rat cancer studies (Nisseiken - Tokyo, Japan) make evaluation of the lesion incidence difficult. With a more robust historical data, the sponsor considers that uterine adenocarcinoma incidence observed with Afidopyropen may have fallen within historical control.

This tumor type only was observed with the F344/DuCrlCrlj rat, a strain that has shown a high and increasing background incidence of this lesion. This mechanism is theoretically available to the mouse, but no afidopyropen-induced increase in uterine adenocarcinomas was observed with the mouse. Dopamine agonist-induced uterine carcinomas have not been reported in literature with the mouse. It may be the F344/DuCrlCrlj rat is uniquely susceptible rodent / rodent strain to the dopamine

agonist effect provided by afidopyropen treatment. Alternatively, mouse PK (not studied) may not exhibit dose-dependent saturation of clearance of afidopyropen or its other dopamine-agonist active metabolites as has been experimentally demonstrated in rats.

Assessment of the postulated mode of action

The data presented cannot with a moderately high degree of confidence adequately explain the development of uterine adenocarcinomas in female rats exposed to high chronic dietary doses of afidopyropen.

Human applicability of the proposed mode of action

The IPCS HRF presents an approach of several questions to lead to a documented logical conclusion regarding the human relevance of the MOA underlying animal tumors.

1. Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?

Due to inconsistencies in the database, including variable prolactin values, lack of measurements on the corpora lutea, lack of proposed precursor lesions at the tumourgenic dose of 1000 ppm and decreased ovarian and uterine weights, the weight of evidence is insuffienct to establish the proposed MOA in animals.

2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

As afidopyropen does not match that of bromocriptine, human relevance can not be ruled out.

3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

In the absence of comparative assays on kinetics / metabolism in rats and other mammalian species, no conclusion can be reached on saturation in rats being species specific.

Therefore the human-relevance of the uterine carcinomas found in the rat can not be excluded.

Conclusions

The Meeting concluded that afidopyropen is carcinogenic in female rats, but not in mice or male rats. Based on the weight of evidence, the Meeting was unable to establish an MOA for the tumours and, therefore, human relevance could not be ruled out.

BUPROFEZIN / ANILINE (addendum)

First draft prepared by
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Explanation

Buprofezin is the ISO-approved name for (*Z*)-2-*tert*-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one (IUPAC), Chemical Abstracts Service (CAS) number 69327-76-0. Buprofezin is an insecticide that acts by the inhibition of chitin synthesis.

Buprofezin was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1991, 1999 and 2008. In 1991 an acceptable daily intake (ADI) of 0–0.01 mg/kg body weight (bw) was established based on a no-observed-adverse-effect level (NOAEL) of 0.9 mg/kg bw per day identified in a two-year study in rats and with a safety factor of 100. In 1999 JMPR considered that the establishment of an acute reference dose (ARfD) was unnecessary. In 2008 the JMPR Meeting established an ADI of 0–0.009 mg/kg bw, based on the same study and NOAEL as the 1991 evaluation. The 2008 JMPR established an ARfD of 0.5 mg/kg bw based on the NOAEL of 50 mg/kg bw per day for ataxia in a 13-week dog study.

Buprofezin was re-evaluated at the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR) to support additional maximum residue limits (MRLs) and in response to a concern raised by a Codex member. This concern related to the production of aniline from residues of buprofezin during the processing of commodities. New information, not previously evaluated, included an in vivo gene mutation study of aniline in transgenic rats and published literature on the toxicity and carcinogenic mode of action of aniline. There were no new data submitted on buprofezin. This

evaluation focuses on the available data for aniline, in particular that for carcinogenicity, genotoxicity and relevance to human exposures.

The new gene mutation study contained a statement of compliance with good laboratory practice (GLP) and complied with the applicable Organisation for Economic Co-operation and Development (OECD) test guideline. The review used both primary sources of information and reviews from national/international organizations. The papers from the published literature did not provide evidence that they complied with GLP or national or international test guidelines.

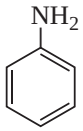
Evaluation for acceptable daily intake

The toxicity of aniline (aminobenzene) has been reviewed extensively by national and international bodies (MAK, 2010; ECB, 2004, IARC, 1982, 1987; EFSA, 2007; ICH, 2015; SCOEL, 2016). These reviews were used to provide some generic information and provide an extensive background to the toxicity of aniline. Original papers and reports were evaluated for critical aspects of the cancer mode of action (MOA).

1. Biochemical aspects

Chemical structure of aniline is shown in Fig. 1.

Figure 1. Structure of aniline (aminobenzene, CAS No. 62-53-3)



1.1 Absorption, distribution and excretion

Aniline is reported to be extensively absorbed when administered orally to F344 rats (ca 90% at 50 or 250 mg/kg bw) and B6C3F$_1$ mice (ca 70% at 100 or 500 mg/kg bw), based on 0–24 h urine samples (McCarthy et al., 1985).

Peak plasma radioactivity levels in rats dosed with ^{14}C-aniline at 10, 30 or 100 mg/kg bw were 0.5, 1 and 2 h respectively (ECB, 2004). Following oral dosing of ^{14}C-aniline to F344 rats, at 100 mg/kg bw for one day, the highest concentration of radiolabel was detected in red blood cells (RBCs) (25 µg equiv./g), with lower levels (0.4 to 4 µg equiv./g) in plasma, liver, spleen, kidney, lung and heart. Following 10 days dosing at 100 mg/kg bw per day, radioactivity in spleen was 12 times higher than after one day; other tissues showed increases of between 1.8- and 3.8-fold. Covalent binding of radioactivity to proteins was six times higher in the spleen than in the liver. (Bus & Sun, 1979).

Radioactivity from ^{14}C-aniline administered to F344 rats at 50 or 250 mg/kg bw intraperitonially (i.p.), after seven unlabelled doses of 50 mg/kg bw per day by gavage, was associated with DNA, RNA and protein. Levels of radioactivity associated with protein and RNA increased in the majority of tissues, with the highest increases in spleen. Radioactivity associated with DNA was not increased by predosing at the low dose level; the highest binding level was associated with the kidney. At the high dose level, the highest covalent binding indices were in the kidney (14.2), large intestine (4.3) and spleen (3.7). In a parallel experiment, B6C3F$_1$ mice received ^{14}C-aniline at 100 or 500 mg/kg bw i.p., after seven unlabelled doses of 100 mg/kg bw per day by gavage. These mice displayed no (or minimal) binding to any of the macromolecules. A comparison of the findings in rats and mice indicated that binding was likely to be associated with a rat-specific metabolite formed predominantly at high doses (McCarthy et al., 1985).

Excretion is rapid in rats and humans with a half-life of approximately three hours (ECB, 2004).

The excretory pathway in rats relies extensively on sulfate conjugation, which is reported to become saturated at doses above 50 mg/kg bw. In mice, conjugation is with glucuronic acid and this pathway is reported not to become saturated, (McCarthy et al., 1985)

1.2 Biotransformation

Metabolism of aniline is via *N*-hydroxylation, *N*-acetylation and/or hydroxylation of the phenyl ring, followed by conjugation with glucuronic acid or sulfate. (ECB, 2004; McCarthy et al., 1985).

In a comparative study of the metabolism of aniline in rats and mice, groups of F344 rats (group size and sex unspecified) received seven gavage doses of unlabelled aniline (50 mg/kg bw per day) followed by a single dose of ^{14}C-aniline (50 or 250 mg/kg bw) or just the single labelled dose. Urine samples were collected via catheter over 24 h and anlaysed for metabolites. Liver samples were analysed for P450 activity and kinetic parameters of *N*-hydroxylase and *p*-hydroxylase enzymes. Protein, RNA and DNA binding in major organs was investigated. In a parallel investigation, B6C3F$_1$ mice received unlabelled doses of 100 mg/kg bw per day and labelled doses of 100 or 500 mg/kg bw. DNA binding indices were higher in rats than mice, particularly in the kidney. The main metabolic reactions were *N*-acetylation, hydroxylation at the 4- or 2- positions on the phenyl ring, and conjugation with glucuronic acid or sulfate (Table 1). There were no notable differences in findings with or without pretreatment. In the rat, sulfate conjugation predominated, with no glucuronidation at the low dose. At the high dose level there was some glucuronidation and a reduction in the proportion of sulfate conjugates from 92% to 67% of total urinary radioactivity, indicating saturation of the primary excretory pathway. In mice, there was no change in metabolite profile with dose level; a high level of unidentified compounds (< 25%) makes determination of the overall pathway in mice impossible. Here glucuronidation was the predominant conjugation reaction (Table 1). The Michaelis constant (K_M) of hepatic *p*-hydroxylase was 6-fold lower in mice than rats, although the maximum rate of reaction (V_{max}) was similar in both species, indicating the mouse would have a greater potential than the rat to hydrolyse the *p*- position on aniline's phenyl ring. There were no species differences in the K_M or V_{max} of hepatic *N*-hydroxylase (McCarthy et al., 1985).

Table 1. Compounds found in the urine of mice or rats administered seven unlabelled doses of aniline followed by a single ^{14}C- dose of aniline (% urinary radioactivity)

Compound	Rat		Mouse	
^{14}C dose (mg/kg bw)	**50**	**250**	**100**	**500**
Aniline	1	5	2	2
p-aminophenol glucuronide	0	2	1	6
p-acetamidophenol glucuronide	0	11	33	33
Total glucuronides	0	13	34	39
o-aminophenol sulfate	10	9	16	15
p-aminophenol sulfate	12	22	0	0
o-acetamidophenol sulfate	0	3	6	4
p-acetamidophenol sulfate	70	33	0	0
Total sulfates	92	67	22	19
Unknowns	1	3	27	32

Adapted from McCarthy et al., 1985

The proportion of hydroxylation or acetylation is reported to vary with species, sex and, in humans, acetylation phenotype (ECB, 2004). The *N*-hydroxy metabolite, *N*-hydroxyaniline (phenylhydroxylamine), is reported to be oxidized to nitrosobenzene in erythrocytes, leading to the production of methaemoglobin (MetHb) and a sulfuric acid amide adduct of haemoglobin (Mellert et al., 2004).

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity, irritancy and skin sensitization of aniline are summarized in Table 2 (from ECB, 2004).

Table 2. Acute toxicity, irritancy and sensitisation of aniline

Species	Route	Purity (%)	Result
Rat	Oral	–	LD_{50} 442 mg/kg bw
Rat	Inhalation	–	LC_{50} 1 mg/L (4 h)
Rabbit	Skin	–	Slightly irritating
Rabbit	Eye	–	Severely irritating
Guinea pig	Skin	–	Some evidence of sensitization
Human	Skin	–	Some evidence of sensitization

LD_{50} Median lethal dose LC_{50} Median lethal concentration

2.2 Short-term studies of toxicity

Many of the published papers on the repeat-dose toxicity lack sufficient detail and/or did not report key investigations such as blood analyses. The studies included below are considered to be acceptably designed and reported or presented useful information.

(a) Oral administration

Mouse

In a preliminary investigation for a carcinogenicity study (NCI, 1978), $B6C3F_1$ mice (five/sex per group) received aniline hydrochloride (ca 100% pure) at 0, 100, 300, 3000 or 10 000 ppm in the diet for eight weeks (equivalent to aniline exposures of 11, 32, 324 or 1080 mg/kg bw per day; assuming 70% conversion from aniline hydrochloride to pure aniline). There were no deaths. Body weight relative to controls was slightly depressed (2–3%) at 10 000 ppm in both sexes. At 3000 ppm and above, all mice had dark, granular and enlarged spleens (NCI, 1978). There is insufficient information to determine a NOAEL.

Rat

Study 1

In a preliminary investigation for a carcinogenicity study (NCI, 1978), Fisher F344 rats (five/sex per group) received aniline hydrochloride (ca 100% pure) at 0, 100, 300, 3000 or 10 000 ppm in the diet for eight weeks (equivalent to aniline exposures of 7, 21, 210 or 700 mg/kg bw per day, assuming 70% conversion from aniline hydrochloride to pure aniline). There were no deaths. Body weight was depressed by over 20% at 10 000 ppm in both sexes. At 3000 ppm and above, all rats had dark, granular and enlarged spleens (NCI, 1978). There is insufficient information to determine a NOAEL.

Study 2

The repeat dose toxicity of aniline was investigated in Fisher F344 rats (six males/group) which received aniline hydrochloride (ca 100% pure) at variable concentrations in the diet for one or four weeks to provide nominal aniline hydrochloride exposures of 0, 10, 30 or 100 mg/kg bw per day. Actual aniline exposures have been calculated to be approximately 0, 4, 12 or 40 mg/kg bw per day. Investigations focused on blood parameters, MetHb, transferrin, iron and iron-binding capacity, haemoglobin adducts (sulfuric acid amide) and pathology of the spleen, liver, kidneys and lymph nodes.

There were no deaths, clinical signs or effects on food consumption or body weight. A regenerative haemolytic anaemia was produced after one week's exposure at 40 mg/kg bw per day (measured as pure aniline) and after four weeks exposure at 12 and 40 mg/kg bw per day (Table 3). MetHb levels were not increased at any dose level. Haemoglobin adducts were increased in all treated groups (Table 3). Spleen weights (absolute and relative to body weight) and the incidence of vascular congestion were increased

in a dose-related manner at both time points in all rats receiving 12 and 40 mg/kg bw per day. Vascular congestion was present in some animals at 4 mg/kg bw per day (Table 3). There was no evidence of proliferation of the sinusoidal endothelial cells or formation of vascular spaces in the red pulp. Focal perisplenitis (Grade 1) was present in one high-dose animal after one week and all high dose animals at four weeks (Grade 2 or 3). Haemosiderin deposition in the spleen was similar in control and test animals. Total iron-binding capacity and transferrin were increased at the top dose level after one week, but showed no progression with duration of dosing. Bone marrow hypercellularity was present in all highest-dose animals after four weeks, but not after one week.

This study demonstrated that changes to red cell parameters and the spleen are produced following one week of dosing with aniline hydrochloride at doses similar to, or lower than, those which produced tumours after exposure for two years. The most sensitive marker was haemoglobin adduct formation. The lowest-observed-adverse-effect level (LOAEL) in this study was 4 mg/kg bw per day, the lowest dose tested, based on vascular congestion of the spleen and increases in haemoglobin adducts (Mellert et al., 2004).

Table 3. Findings in F344 rats (n = 6) exposed to aniline for one or four weeks

Dose (mg aniline/kg bw per day)	Week	0	4	12	40
RBC (10^9 /L)	1	8.4	8.3	8.3	7.2**
	4		8.2	7.8**	7.1**
Reticulocytes (%)	1	2.8	2.9	3.8	8.1**
	4		3.7	4.3**	10.3**
MetHb (%)	1	0.7	0.5	0.8	1.2
	4		0.6	0.8	1.1
Haemoglobin (mM)	1	9.0	9.0	8.6**	7.6**
	4		8.7	8.6**	7.9**
Heinz bodies (%)	1	0	0.1	2.7**	47**
	4		0.4	4.4**	28.9**
Transferrin (g/L)	1	3.8	3.8	3.9	4.2**
	4		3.8	3.9	4.0*
Total iron binding capacity (µM)	1	88	88	91	95**
	4		87	90	93**
Haemoglobin adducts (nmol/kg)	1	0.8 ± 0.3	45 ± 4**	131 ± 17**	350 ± 51**
	4		85 ± 8**	198 ± 25**	352 ± 18**
Spleen weight (g)	1	0.57	0.59	0.62*	1.0**
	4		0.59	0.68**	1.2**
Bone marrow hypocellularity; rats showing Grade 2	1	0	0	0	0
	4		0	0	6 *

Dose (mg aniline/kg bw per day)	Week	0	4	12	40
Spleen haemosiderin; rats showing Grade 1	1	0	0	0	0
	4		0	0	6*
Spleen focal perisplenitis; rats showing any signs (at median grade)	1	0	0	0	1 (1)
	4		0	0	6 (3)*
Spleen vascular congestion; rats showing any signs (at median grade)	1	1 (1)	2 (1)	6 (1)*	6 (3)*
	4		4 (1)	6 (2)*	6 (3)*

* Significant at $p < 0.05$ ** Significant at $p < 0.01$

Source: Mellert et al., 2004

Study 2

Aspects of the repeat-dose toxicity of aniline were investigated in a 28-day study of genotoxicity. Groups of transgenic Fischer 344 Big Blue® male rats ($n = 6$ for the vehicle control or $n = 8$ for the aniline-treated groups) were administered aniline (purity 99.94%; batch SHBH0581V) at doses of 0, 25, 50 or 100 mg/kg per day. Treatment was performed once daily via oral gavage for 28 consecutive days, in a vehicle of 1% carboxymethyl cellulose. Positive control animals ($n = 6$ per group) for the gene mutation assay were treated with *N*-ethyl-*N*-nitrosourea (ENU) at 20 mg/kg per day, on days 1, 2, 3, 12, 19 and 26. For the micronucleus (MN) assay ($n = 6$) they received cyclophosphamide (CP) at 15 mg/kg per day on days 1 and 2, and at 12 mg/kg per day on days 26 and 27. All doses were administered at a dose volume of 10 mL/kg bw.

Surviving animals were bled from the retro-orbital sinus on days 3 and 28 for haematology, methaemoglobin (metHb) and toxicokinetic (TK) evaluation; and on days 4 and 29 for MN evaluation via flow cytometry. Surviving animals in all groups were then sacrificed by CO_2 overdose on day 31. Liver, spleen, bone marrow and testes were collected, weighed (except for bone marrow), flash frozen and stored at or below −60°C. Liver, spleen and bone marrow from five animals per group were processed for DNA isolation and analysis of cII mutants.

Dose levels for the mutation assay were determined from the results of a five-day range-finding study conducted at aniline doses of 25, 50, 100 and 250 mg/kg bw per day. At 250 mg/kg bw per day, all animals lost weight over the five-day treatment period; mean body weight gains were also significantly lower at 100 and 250 mg/kg bw per day. In all treated groups, aniline was detected in rat plasma isolated approximately one hour after dosing on day 5. Based on these results dose levels of 25, 50 and 100 mg/kg bw per day were selected for testing in the mutation assay.

In the main study, one vehicle control male was found dead on day 24; the cause of death could not be identified. All other animals survived to terminal sacrifice on day 31. No toxicologically significant differences were noted for feed consumption, clinical signs, body weights and body weight changes between the aniline-treated groups and the vehicle control group. Aniline was detected in plasma isolated approximately one hour after dosing on day 3 and day 28 (Table 4). Increases in MetHb, decreases in RBC count and haemoglobin, and a compensatory increase in reticulocytes, were seen in all treated groups on day 28. Methaemoglobin was increased markedly on day 3, indicating a possible single-dose effect (Table 4). Spleen weights and iron deposition in the spleen were increased significantly in all aniline treatment groups.

A NOAEL could not be determined from this study as changes in the blood (increased MetHb and reticulocytes) and spleen (increased weight and iron deposition) were noted at all dose levels. The LOAEL was 25 mg/kg bw per day (McKeon & Ciubotaru, 2018).

Table 4. Findings in male Big Blue rats (n=8) exposed to aniline for up to 28 days

Dose (mg/kg bw per day)		0	25	50	100
Day 3					
Plasma aniline (ng/mL) day 3		BLQ	1596	5244	12 425
RBC (10^9/mL)		8.4	8.5	8.3	8.2
Haemoglobin (g/dL)		15.2	15.2	14.9	14.58
Reticulocytes (10^6/mL)		245	266	248	337*
Met-Haemoglobin (%)		0.1	11**	16**	22**
Day 28					
Plasma aniline (ng/mL)		BLQ	1812	8320	18 400
RBC (10^9/mL)		9.2	8.5**	7.8**	6.3**
Haemoglobin (g/dL)		15.6	14.4**	13.7**	12.9**
Reticulocytes (10^6/mL)		248	347**	606**	840**
Met-Haemoglobin (%)		2	18**	22**	21**
Spleen wt (g)		0.6	0.8**	1.1**	1.5**
Splenic iron deposition	Minimal	0	8**	0	0
	Mild	0	0	8**	8**

* Significant at $p < 0.05$ ** Significant at $p < 0.01$

Source: McKeon & Ciubotaru, 2018

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study (NCI, 1978), $B6C3F_1$ mice (50/sex/group; 49 females at 12 000 ppm) received aniline hydrochloride (ca 100% pure) at 0, 6000 or 12 000 ppm in the diet for 103 weeks, followed by a four week observation period. Doses were equivalent to aniline exposures of 600 or 1200 mg/kg bw per day.

Survival was not adversely affected by treatment and was > 60% in all groups. There were no effects on clinical signs or terminal body weights, but body weights in treated males were lower than controls from weeks 30 to 90. There were no statistically significant adverse pathology findings reported, neither non-neoplastic nor neoplastic. The only finding in the spleen was erythropoiesis in 4, 6 and 13 female mice from the control, low- and high-dose groups respectively ($p = 0.03$ by Fisher's exact test in the high-dose group). The only notable finding in tumour incidence was an increase in liver carcinoma in both female groups (5/48 compared to 1/46 in controls; $p = 0.05$) but not in males, which had an inverse dose relationship (12/39, 9/49 and 7/49 in controls, low- and high-dose groups respectively). It is concluded that aniline was not carcinogenic to mice and the NOAEL for carcinogenicity is 12 000 ppm (equivalent to 1200 mg/kg bw per day), the highest dose tested (NCI, 1978) As no blood samples were taken, there was insufficient information to determine a NOAEL for general toxicity.

Rat

Study 1

In a long-term toxicity/carcinogenicity study (NCI, 1978), F344 rats (50/sex per test group; 25/sex as controls) received aniline hydrochloride (ca 100% pure) at 0, 3000 or 6000 ppm in the diet for 103 weeks followed by a four-week observation period. Doses were equivalent to aniline exposures of 105 or 210 mg/kg bw per day.

Survival was slightly lower for the top-dose group males than controls in the last quarter of the study, but survival was > 50% in all groups. There were no effects on clinical signs. Body weights of top-

dose females were lower than those of the controls by approximately 15% at termination. Haemosiderosis was significantly increased in the kidney and liver of top-dose males and kidney of both treated female groups. Papillary hyperplasia of the splenic capsule was increased in males (0/24, 18/50 and 7/50 in controls, low- and high-dose groups respectively) and in females (0/23, 23/50 and 28/50 in controls, low- and high-dose groups respectively). Erythropoiesis was present in the spleens of the majority of treated females (compared with 1/23 of the controls) but in only a small number of males (12%). Fibrosis and necrosis of the spleen were present in < 5% of treated animals based on the original report, but a subsequent repeat of the review concluded that there were increases in fibrosis (Weinberger et al., 1985; see Table 7). Significant increases in mesenchymal tumours of the spleen were seen in males at both dose levels (Table 5). The incidence of these tumours in females was not statistically significant *individually* (Fisher's exact test), but was considered by the report's authors to be indicative of a treatment-related effect given the low background incidence. Findings of sarcomas in multiple organs or the body cavity, were considered to be secondary to the splenic tumours. The incidence of adrenal phaeochromocytoma was also increased in males (2/24, 6/50 and 12/44 in controls, low- and high-dose groups respectively) but its statistical significance was equivocal; positive in a Cochran-Armitage test but with $p > 0.05$ in a Fisher's exact test. Nor was the effect reproduced in a second study (see below), therefore it is considered not to be treatment-related.

Aniline hydrochloride is carcinogenic to F344 rats dosed at 3000 ppm and above for two years. The total numbers of rats with tumours of the spleen (for example, fibrosarcoma, sarcoma, hemangiosarcoma; Table 5) was increased in both male groups and high-dose females. The LOAEL for carcinogenicity is 3000 ppm (equivalent to 105 mg/kg bw per day, pure aniline) the lowest dose tested, based on increases in tumours of the spleen in males at this dose level. The LOAEL for toxicity is 3000 ppm (equivalent to 105 mg/kg bw per day) the lowest dose tested, based on papillary hyperplasia of the spleen in females. (NCI, 1978).

Table 5. Tumour incidence in the NCI carcinogenicity study of aniline in rats (control incidences 0/23−25)

	Male		Female	
	3000 ppm	**6000 ppm**	**3000 ppm**	**6000 ppm**
Spleen	(*N* = 50)	(*N* = 46)	(*N* = 50)	(*N* = 50)
Sarcoma: NOS	4	2	0	3
Fibroma	7	6	0	0
Fibrosarcoma	3	7	0	0
Hemangioma	0	0	0	1
Hemangiosarcoma	19	20	1	2
Lipoma	0	0	0	1
Body cavity/multiple organ	(*N* = 50)	(*N* = 48)	(*N* = 50)	(*N* = 50)
Sarcoma: NOS	0	1	0	1
Fibrosarcoma	2	8	1	3
Leiomyosarcoma	0	2	0	0
Haemangiosarcoma	0	1	0	0

NOS Not otherwise specified

Study 2

In a second long-term toxicity/carcinogenicity study, Fischer 344 rats (90/sex per group) were treated via diet with aniline hydrochloride (ca 100% pure), at variable concentrations, for two years to give nominal exposures of 10, 30 or 100 mg/kg bw per day, corresponding to 7, 22 or 72 mg/kg bw per day as pure aniline. There were three additional satellite groups for interim kills at 26, 52 (10 rats/sex per dose) and 78 weeks (20 rats/sex per dose). Haematology was performed on all animals at scheduled kills. A wide range of organs and tissues was examined from control and top-dose groups, plus spleen and abnormal findings from the mid- and low-dose groups.

Survival was slightly reduced in top-dose males, but overall survival was good at over 70% in all groups. Evidence of regenerative anaemia was evident from week 26 in the top- and mid-dose groups of both sexes (Table 6). The only organ/tissue showing any adverse effects of treatment was the spleen. Spleen weight was increased in both top dose groups at week 78 and in top-dose females at termination. Control males had a spuriously high spleen weight at termination. Spleens from all aniline treated groups showed gross and non-neoplastic changes at increased incidences and/or severity compared with controls; these showed progression with duration of treatment and increasing dose (Table 6). The only organ/tissue with a significant increase in tumour incidence was the spleen of top-dose males. One mid-dose male had a stromal sarcoma of the spleen, which is considered treatment-related given its low background incidence. Although females exhibited a similar range of non-neoplastic changes to males, these did not progress to tumours, other than in one top-dose animal. The splenic tumours were not seen in any animals examined on or before week 78. No increase in adrenal phaeochromocytoma incidence was seen in treated males or females. It was noted by the Meeting that the tumour types reported in this study differ from those of the NCI study – it is unclear if this is merely a nomenclature issue as they are all 'sarcomas'.

Aniline hydrochloride induced splenic tumours in male F344 rats dosed at 21 mg/kg bw per day (as pure aniline) for two years. Tumours of the spleen were increased in males in the mid- and high-dose groups, but not in females. The LOAEL for aniline for general toxicity was 7 mg/kg bw per day based on reduced erythrocyte counts, and pathological changes in the spleen at this dose level. The NOAEL for aniline for carcinogenicity was 7 mg/kg bw per day (CIIT, 1982).

Table 6. Effects on F344 rats exposed to aniline in the diet for up to two years (CIIT study)

Dose (mg/kg bw per day) adjusted for pure aniline		**Males**				**Females**			
	Week	**0**	**7**	**21**	**70**	**0**	**7**	**21**	**70**
Survival (%)	104	83	88	86	71*	78	82	87	83
RBC (10^6/mm^3)	26	9.2	8.7	8.4	7.8*	9.0	8.5	8.0*	7.4*
	78	9.6	8.5*	7.7*	7.2*	8.3	8.3	7.5*	7.0*
Reticulocytes (%)	26	1.7	2.1	2.4*	4.4*	0.8	1.1	1.7*	3.8*
	78	2.0	2.4	3.4*	4.5*	1.8	2.5*	3.6*	5.1*
MetHb (%)	26	1.1	1.5	2.5*	2.6*	2.1	2.5	2.4	3.0*
	78	1.0	1.5	2.5*	2.4*	2.0	1.1	2.0	2.4
Heinz bodies (%)	26	0	0	0	2.8*	0	0	0	0.2
	78	0	0	0	1.2*	0	0	0	0.2*
Spleen weight (g) [§]	78	0.75	0.77	0.89	2.4*	0.52	0.50	0.59	1.2*
	104	2.0	1.0	1.3	2.1	0.71	0.76	0.67	1.3*
Spleen 'dark' (*n* =)	26	0	1	6*	10*	0	0	1	7*
	78	0	0	0	4	0	0	0	5*
Spleen granular/rough surface (*n* =)	26	0	0	2	10*	0	0	1	7*
	78	0	0	0	18*	0	0	0	19*
Spleen haemosiderosis/pigment (median grade) *n* =	26	8 (2)	10(1)	10(1)	10(2)	7(3)	10(2)	10(2)	10(3)
	78	20(2)	19(3)	20(3)	18(3)	20(2)	20(3)	20(4)	20(3)

Dose (mg/kg bw per day) adjusted for pure aniline	Week	Males				Females			
		0	7	21	70	0	7	21	70
Spleen haematopoiesis (median grade) *n* =	26	5(1)	5(1)	9(2)	10(3)	7(2)	5(2)	10(2)	10(2)
	78	17(1)	20(3)	20(3)	20(3)	20(2)	20(3)	20(3)	20(3)
Spleen capsulitis (median grade) *n* =	26	0	7(1)*	8(2)*	10(4)*	0	5(2)*	4(2)	8(2)*
	78	0	0	6(1)*	20(4)*	0	0	3(1)	18(2)*
Spleen stromal hyperplasia (median grade) *n* =	26	0	0	0	0	0	0	0	0
	78	0	0	1(2)	6(5)*	0	0	0	1(1)
Spleen congestion (median grade) *n* =	26	0	0	0	0	0	0	0	0
	78	0	20(3)*	20(4)*	20(4)*	0	3(2)	17(3)*	18(4)*
Spleen tumours (*n* =)	104								
Fibrosarcoma		0	0	0	3*	0	0	0	0
Stromal sarcoma		0	0	1	21*	0	0	0	0
Haemangiosarcoma		0	0	0	6*	0	0	0	1
Osteogenic sarcoma		0	0	0	3*	0	0	0	0
Adrenal phaeochromocytoma	104	3	3	1	3	5	1	0	1

N = 10 at week 26, *N* = 20 at week 78 and *N* = 90 at week 104

* Significant at $p < 0.05$

§ Spleen weights not measured prior to week 78

Source: CIIT, 1982

Benchmark dose (BMD) modelling using Proast v66.39 and model averaging gave the following values:

Lower confidence limit on the benchmark dose for a 1% response ($BMDL_1$)

- $BMDL_1$ for all spleen tumours = 13.5 mg/kg bw per day
- $BMDL_1$ spleen stromal sarcoma = 16.4 mg/kg bw per day

Lower confidence limit on the benchmark dose for a 10% response ($BMDL_{10}$)

- $BMDL_{10}$ for all spleen tumours = 38.6mg/kg bw per day
- $BMDL_{10}$ spleen stromal sarcoma = 43.4 mg/kg bw per day

Study 3

A review of the histopathology slides from the NCI study was reported by Weinberger et al., (1985). The incidences of animals with tumours of the spleen were broadly consistent with those of the NCI report, but the description/categorization of the tumours was different (Table 7). The pre-neoplastic findings were categorized, against defined criteria, into six classes, which differed from those used in the NCI report (Table 7). The only pre-neoplastic lesion which correlated well with tumours was fatty metamorphosis, as all the others were present at similar incidences and severities in males and females, and in both dose groups.

Table 7. Findings of the histopathology review of the spleen slides from the NCI study of F344 rats (Weinberger et al., 1985)

	Males			Females		
Dose (ppm)	**0**	**3000**	**6000**	**0**	**3000**	**6000**
N =	25	50	50	25	50	50
Fatty metamorphosis (*n* =)	0	18**	23**	0	2	4
Mean severity	0	0.41**	0.46**	0	0.09	0.18
Fibrosis (*n* =)	3	46**	46**	1	45**	48**
Mean severity	0.16	2.4**	2.7**	0.02	1.3**	1.1**
Capsule hyperplasia (*n* =)	0	44**	40**	1	49**	46**
Mean severity	0	1.4**	1.6**	0.2	1.4**	1.7**
Haemorrhage (*n* =)	1	42**	44**	1	43**	48**
Mean severity	0.02	2.2**	2.0**	0.02	1.2**	2.1**
Haemosiderosis (*n* =)	21	45	46	21	45	49
Mean severity	1.1	1.7**	1.3	1.6	1.8	1.6
Haematopoiesis (*n* =)	22	42	37	21	45	45
Mean severity	0.9	0.8	0.7	1.4	1.4	1.2
Tumours (*n* =)						
Fibroma	0	6	2	0	0	0
Fibrosarcoma	0	7	26*	0	2	4
Well differentiated	0	2	12	0	0	0
With osseous metaplasia	0	4	6	0	0	0
Poorly differentiated	0	1	7	0	2	4
Haemangioma	0	0	0	0	0	1
Total	0	13*	28**	0	2	5

* Significant at $p < 0.05$ ** Significant at $p < 0.01$ (Fisher's exact test for incidence, Student's *t*-test for severity)

Weinberg et al. (1985) proposed that the splenic tumours produced by oral aniline exposures progressed from an initial event or events (which could not be confirmed from the available data) resulting in acute vascular congestion→haemorrhage→fibrosis→transformed cell→fatty metamorphosis→tumours.

Although the NCI and CIIT studies and the re-evaluation of the NCI slides categorize the splenic tumours differently, the conclusions are consistent: aniline is carcinogenic to the spleen of F344 male rats at doses also producing a range of non-neoplastic lesions.

2.4 Genotoxicity

The genotoxicity of aniline has been studied extensively and reported mainly in the published literature, and reviewed by a number of national and international bodies (ECB, ICH, IARC, EFSA) and in the published literature (Bomhard et al., 2005) Conclusions in many (but not all) assays are consistent, indicating that it exhibits weak genotoxic potential both in vitro (with and without metabolic activation) and in vivo. The positive results are generally found at concentrations/doses producing significant

cytotoxicity or general toxicity. The most consistent finding is of clastogenicity, seen not only in specific assays for clastogenicity but also as increases in "small colonies" in a number of the mouse lymphoma assays for mammalian cell gene mutation. The summary below is taken from the review by the International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use (ICH, 2015):

> Aniline is not mutagenic in *Salmonella* with or without S9 or in *E. coli* WP2 uvrA with S9 up to 3000 µg/plate. Further Ames study data are described in databases and show aniline to be negative in all 5 standard strains.
>
> Aniline was mutagenic in the mouse lymphoma L5178Y cell tk assay with and without S9 at quite high concentrations.
>
> Chromosomal aberration tests gave mixed results; both negative and some weakly positive results are reported in hamster cell lines at very high, cytotoxic concentrations, e.g., about 5 to 30 mM, with or without S9 metabolic activation.
>
> In vivo, chromosomal aberrations were not increased in the bone marrow of male CBA mice after two daily i.p. doses of 380 mg/kg, but a small increase in chromosomal aberrations 18 h after an oral dose of 500 mg/kg to male PVR rats was reported.
>
> Most studies of micronucleus induction are weakly positive in bone marrow after oral or i.p. treatment of mice or rats and most commonly at high doses, above 300 mg/kg. Dietary exposure to 500, 1000 and 2000 ppm for 90 days was associated with increases in micronuclei in peripheral blood of male and female B6C3F1 mice.
>
> In vivo, a weak increase in Sister Chromatid Exchanges (SCE), reaching a maximum of 2-fold increase over the background, was observed in the bone marrow of male Swiss mice 24 h after a single intraperitoneal dose of 61 to 420 mg/kg aniline. DNA strand breaks were not detected in the mouse bone marrow by the alkaline elution assay.

(a) In vitro

No new data submitted

(b) In vivo

To investigate whether the mutagenicity of aniline was evident in the spleen, and its potential relevance to the cancer MOA, a study was performed in transgenic rats.

In a 28-day study of genotoxicity, groups of transgenic Fischer 344 Big Blue® male rats ($n = 6$ for the vehicle control or $n = 8$ for the aniline-treated groups) were administered aniline (purity 99.94%; batch SHBH0581V) at doses of 0, 25, 50 or 100 mg/kg bw per day. Treatment was performed once daily via oral gavage for 28 consecutive days in a vehicle of 1% carboxymethyl cellulose. Positive control animals ($n = 6$ per group) for the gene mutation assay were treated with *N*-ethyl-*N*-nitrosourea (ENU) at 20 mg/kg bw per day, on days 1, 2, 3, 12, 19 and 26. For the micronucleus (MN) assay ($n = 6$) they received cyclophosphamide (CP) at 15 mg/kg bw per day on days one and two, and at 12 mg/kg bw per day on days 26 and 27. All doses were administered at a dose volume of 10 mL/kg bw.

Surviving animals were bled from the retro-orbital sinus on days 4 and 29, for MN evaluation via flow cytometry. Surviving animals in all groups were sacrificed by CO_2 overdose on day 31. Liver, spleen, bone marrow and testes were collected, weighed (except for bone marrow), flash frozen and stored at or below −60°C. Liver, spleen and bone marrow from five animals per group were processed for DNA isolation and analysis of cII mutants.

General toxicity findings on the spleen and blood parameters are described above. No increase in mutant frequency was seen in the liver, spleen or bone marrow of any aniline-treated animals (Table 8). Statistically significant increases in the proportion of micronucleated reticulocytes were seen in all aniline-treated groups on day 29, together with increases in total reticulocyte counts (Table 8).

Aniline increased the incidence of micronuclei in the peripheral blood of male F344 Big Blue® rats in a time- and dose-dependent manner and met the overall criteria for a positive result. These exposures also produced significantly increased levels of MetHb and stimulated the bone marrow to increase the production of reticulocytes (Table 8). Tweats et al. (2007) reported that stimulation of

erythropoiesis (for example, with recombinant erythropoietins) and the resulting increased levels of immature erythrocytes in the blood, can lead to increases in the proportion of cells with micronuclei. Authors of the study report concluded that the micronuclei findings at 25 and 50 mg/kg bw per day were not "positive", as the results were within the 95% upper control limit (non-concurrent data, 2013–2015). However the Meeting considered the results at 50 mg/kg bw per day on day 29 to be positive as they are the same as the positive control, and the the group mean value is also outside the 95% range of the historical control data (HCD). The results at 25 mg/kg bw per day are considered to be equivocal as they are statistically significant and part of a dose–response relationship, but only slightly above the historic control mean.

Table 8. Findings in male Big Blue rats exposed to aniline for up to 28 days

Dose (mg/kg bw per day)	0	25	50	100	Positive control
Plasma aniline (ng/mL; day 28)	BLQ	1812	8320	18 400	NA
Reticulocytes (10^6/mL; day 29)	248	347**	606**	840**	
Reticulocytes with micronuclei (%)					
Day 4	0.06 ± 0.02	0.08 ± 0.03	0.11 ± 0.02*	0.26 ± 0.06**	0.37 ± 0.13**
Day 29	0.06	0.13 ± 0.02**	0.18 ± 0.03**	0.28 ± 0.04**	0.18 ± 0.07**
Mutant cII frequency (per 10^6 cells)					
Liver	34	30	28	24	288**
Spleen	25	23	17	19	313**
Bone marrow	19	20	20	16	365**

* Significant at $p < 0.05$ ** Significant at $p < 0.01$

BLQ Below limit of quantitation (300 ng/mL)

Aniline did not increase the frequency of cII mutants in the spleen, liver or bone marrow of F344 Big Blue® rats exposed orally at 100 mg/kg bw per day for 28 days (Table 8).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

There are no guideline-compliant studies of the potential reproductive toxicity of aniline.

(b) Developmental and postnatal toxicity

In a study of developmental and postnatal toxicity, presumed-pregnant F344 rats (12–15/group) were dosed by gavage with aniline hydrochloride in distilled water at 0, 10, 30 or 100 mg/kg bw per day (equivalent to 7, 21 or 70 mg/kg bw per day as pure aniline) on days 7–20 of gestation. A positive control group of hydroxyurea (200 mg/kg bw per day) was included. Dams were allowed to deliver naturally (up to gestation day (GD) 24) and then nurse the pups until postnatal day (PND) 30; pups were killed on PNDs 10, 25, 50 or 60. Attainment of developmental landmarks and functional observations were performed during pup growth. In dams receiving 100 mg/kg bw per day, MetHb levels, relative spleen weights and RBC mean cell volume were increased. There were no adverse effects on gestational parameters, developmental landmarks or functional observations. More pups died in the aniline-treated groups: 3, 6, 15 and 13 in the control, low-, mid- and high-dose groups respectively, but the total number of pups alive or days of death are not given. Sporadic increases in relative spleen and liver weights of pups were reported but exhibited no consistent pattern. Changes in haematological parameters (increased mean corpuscular volume, MCV) were reported for top-dose pups on PND 0 but not subsequently; the magnitude was < 10% and was considered not to be adverse. The positive control increased the incidences of resorptions and malformations.

The NOAEL for maternal toxicity was 21 mg/kg bw per day (as pure aniline), based on increases in MetHb and relative spleen weights. The NOAEL for offspring toxicity was 7 mg/kg bw per day, based on increased pup mortality at 21 mg/kg bw per day. (Price et al., 1985).

Rat

In a study of developmental toxicity, presumed-pregnant F344 rats (21–24/group) were dosed with aniline hydrochloride by gavage at 0, 10, 30 or 100 mg/kg bw per day (equivalent to pure aniline at 7, 21 or 70 mg/kg bw per day) on GDs 7–20. In addition to routine maternal and fetal examinations, haematological investigations were performed on dams and fetuses in the high-dose group. At 100 mg/kg bw per day, maternal body weight gains were reduced (by 25%), MetHb levels were increased (2–3-fold), RBC counts were reduced (by 28%) and reticulocytes counts were increased (4-fold). Spleen weight relative to body weight was increased significantly, by > 25% and in a dose-related fashion, in the dams of all dose groups. There were no adverse effects on gestational parameters or on the fetuses; statistically significant increases (< 10%) in relative liver weight and MCV are considered not to be adverse.

The LOAEL for maternal toxicity was 7 mg /kg bw per day (as pure aniline), the lowest dose tested, based on increased relative spleen weights. The NOAEL for aniline for embryo/fetal toxicity was 70 mg/kg bw per day, the highest dose tested (Price et al., 1985).

2.6 Special studies

(a) Methaemoglobin production

MetHb production following aniline exposure has been reported in a number of studies, including some described above.

Study 1
Jenkins et al. (1972) investigated the production of MetHb in human volunteers and rats administered aniline, and the effects of aniline metabolites on rats and human and rat erythrocyte preparations.

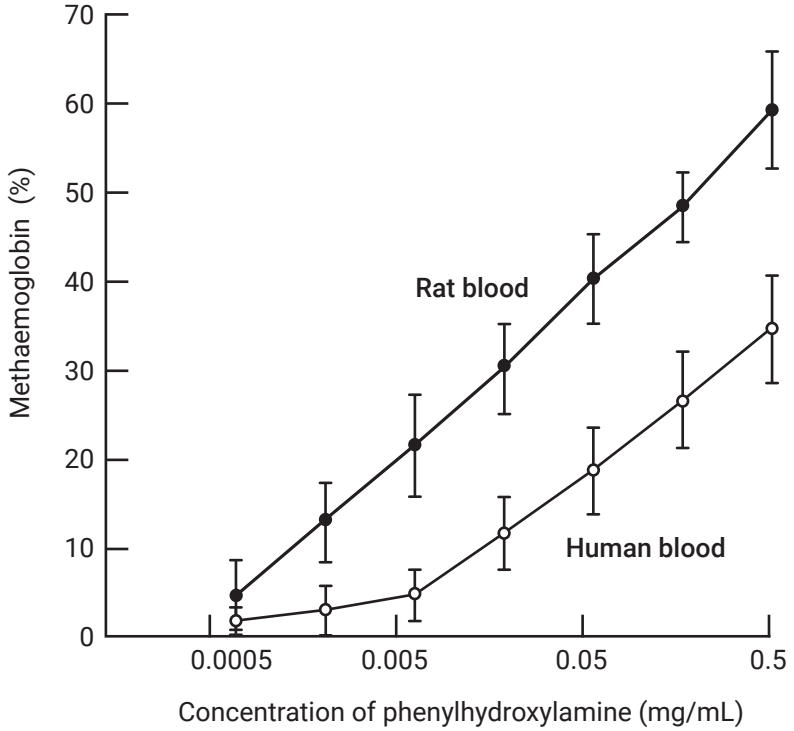
Human volunteers (17 males, three females; ages 22–24 years; weights not specified) received oral doses of aniline at 5, 15 or 25 mg/person per day on three consecutive days. Five volunteers received 35 or 45 mg/person per day, two volunteers received 55 mg/person per day and one volunteer received 65 mg per day. Pinprick blood samples were taken 1, 2, 3 or 4 h after each dose for MetHb analysis. Venipuncture samples were taken 24 h post dose for a range of haematology and clinical chemistry investigations. There were no treatment-related changes to any measured parameters except MetHb levels. Volunteers exposed to 5 or 15 mg of aniline had small, (and not statistically significant) increases in MetHb levels of < 2%. Volunteers receiving 25 to 55 mg aniline had statistically significant increases in MetHb (2.5–7%). The volunteer receiving 65 mg had a peak MetHb level of 16% at two hours post dose, but this had returned to normal at the three-hour sample, showing rapid recovery.

Study 2
Groups of Wistar rats received aniline i.v. at 5 to 200 mg/kg bw or orally at 20, 40, 100, 200, 300 or 1000 mg/kg bw. Blood samples (tail snip) were taken at 0, 30 or 60 minutes after dosing for MetHb determination. Rats dosed i.v. with 5 or 10 mg/kg bw had no increase in MetHb; 20 mg/kg bw resulted in a MetHb level of 10%; 200 mg/kg bw produced a MetHb level of 46%. Oral exposure at 20 mg/kg bw produced a minor increase in MetHb (3.3% versus 2.4% in controls); 40, 300 and 1000 mg/kg bw resulted in MetHb levels of 17% , 18 and 47% respectively.

Study 3
Groups of Wistar rats received aniline in the diet at 930 ppm for 13 days, or its metabolites 4-aminophenol or phenylhydroxylamine at 1090 ppm (equimolar doses). Spleen weights were increased in rats receiving aniline (60%) and phenylhydroxylamine (450%). Rats exposed to aniline exhibited increased erythopoietic activity, engorgement and haemosiderosis of the spleen. Those exposed to phenylhydroxyalamine had more marked effects on the spleen than those exposed to aniline, together with haemosiderosis and necrosis of the kidney and liver. Rats fed 4-aminophenol exhibited no adverse effects.

Rat and human erythrocytes were exposed to phenylhydroxylamine at 0.0005–0.5 mg/mL. Rat erythrocytes demonstrated a linear increase in MetHb, but human cells were less sensitive and demonstrated an initial lag phase (Fig. 2). Rat erythrocytes exposed to nitrosobenzene (0.002%) in the presence of glucose had MetHb levels 2 to 3-fold higher than cells without added glucose; after one hour erythrocyte preparations incubated with glucose had a MetHb level of over 50%.

Figure 2. MetHb formation in rat and human erythrocyte preparations exposed to phenylhydroxylamine



Redrawn from Jenkins et al., 1972

This study shows that, for phenylhydroxylamine, humans are less sensitive than rats to acute exposure, judged by the resulting production of MetHb. Human recovery from aniline-induced MetHb at 16% was reported to be very rapid, essentially complete within one hour. The aniline metabolite phenylhydroxylamine is more toxic than aniline to rats.

The NOAEL for MetHb production in humans was 15 mg/person (equivalent to 0.2 mg/kg bw per day) based on significant increases in MetHb at 25 mg/person (equivalent to 0.35 mg/kg bw per day) (Jenkins et al., 1972).

Study 4

In an in vitro investigative study, rat erythrocytes were exposed for two hours to aniline at 3.2 mM or phenylhydroxylamine at 0.1 mM. Levels of free iron, MetHb and glutathione (GSH) were determined. Aniline treatment did not affect free iron or MetHb levels, while GSH levels were decreased by approximately 20%. Phenylhydroxylamine-treated preparations had significantly higher levels of free iron and MetHb (5.5-fold and 7-fold respectively) than aniline-treated or control cells; GSH levels were approximately 50% of those in controls. This study indicates that in the absence of metabolism, aniline concentrations of 3.2 mM do not induce MetHb in rat erythrocytes, but that aniline's metabolite phenylhydroxylamine at 0.1 mM does do so (Ciccoli et al., 1999).

Study 5

In an in vitro study to investigate activities of MetHb reductase and levels of MetHb, erythrocyte preparations were sourced from rats, mice, beagle dogs, hamsters and mastomys. MetHb reductase activities were lowest in beagles and rats (0.05 and 0.1 nmol/min. per mg protein, respectively) with the other species higher at 0.17 to 0.24 nmol/min. per mg protein. Despite these differences in MetHb

reductase activities, there were no differences in the levels of MetHb produced after erythrocytes from rats, mice, hamsters and mastomys were incubated with sodium nitrate (0.25–1 mM) or primaquine (1–5 mM). The findings in beagles are not reported clearly (Srivastava et al., 2002). This work indicates that the two-fold higher level of MetHb reductase seen in mice compared to rats might not be the primary reason for the species differences in response to aniline.

(b) Binding to haemoglobin

In an investigative study to derive a biomonitoring method for aromatic amine exposures, two female Wistar rats received aniline hydrochloride (99% pure) by gavage at 0.47 mmol/kg bw (60 mg/kg bw). Blood samples were taken (timing not given), the haemoglobin extracted and analysed for aducts. The haemoglobin binding indices (HBIs), measured as (mmol substance/mol Hb)/dose (mmol/kg bw), were 22.8 and 21.6 in the two animals. Related work on equimolar doses of acetanilide and nitrosobenzene was reported, showing that the HBI for nitrobenzene was 3 to 4-fold higher than that of aniline, and that aniline and acetanilide could not be distinguished in terms of haemoglobin adducts, indicating a common pharmacokinetic pathway (Albrecht & Neumann, 1985). Subsequent work with a range of aromatic amines (Birner & Neumann, 1988) reported that there was a correlation between the HBI and levels of MetHb. Aniline was of relatively low potency (fifth out of six; HBI 22, MetHb 1.7%). The most potent was 4-chloroaniline (HBI 569, MetHb 49%).

(c) Effect on lipid peroxidation, protein oxidation and iron

Study 1

The potential for aniline to increase lipid peroxidation, oxidize proteins and alter iron levels in the spleen was investigated in groups of Wistar rats (five males/group) receiving aniline in their drinking water to give doses of 65 mg/kg bw per day for one, two or three months. There were time-dependent increases in lipid peroxidation (by 40–60%), protein oxidation (by 20–70%) and splenic iron content (2 to 4-fold). Associated pathology findings included expansion of the red pulp, dilated sinusoids, vascular congestion, capsular thickening with hyperplastic change and fibrosis of the red pulp, especially around the splenic trabeculae (Khan et al., 1999a).

Study 2

Groups of five male SD rats received 0.5 mmol aniline hydrochloride(ca 60 mg/kg bw per day) by gavage, 3% carbonyl iron-supplemented diet, or a combination, for 30 days. Controls received a basal diet only. On day 30, the spleens were investigated for iron content (total and free/chelatable), lipid peroxidation (malondialdehyde), protein oxidation and morphology. The findings showed that both aniline and iron increased the levels of iron and lipid peroxidation; protein oxidation was increased by aniline but not iron administration. There was evidence of some additive effects with coadministration of aniline and iron (Table 9). Splenic morphology of aniline-treated rats was reported to show vascular congestion, red pulp expansion and thickening of the capsule, which was more pronounced in rats receiving aniline and the iron-supplemented diet. Iron deposition was more extensive in the rats receiving the combination (Khan et al., 1999b). The increase in levels of free (chelatable) iron could lead to oxidative stress from free radical production (Papanikolaou & Pantopoulos, 2005).

Table 9. Levels of splenic iron, lipid peroxidation and protein oxidation (all as % relative to controls) in rats receiving aniline, iron supplementation or a combination, for 30 days

	Control	Aniline hydrochloride (0.5 mmol/kg bw per day)	Carbonyl iron diet	Combination
Total iron	100	422	590	916
Chelatable iron	100	207	244	285
Lipid peroxidation	100	129	139	165
Protein oxidation	100	167	ca 115[§]	185

[§] Exact value not given; figure based on a graphical presentation

2.7 Observation in humans

Suicidal oral intake of 60 ml aniline resulted in death at day 4 after intake. Initially, MetHb formation had increased to 85% then decreased to 27% at day 4. During this time interval *p*-aminophenol excretion was approximately 8.4 mg/h. Pathologic assessment revealed degenerative changes in myocardium, liver and kidney, oedema in lung and brain and haemorrhages in the medulla oblongata (cited by ECB, 2004).

Human volunteers (17 male, 3 female, ages 22–24 years, weights not specified) received an oral dose of aniline of 5, 15 or 25 mg/person per day on three consecutive days. Five volunteers received 35 or 45 mg/person per day, two received 55 mg/person per day and one volunteer received 65 mg per day. Pin-prick blood samples were taken at 1, 2, 3 or 4 h after each dose for MetHb analyses. Venipuncture samples were taken at 24 h post dose for a range of haematology and clinical chemistry investigations. There were no treatment-related changes in any of themeasured parameters except MetHb levels. Volunteers exposed to 5 or 15 mg aniline had small, not statistically significant, increases in MetHb levels (< 2%). Volunteers receiving 25–55 mg aniline had statistically significant increases in MetHb (2.5–7%). The volunteer who received 65 mg had a peak MetHb level of 16% at 2 h post dose, but this had returned to normal at the 3 h sample, showing rapid recovery (Jenkins et al., 1972). The NOAEL was 15 mg/person (equivalent to 0.2 mg/kg bw per day) based on significant increases in MetHb at 25 mg/person (equivalent to 0.35 mg/kg bw per day) (Jenkins et al., 1972).

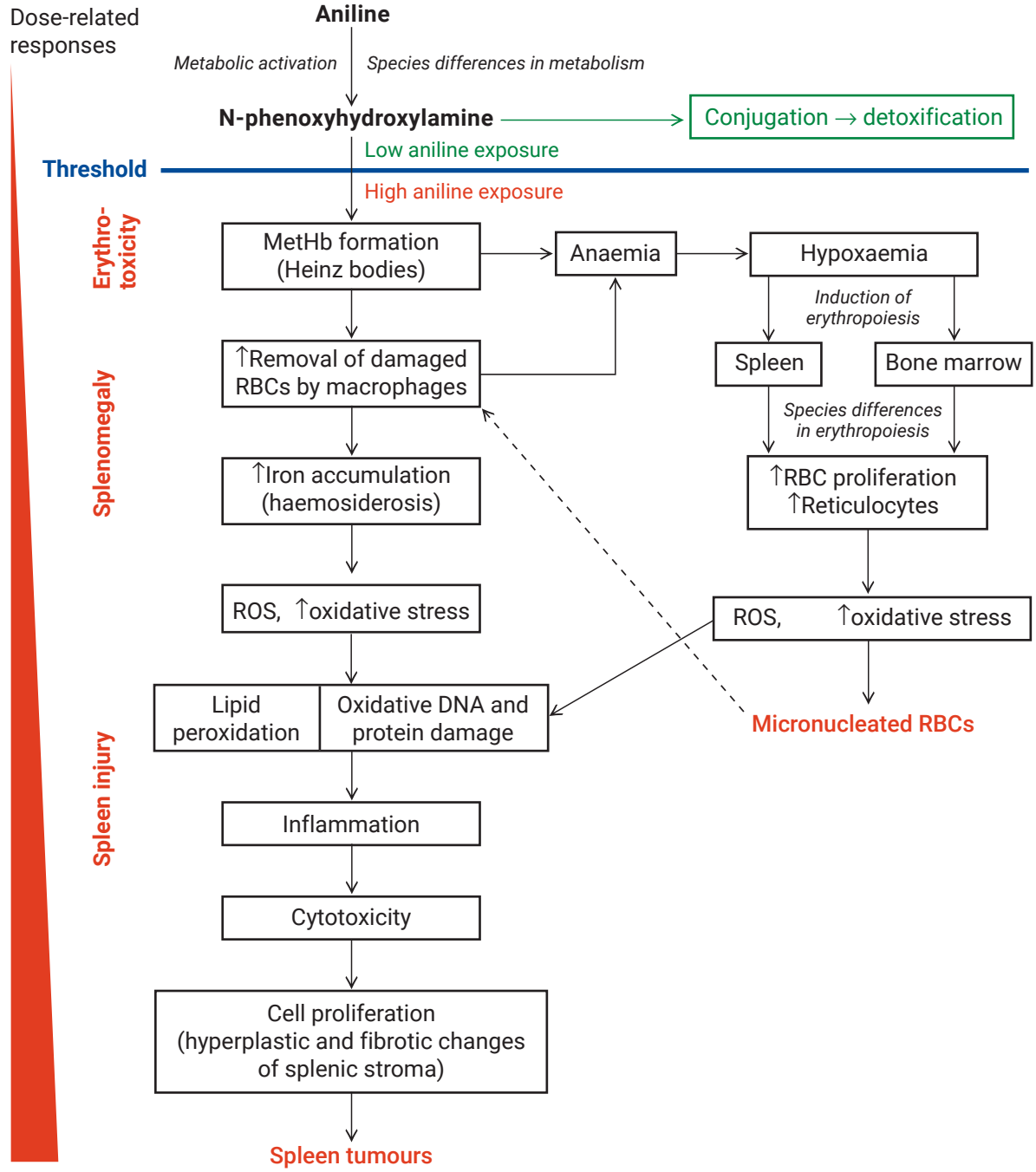
Data from investigations of skin sensitization in volunteers and dermatitis patients has indicated that aniline has skin sensitizing potential in humans (ECB, 2004).

The excess of bladder cancer deaths, observed in clusters of cases of workers in the aniline dye industry, has been attributed to exposure to chemicals other than aniline. Epidemiological studies of workers exposed to aniline, but not to other known bladder carcinogens, have shown little evidence of increased risk. A mortality study of 342 men employed in the manufacture of organic dyes, in which two of the three processes involved aniline as a raw material, showed no death from bladder cancer (IARC, 1987).

2.8 Mode of action proposal for splenic tumours

A MOA for the splenic tumours seen in rats has been proposed and is outlined in Fig 3. The sponsor provided a detailed document to support the MOA, which is presented in the Annex 1. Extensive considerations of this MOA can also be found in the reviews by the ECB, (2004), MAK (2010) and SCOEL (2016). The line of argumentation behind the MOA is not intended to demonstrate that humans are not at any risk from splenic tumours following any level of aniline exposure, but that there is a threshold below which human exposures would likely be expected not to result in spleen tumours.

Figure 3. Overview of events involved in the AOP for spleen tumour development of aniline



Redrawn from sponsor's original

Key elements of the MOA include:-

- A threshold for spleen tumours was seen in rats (at 7 mg/kg bw per day, zero tumours out of 90 rats examined).
- Spleen tumours have a very low background rate in humans and have not been reported in epidemiology studies of aniline exposures (however, bladder tumours were the focus of most studies).
- A dose- and time-related progression from redox associated erythrocyte damage, MetHb production, filtering of damaged erythrocytes by the spleen, increased iron levels in the spleen, oxidative stress and oxidation of macromolecules, development and progression of pre-neoplastic lesions (haemosiderosis, congestion, fatty metamorphosis, capsule thickening), to late onset of tumours, has been identified.

- MetHb production is seen in humans exposed to aniline, indicating that relevance to humans cannot be discounted.
- Aniline produced no increase in mutations in the spleen of the same strain of rats used in the cancer studies. The mutation study was well performed and used an upper dose level which produced a significant increase in splenic tumours. The binding of aniline-derived radiolabel to DNA was higher in other organs/tissues than in the spleen, which was the only site of tumours. An alternative mode of action based on mutagenicity/direct DNA reactivity is therefore unlikely to apply to the spleen tumours seen in rats following exposure to aniline.
- Although some aspects have not been fully investigated (for instance, species and sex differences in spleen tumours) these are not considered crucial to determining if a threshold-based MOA applies.

The mode of action behind the spleen tumours has been adequately investigated. Aniline was clastogenic in a number of assays but was not mutagenic in vivo to the liver, bone marrow or spleen of male rats. It is considered that the low level of DNA binding of aniline metabolites seen in a number of tissues was unlikely to be the cause for the significant increase of spleen tumours in male rats observed at moderate doses. This is supported by the absence of a tumour response in other organs which also showed high levels of binding. A range of studies support the proposed MOA based on redox-associated damage to erythrocytes, increases in Heinz bodies, and stimulation of erythropoiesis. The damaged erythrocytes are removed by the spleen, which leads to an increase in free iron deposition in the spleen, production of reactive oxygen species, protein oxidation and lipid peroxidation. These changes result in a progression of pathological lesions in the spleen from congestion, capsulitis, to hyperplasia and tumours. Although the available studies do not address all the tumour findings (for example the sensitivity of male rats versus females and absence of splenic tumours in mice), the proposed MOA is plausible, and taken with the absence of mutations in the spleen in the in vivo gene mutation study, support a threshold MOA secondary to erythrocyte damage. Humans exposed to aniline produce significant levels of MetHb (a biomarker of redox damage) therefore the MOA is of relevance to humans.

If human exposures to aniline are at or below those which cause minimal increases in MetHb, splenic tumours are unlikely to be produced.

Comments

Biochemical aspects

Aniline is extensively absorbed when administered orally to rats (ca 90% at 50 or 250 mg/kg bw) and mice (ca 70% at 100 or 500 mg/kg bw), based on 0–24 h urine samples (McCarthy et al., 1985).

Peak plasma radioactivity levels in rats dosed with ^{14}C-aniline at 10, 30 or 100 mg/kg bw were 0.5, 1 and 2 h respectively (ECB, 2004). Following oral dosing of ^{14}C-aniline to rats, at 100 mg/kg bw for one day, the highest concentration of radiolabel was detected in erythrocytes (25 µg equiv./g), with lower levels (0.4– 4 µg equiv./g) in plasma, liver, spleen, kidney, lung and heart. Following 10 days dosing at 100 mg/kg bw per day, radioactivity in spleen was 12 times higher than after one day; other tissues had increases of 1.8- to 3.8-fold. Covalent binding of radioactivity to proteins was six times higher in the spleen than in the liver (Bus & Sun, 1979).

Excretion is rapid in rats and humans with a half-life of approximately three hours (ECB, 2004).

The metabolism of aniline is via *N*-hydroxylation, *N*-acetylation and/or hydroxylation of the phenyl ring, followed by conjugation with glucuronic acid or sulfate (ECB, 2004; McCarthy et al., 1985). The excretory pathway in rats relies extensively on sulfate conjugation, which is reported to become saturated at doses above 50 mg/kg bw. In mice, conjugation is with glucuronic acid and this pathway is reported not to become saturated (McCarthy et al., 1985). The proportion of *N*-hydroxylation or *N*-acetylation is reported to vary with species, sex and, in humans, acetylation phenotype (ECB, 2004). The *N*-hydroxy metabolite, *N*-hydroxyaniline (phenylhydroxylamine), is reported to be oxidized to nitrosobenzene in erythrocytes, leading to the production of MetHb and a sulfuric acid amide adduct of haemoglobin (Mellert et al., 1984).

Toxicological data

Aniline has a median lethal dose (LD_{50}) of 442 mg/kg bw in rats and a median lethal concentration (LC_{50}) of 1 mg/L in rats. Aniline is slightly irritating to the skin, severely irritating to the eye and exhibits skin sensitizing potential in guinea pigs and humans.

In an eight-week range-finding study, mice received aniline hydrochloride at 0, 100, 300, 3000 or 10 000 ppm in the diet weeks (equivalent to aniline exposures of 11, 32, 324 or 1080 mg/kg bw per day). At 3000 ppm (equivalent to 324 mg/kg bw per day) and above, all mice had dark, granular and enlarged spleens (NCI, 1978). There is insufficient information to determine a NOAEL.

In an eight-week range-finding study, rats received aniline hydrochloride at 0, 100, 300, 3000 or 10 000 ppm in the diet (equivalent to aniline exposures of 7, 21, 210 or 700 mg/kg bw per day). At 3000 ppm (equivalent to 210 mg/kg bw per day) and above, all rats had dark, granular and enlarged spleens (NCI, 1978). There is insufficient information to determine a NOAEL.

In a repeat dose toxicity study, male rats received aniline hydrochloride at variable concentrations in the diet for one or four weeks to provide nominal aniline exposures of 0, 4, 12 or 40 mg/kg bw per day. The LOAEL in this study was 4 mg/kg bw per day, the lowest dose tested, based on vascular congestion of the spleen and haemoglobin adducts (Mellert et al., 2004).

Aspects of the repeat-dose toxicity of aniline were investigated in a 28-day study of genotoxicity. Groups of transgenic Fischer 344 Big Blue® male rats were administered aniline at doses of 0, 25, 50 or 100 mg/kg bw per day, once daily via oral gavage. A NOAEL could not be determined from this study as changes in blood (increased MetHb and reticulocytes) and spleen (increased weight and iron deposition) were noted at all dose levels. The LOAEL was 25 mg/kg bw per day (McKeon & Ciubotaru, 2018).

In a long-term toxicity and carcinogenicity study, mice received aniline hydrochloride at 0, 6000 or 12 000 ppm in the diet for 103 weeks followed by a four-week observation period. Doses were equivalent to aniline exposures of 600 or 1200 mg/kg bw per day. Investigation of general toxicity was limited, and a NOAEL for general toxicity could not be derived. The NOAEL for carcinogenicity was 12 000 ppm (equivalent to 1200 mg/kg bw per day), the highest dose tested (NCI, 1978).

In a long-term toxicity and carcinogenicity study, rats received aniline hydrochloride at 0, 3000 or 6000 ppm in the diet for 103 weeks followed by a four-week observation period. Doses were equivalent to aniline exposures of 105 or 210 mg/kg bw per day. Tumours of the spleen (fibrosarcoma, stromal sarcoma, haemangiosarcoma, osteogenic sarcoma) were increased in both male groups and high-dose females. The LOAEL for carcinogenicity was 3000 ppm (equivalent to 105 mg/kg bw per day) the lowest dose tested, based on increases in tumours of the spleen in males at this dose level. The LOAEL for toxicity was 3000 ppm (equivalent to 105 mg/kg bw per day) the lowest dose tested, based on papillary hyperplasia of the spleen in females (NCI,1978). A review of the histopathology slides confirmed the carcinogenicity and general toxicity to the spleen (Weinberger et al., 1985).

In a second long-term toxicity and carcinogenicity study, in the same strain, rats received aniline hydrochloride in the diet at variable concentrations, for two years to give nominal aniline exposures of 7, 22 or 72 mg/kg bw per day. Tumours of the spleen were increased in males in the mid- and high-dose groups, but not in females. The LOAEL for general toxicity was 7 mg/kg bw per day, based on reduced erythrocyte counts, and pathological changes in the spleen at this dose level. The NOAEL for carcinogenicity was 7 mg /kg bw per day (CIIT, 1982). The Meeting calculated $BMDL_{10}$ values for all spleen tumours of 38.6 mg/kg bw per day and 43.4 mg/kg bw per day for stromal sarcoma of the spleen.

The Meeting concluded that aniline is not carcinogenic in mice but carcinogenic in rats.

Aniline has been investigated in a wide range of in vitro and in vivo genotoxicity studies of varying quality. It was negative in bacterial mutation assays but some positive results, with and without metabolic activation, have been reported in in vitro mammalian cell gene mutation assays and clastogenicity studies in vitro and in vivo. The most consistent finding was of clastogenicity, which was supported by colony size analysis in the mouse lymphoma gene mutation assays. Low levels of aniline-derived radioactivity binding to DNA in some organs were seen at high doses of aniline (500 mg/kg bw), possibly via the production of reactive intermediates such as *N*-hydroxyphenylamine

and *p*-hydroxyacetanilide. In a 28-day study of genotoxicity, groups of transgenic Fischer 344 Big Blue® male rats were administered aniline at doses of 0, 25, 50 or 100 mg/kg bw per day, via oral gavage. A dose-related increase in micronucleated erythrocytes from the peripheral blood was seen at four and 29 days, together with increases in erythropoiesis and numbers of reticulocytes. There was no increase in cII mutants in the spleen, liver or bone marrow at any dose level (McKeon & Ciubotaru, 2018).

There is evidence that aniline is clastogenic in vitro and in vivo but not mutagenic in vivo.

The Meeting considered that the clastogenicity of aniline was due to a mechanism secondary to reactive oxygen production and that a threshold would apply. The Meeting concluded therefore that aniline is unlikely to be genotoxic at estimated dietary exposure levels.

The mode of action for formation of spleen tumours

The MOA behind the spleen tumours has been investigated. The meeting considered that the low level of DNA binding of aniline metabolites was unlikely to be the cause for the significant increase of spleen tumours in male rats observed at moderate doses. This is supported by absence of tumour response in other organs. A range of studies support the proposed mode of action based on redox-associated damage to erythrocytes, increase in Heinz bodies, and stimulation of erythropoiesis. The damaged erythrocytes are removed by the spleen, which leads to an increase in free iron deposition in the spleen, production of reactive oxygen species, protein oxidation and lipid peroxidation. These changes result in a progression of pathological lesions in the spleen from congestion, capsulitis, to hyperplasia and tumours. Although the available studies do not address all the tumour findings (e.g. sensitivity of male rats versus females and no splenic tumours in mice), the proposed MOA is plausible and taken with the absence of mutations in the spleen in the in vivo gene mutation study, it supports a threshold MOA secondary to erythrocyte damage. Humans exposed to aniline produce significant levels of MetHb (a biomarker of redox damage) therefore the MOA is of relevance to humans.

The Meeting concluded that, based on the absence of gene mutations in the spleen and a clear threshold for splenic tumours by the established MOA, aniline is unlikely to be carcinogenic to humans at estimated dietary exposure levels.

In a study of developmental and postnatal toxicity, rats were dosed with aniline hydrochloride by gavage at 0, 7, 21 or 70 mg/kg bw per day (as aniline) on days 7– 20 of gestation. Dams were allowed to deliver naturally (up to day 24 of gestation) and then nurse the pups until PND 30. The NOAEL for maternal toxicity was 21 mg/kg bw per day, based on increases in MetHb and relative spleen weights at 70 mg/kg bw per day. The NOAEL for offspring toxicity was 7 mg/kg bw per day, based on increased pup mortality at 21 mg/kg bw per day (Price et al., 1985).

In a study of developmental toxicity, rats were dosed with aniline hydrochloride by gavage at 0, 7, 21 or 70 mg/kg bw per day on days 7–20 of gestation. The LOAEL for maternal toxicity was 7 mg/kg bw per day, the lowest dose tested, based on increased relative spleen weights at this dose level. The NOAEL for embryo/fetal toxicity was 70 mg/kg bw per day, the highest dose tested (Price et al., 1985).

The Meeting concluded that aniline is not teratogenic in rats.

Microbiological data

No data are available.

Human data

Human volunteers received an oral dose of aniline of 5, 15, 25, 35, 45, 55 or 65 mg/person per day on three consecutive days. Volunteers exposed to 5 or 15 mg of aniline had small, not statistically significant, increases in MetHb levels (< 2%). Volunteers receiving 25 mg to 55 mg aniline had statistically significant increases in MetHb (2.5% to 7%). The volunteer receiving 65 mg of aniline had a peak MetHb level of 16% at 2 h post dose but this had returned to normal at the 3 h sample, showing rapid recovery. The NOAEL was 15 mg/person (equivalent to 0.2 mg/kg bw per day) based on significant increases in MetHb at 25 mg/person (equivalent to 0.35 mg/kg bw per day) (Jenkins et al., 1972).

The excess of bladder cancer deaths observed in clusters of cases of workers in the aniline-based dye industry has been attributed to exposure to chemicals other than aniline (IARC, 1987). Epidemiological studies of workers exposed to aniline, but not to other known bladder carcinogens, have shown little evidence of increased risk. A mortality study of 342 men employed in the manufacture of organic dyes, in which two of the three processes involved aniline as a raw material, showed no death from bladder cancer (IARC, 1987).

Toxicological evaluation

The Meeting established an ADI of 0–0.02 mg/kg bw on the NOAEL of 0.2 mg/kg bw per day for increases in methaemoglobin levels in a human volunteer study. As this observation was made in humans no interspecies safety factor was necessary and a safety factor of 10 was applied. There is a margin of 1100 between the upper bound of the ADI and the LOAEL for spleen tumours in the rat.

An ARfD of 0.02 mg/kg bw was established on the same basis as the ADI.

Levels relevant to risk assessment of aniline

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a]	Toxicity	-[b]	-[b]
		Carcinogenicity	12 000 ppm, equivalent to 1200 mg/kg bw per day [c]	-
Rat	28-day oral toxicity study [a]	Toxicity	-	Variable ppm to give 4 mg/kg bw per day[d]
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	-	Variable ppm to give 7 mg/kg bw per dayd
		Carcinogenicity	Variable ppm to give 7 mg/kg bw per day	Variable ppm to give 22 mg/kg bw per day
	One-generation study of developmental and post-natal toxicitye	Reproductive toxicity	-[b]	-[b]
		Parental toxicity	21 mg/kg bw per day	70 mg/kg bw per day
		Offspring toxicity	7 mg/kg bw per day	21 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	-	7 mg/kg bw per day [d]
		Embryo and fetal toxicity	70 mg/kg bw per day [c]	-
Rabbit	No data			
Dog	No data			
Human	Three-day study of toxicity [e]	MetHb production	0.2 mg/kg bw per day	0.35 mg/kg bw per day

[a] Dietary administration

[b] Inadequate investigation

[c] Highest dose tested

[d] Lowest dose tested

[e] Gavage administration

Acceptable daily intake (ADI) for aniline

0–0.02 mg/kg bw

Acute reference dose (ARfD) for aniline

0.02 mg/kg bw

Information that would be useful for the continued evaluation of aniline

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to aniline

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of absorption	Rapid (T_{max} 2 h) and extensive (70–90% based on urine)
Dermal absorption	No data
Distribution	Extensive; highest concentrations in RBC, liver, kidney, spleen
Potential for accumulation	High in spleen
Rate and extent of excretion	Rapid (half-life 3 h in rats, mainly in urine)
Metabolism	*N*-acetylation, *N*-hydroxylation, *C*-hydroxylation; conjugation with glucuronic acid or sulfate.
Toxicologically significant compounds in animals and plants	Aniline
Acute toxicity	
Rat, LD_{50}, oral	442 mg/kg bw
Rat, LC_{50}, inhalation	1 mg/L
Rabbit, dermal irritation	Slight irritant
Rabbit, ocular irritation	Severe irritant
Guinea pig/human, dermal sensitization	Evidence of sensitization
Short-term studies of toxicity	
Target/critical effect	MetHb formation, erythrocyte damage, toxicity to spleen
Lowest relevant oral LOAEL	4 mg/kg bw per day (rats)
Lowest relevant oral NOAEL	0.2 mg/kg bw per day (humans)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Carcinogenic to the spleen of rats MetHb formation, erythrocyte damage, toxicity to spleen
Lowest relevant LOAEL (toxicity)	7 mg/kg bw per day, lowest dose tested (rats)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans at estimated dietary exposure levels.
Carcinogenicity lowest NOAEL [a]	7 mg/kg bw per day (rats)
Genotoxicity [a]	Clastogenic in vitro and in vivo. Not mutagenic in vivo in the spleen, liver or bone marrow. Unlikely to be genotoxic at estimated dietary exposure levels.
Reproductive toxicity	
Target/critical effect	Reduction of pup survival
Lowest relevant reproductive NOAEL	7 mg/kg bw per day (rats)
Developmental toxicity	
Target/critical effect	None
Lowest relevant NOAEL	70 mg/kg bw per day, highest dose tested (rats)
Neurotoxicity	No data

Immunotoxicity	No data
Studies on toxicologically relevant metabolites	No data
Human data	NOAEL for MetHb in a three-day gavage study in volunteers is 0.2 mg/kg bw per day Reports of bladder cancer in workers exposed to multiple aromatic amines considered not to be related to aniline; no reports of spleen tumours

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Human volunteer	10
ARfD	0.02 mg/kg bw	Human volunteer	10

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Annex 1: Sponsor's MOA proposal for spleen tumours

Appendix-B

Aniline - Mode of Action Analysis using the WHO/IPCS MoA Framework

1. Summary

Chronic toxicity studies with aniline in laboratory animals are associated with the development of rare tumours in the spleen (haemangiosarcomas, fibrosarcomas, stromal sarcoma, oesteogenic sarcoma and fibroma). The tumours are species and sex-specific. Spleen tumours were observed only in male rats, but not in mice. The tumours occurred only at high-dose levels of aniline.

The tumour formation in the spleen due to aniline exposure is a complex process, which includes a primary damaging effect on erythrocytes (RBCs) and several successive effects, caused by the erythrotoxicity. Several of these effects are mandatory for the tumour formation in the spleen. The proposed Mode of Action includes a molecular initiating event and seven key events, as well as three associated events related to the erythrotoxicity.

Molecular initiating event	**Met-Hemoglobin (Met-Hb)-formation**
Key event 1	Increased splenic removal of damaged RBCs
Key event 2	Iron accumulation
Associated event 1	Anemia / hypoxemia
Associated event 2	Induction of erythropoiesis (extramedullary in spleen and medullary in bone marrow)
Associated event 3	Micronuclei formation
Key event 3	Formation of reactive oxygen species (ROS), oxidative stress
Key event 4	Oxidative damage of macromolecules (lipids, proteins, DNA) due to oxidative stress
Key event 5	Inflammation
Key event 6	Cytotoxicity
Key event 7	Cell proliferation in spleen

This chain of events can finally lead to tumour formation in the spleen.

Based on the adverse outcome pathway (AOP) described above the primary effect (molecular initiating event [MIE]) is the formation of Met-Hb. Aniline concentrations that do not lead to an exceedance of the metabolic detoxification pathways via conjugation, and which will not overwhelm the Met-Hb reductase capacity will not lead to toxic concentrations of Met-Hb, and therefore will not lead to exaggerated erythrotoxicity and successive events of the described AOP. Thus, a clear threshold exists for the Met-Hb formation, as well as for the tumour formation, which is most likely due to an iron-mediated toxicity.

Based on the available data the MIE and successive key events of the proposed MoA for tumour formation have been observed in rats. For humans not all key events have been observed, but this is mainly due to the fact that key events 3 to 7 can only be determined in tissue samples, which are

normally not taken from living persons. However, the MIE, as well as Key events 1 and 2, and the associated events anemia and increased erythropoiesis have been observed in humans.

From the available data a clear dose- and time-relation for the events leading to tumour formation can be derived. Aniline concentrations that do not lead to an exceedance of the metabolic detoxifying pathways, and which will not overwhelm the Met-Hb reductase capacity will not lead to toxic concentrations of Met-Hb, and therefore will not lead to erythrotoxicity and successive events of the described AOP. Thus, a clear threshold exists for the Met-Hb formation, as well as for the tumour formation, which is most likely due to an iron-mediated toxicity.

Due to known species-differences in metabolism, detoxification (e.g. Met-Hb-reductase capacity), as well as erythropoiesis (especially the extramedullary erythropoiesis) there are some uncertainties with regard to the sensitivity and extrapolation of dose-responses to humans. But these uncertainties can be covered by the application of the standard safety factor of 100 (10 for inter and 10 for intra-species differences), to a derived threshold value based on Met-Hb formation. Overall, if a derived threshold value is not exceeded it is very unlikely that humans develop spleen tumours. This is supported by experience in workers that have been exposed to aniline for long time periods, which gave no indications for any tumour formation in the spleen.

2. Problem Formulation

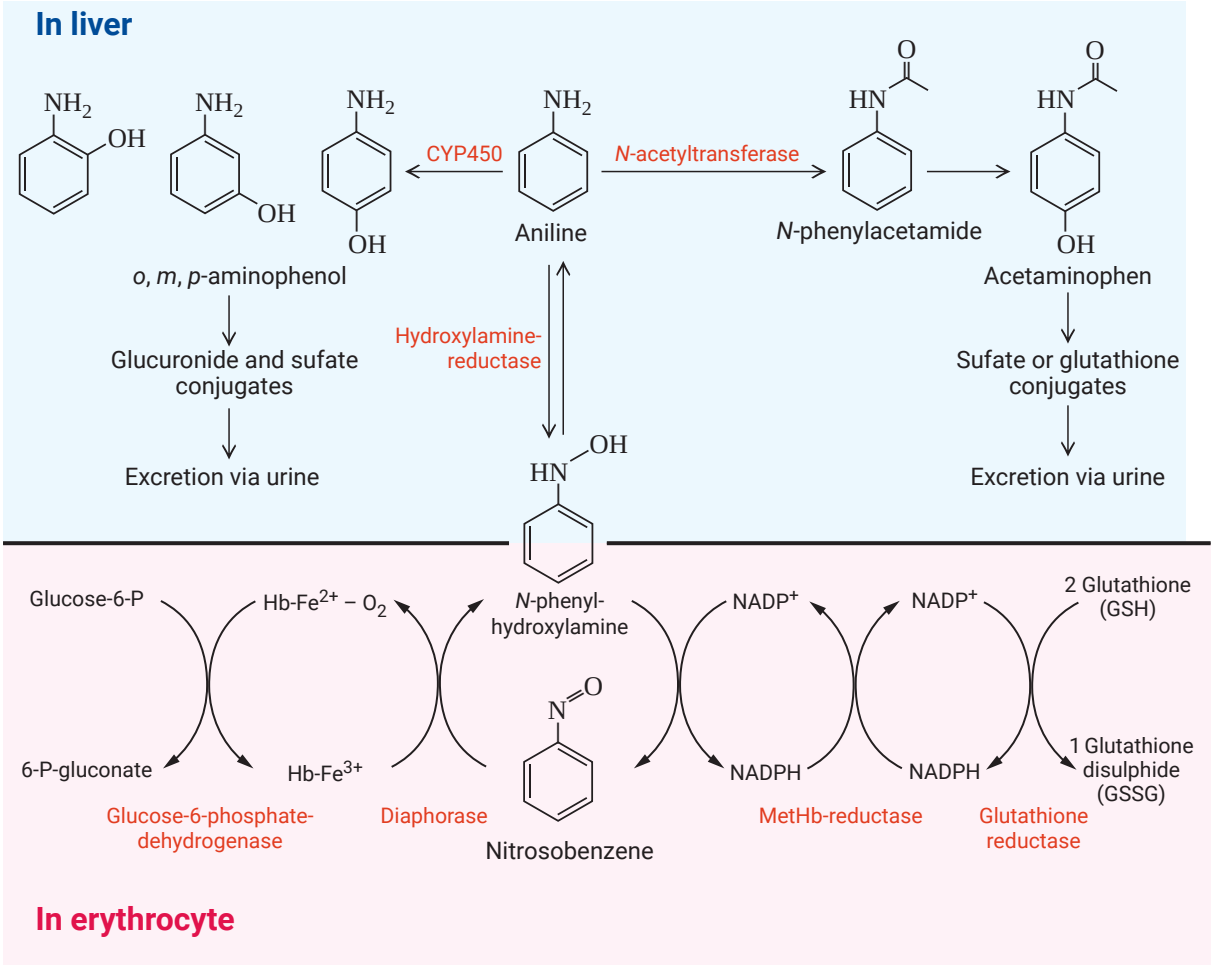
Chronic toxicity studies with aniline in laboratory animals are associated with the development of rare tumours in the spleen (haemangiosarcomas, fibrosarcomas, stromal sarcoma, oesteogenic sarcoma and fibroma). The tumours are species and sex-specific. Spleen tumours were observed only in male rats, but not in mice. The tumours occurred only at high-dose levels of aniline.

This document has formulated a proposed Mode of Action (MoA) for the spleen tumour development in male rats and the potential relevance to humans.

3. Hypothesized Mode of Action (MoA) statement

The tumour formation in the spleen due to aniline exposure is a complex process, which includes a primary damaging effect on erythrocytes (RBCs) and several successive effects, caused by the erythrotoxicity. Several of these effects are mandatory for the tumour formation in the spleen.

The erythrotoxicity is not caused by aniline itself but by metabolic activation to *N*-phenylhydroxylamine (Khan et al., 1998). However, formation of *N*-phenylhydroxylamine is not the main metabolic pathway for aniline degradation (see Figure 1). In the liver, aniline is primarily metabolized by ring-hydroxylation by cytochrome P450 resulting in ortho-, meta- or para-aminophenol or *N*-acetylation by *N*-acetyltransferase leading to formation of *N*-phenylacetamide. *N*-phenylacetamide is further hydroxylated to acetaminophen which is conjugated with sulfate or glutathione and excreted via urine. The aminophenols are conjugated with glucuronide or sulfate and are also excreted via urine. A minor metabolic pathway for aniline is the *N*-hydroxylation resulting in *N*-phenylhydroxylamine. This pathway however, predominates only if aniline exposure exceeds the capacity of the main detoxification pathways via conjugation. In that case increasing levels of *N*-Phenylhydroxylamine are formed.



$Hb\text{-}Fe^{2+}$ = Haemoglobin; $Hb\text{-}Fe^{3+}$ = Methaemoglobin

Figure 1: Pathways for metabolic degradation of aniline [redrawn from sponsor's original]

Inside the erythrocytes N-phenylhydroxylamine oxidizes the ferrous iron (Fe^{2+}) of hemoglobin (Hb) to ferric iron (Fe^{3+}) resulting in the formation of Met-Hemoglobin (Met-Hb). The ferric iron in Met-hemoglobin can be reduced back to its ferrous state by the Met-Hemoglobin-reductase (also known as NADH-dependent cytochrome b5 reductase (also called NADH-diaphorase)). However, if

Met-Hb levels exceeding the capacity of the Met-Hb-reductase (due to depletion of NADP-generating pathways in the RBCs) increasing levels of Met-Hb can lead to formation and precipitation of haemichromes and finally to formation of Heinz bodies (precipitates of damaged hemoglobin in erythrocytes). The Heinz bodies reduce the flexibility and function of the erythrocytes (due to impairment of membrane integrity and reduced cell deformability). In severe cases hemolysis of the erythrocyte can occur, resulting in hemolytic anemia. In addition, the oxidation of Fe^{2+} to Fe^{3+} can produce reactive oxygen species (ROS) causing oxidative stress and if the antioxidant defense is overwhelmed also oxidative damage inside the RBCs. Damaged and/or destroyed RBCs are removed from the blood by the spleen (or other organs), specifically in the splenic sinuses (sinusoids). Increasing levels of damaged RBCs or RBC fragments accumulate in the sinusoids and may lead to obstruction and swelling of the sinusoids. This can lead to inflammation, and in long-term to regenerative cell proliferation causing hyperplastic and fibrotic changes of the splenic stroma, which can finally lead to tumour formation (CIIT, 1982). Increasing levels of damaged RBCs that are removed by the spleen result also in iron accumulation in the spleen (mainly in the macrophages located in the red pulp (Khan et al., 1993, 1995a, 1997a, Pauluhn, 2004, Wu et al., 2005, MAK, 2010). Increased iron levels can stimulate the iron cycle and cell proliferation and result in formation of reactive oxidative species (ROS) leading to oxidative stress. The oxidative stress can cause oxidative damage to macromolecules (lipids, proteins, DNA) in splenic tissues. The resulting inflammatory reactions cause cytotoxicity, which in turn stimulate cell proliferation leading to hyperplastic and fibrotic changes in the splenic stroma in the red pulp. After

long-term exposure to aniline dose levels that are causing severe Met-Hb formation the above described events can lead finally to the development of spleen tumours (fibrosarcomas, stromal sarcomas, haemangiosarcomas).

In addition to the primary erythrotoxicity, anemia and hypoxemia can develop if the Met-Hb-reductase capacity is overwhelmed. Compensatory erythropoiesis is stimulated by hypoxemia in the bone marrow, and in the spleen. This leads to stimulation of RBC-proliferation, including the iron cycle (see above) which can further increase the formation of ROS and oxidative stress. In the spleen the oxidative stress adds to the oxidative damage of macromolecules. In addition, the increased rate of DNA-replications (due to enhanced RBC production) results in an increase in spontaneous errors in DNA increasing the risk that micronucleated RBCs can be build. Micronucleated RBCs can also be removed by the spleen, potentially adding further to the above described events.

Based on the adverse outcome pathway (AOP) described above the primary effect (molecular initiating event (MIE)) is the formation of Met-Hb. Aniline concentrations that do not lead to an exceedance of the metabolic detoxification pathways via conjugation, and which will not overwhelm the Met-Hb reductase capacity will not lead to toxic concentrations of Met-Hb, and therefore will not lead to exaggerated erythrotoxicity and successive events of the described AOP. Thus, a clear threshold exist for the Met-Hb formation, as well as for the tumour formation, which is most likely due to an iron-mediated toxicity.

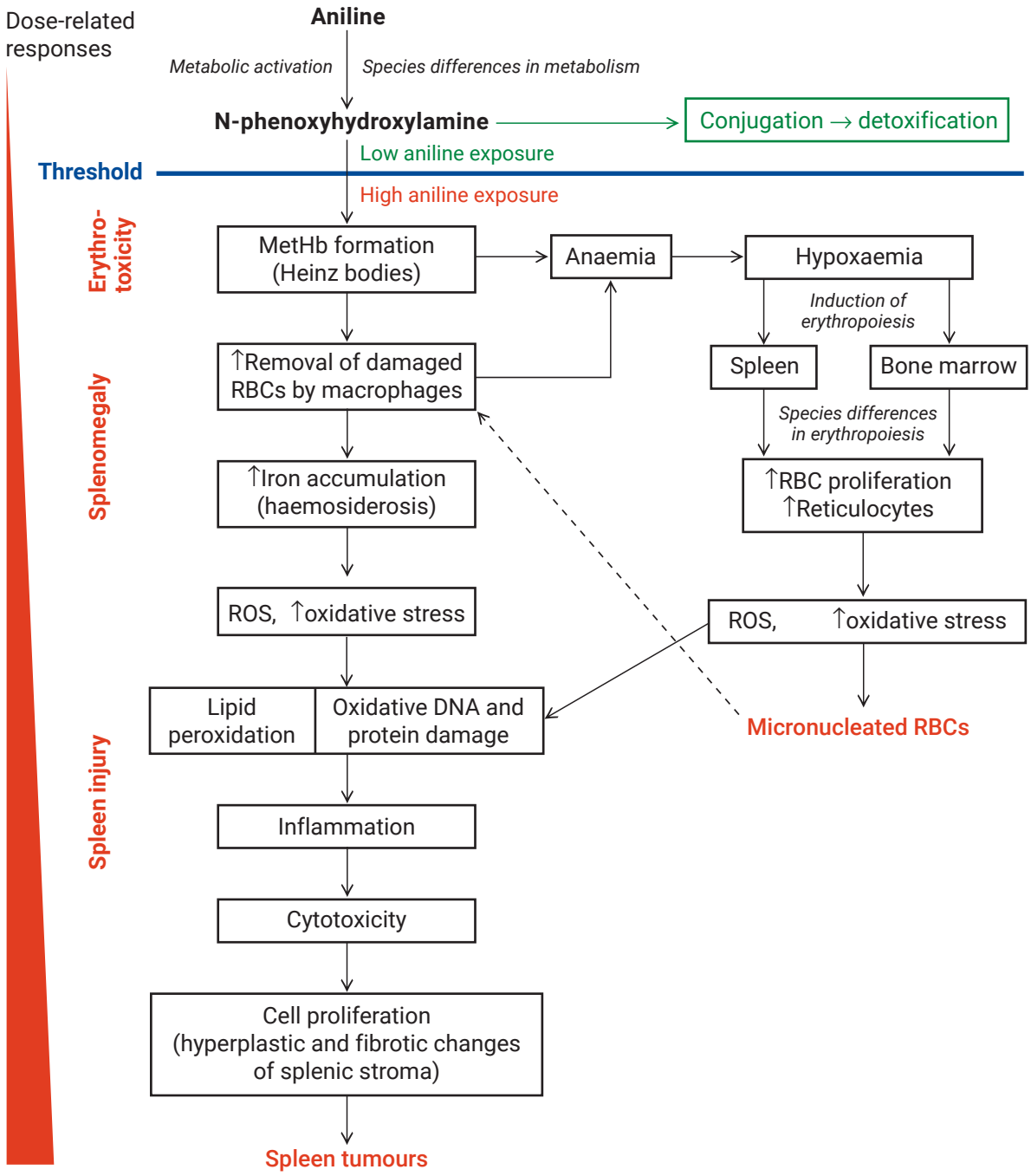


Figure 2: Overview of events involved in the AOP for spleen tumor development of aniline
[redrawn from sponsor's original]

4. Other possible MoAs for aniline induced tumor formation

In addition, to the carcinogenic potential aniline is also classified for mutagenicity, based on some positive results observed in genotoxicity assays.

Based on the AOP presented above, it is obvious that chromosome-damage may also experimentally occur by marked oxidative stress in the high toxic dose range, i.e. when a strong methemoglobin formation is induced once the detoxification capacity towards aniline becomes exhausted.

In vitro and *in vivo* genotoxicity assays with aniline were mostly negative. Positive results are restricted to high toxic concentrations or high dose levels. Detailed reviews on the genotoxicity of aniline can be found elsewhere (see section B2 of this document; Bomhard & Herbold, 2005).

Based on the new available Transgenic Rodent assay with aniline in Big Blue Transgenic rats (McKeon & Ciubotaru, 2018) a direct mutagenic effect of aniline in liver, spleen and bone marrow can be excluded. In that study a dose-and time-related induction of micronuclei was also observed. These were correlated to dose- and time-related Met-hemoglobinemia formation (increased Met-Hb, decreased RBC and hemoglobin (Hb)), stimulation of the erythropoiesis (increased reticulocytes (RETI)). As described above the increased rate of erythropoiesis in tissues under oxidative stress can lead to the formation of micronuclei. Micronucleated RBCs will also be removed by the spleen and thus can theoretically add to the accumulation of RBCs in the splenic sinusoids. However, since the responses in all available micronucleus assays were mostly weak, it is not considered that this has a strong influence on the AOP described above. This is supported by the fact that micronuclei were also observed in mice after high doses of aniline, but no spleen tumors occurred in mice.

In addition, there were also *in vivo* studies showing oxidative DNA-damage (8-OH-dG-formation; Ma et al.., 2008, Wu et al.., 2005) after aniline exposure. However, additional examinations could demonstrate that this was correlated to accumulation of free unchelated iron and oxidative stress and not due to a direct genotoxic effect (Khan et al., 1997a, Khan et al., 1999b).

5. Compilation of data used in the MoA analysis for aniline

The following Table summarises the data used for the MoA analysis. Most of the data are taken from the public literature. Since there are several very detailed reviews for aniline available, sometime reference is made to these reviews instead to the original publications.

Table 5-1: Data used for MoA analysis

Study type / Species (Comments)	Dose levels	Findings*	References
104 weeks, diet Rat, F344 (130 ♂/130 ♀ (interim sacrifices after 26 and 52 weeks (10/sex) and after 78 weeks (20/sex))	0, 10, 30, 100 mg/kg body weight and day in the diet (aniline hydrochloride; about 7, 22, 72 mg/kg bw/d aniline	≥ 7 mg/kg bw/d: hemosiderosis and hematopoiesis in spleen with increasing severity (at ≥52 weeks) ↓RBC, Hb,HCT; ↑MCV, RETI ≥ 22 mg/kg bw/d: ↑ spleen weight, ↑extramedullary hematopoiesis in spleen (dose-related from week 26); ↑ Met-Hb 72 mg/kg bw/d: ↑ deaths spleen: solid, enlarged, irregular surface; ↑ liver weights, Heinz bodies and MCH, spleen: chronic capsulitis (from week 26), stromal hyperplasia and fibrosis (week 104), lymphoid atrophy (week 104), fatty degeneration, tumour formation; bone marrow: erythroid and myeloid hyperplasia increased; pigment accumulation in pancreatic lymph nodes, adrenals and liver Neoplastic lesions spleen (incidences given for 0-7-22-72 mg/kg bw/d dose group) Males: Fibrosarcoma: 0/123-0/129-0/128-3/130 Stromal sarcoma: 0/123-0/129-1/128-21/130 Capsular sarcoma: 0/123-0/129-0/128-1/130 Hemangiosarcoma: 0/123-0/129-0/128-6/130 osteogenic sarcoma: 0/123-0/129-0/128-3/130 Females: Fibrosarcoma: 0/129-0/129-0/130-0/130 at Stromal sarcoma: 0/129-0/129-0/130-0/130 Capsular sarcoma: 0/129-0/129-0/130-0/130 Hemangiosarcoma: 0/129-0/129-0/130-1/130 osteogenic sarcoma: 0/129-0/129-0/130-0/130	CITT, 1982
4 weeks, diet Rat, F344 10 ♂/10 ♀	0-30-100-300-1000 mg/kg bw/d aniline hydrochloride for 30 days 0-21-72-210 mg/kg bw/d aniline	no histopathology 30 mg/kg bw/d and above: ↑MetHb, ↓ Heinz bodies, ↑ RETI 100 mg/kg bw7d and above: spleen and liver enlarged and with irregular surface; spleen and kidney discoloured 300 mg/kg bw/d and above: cyanosis 1000 mg/kg bw/d: 10 premature deaths in ♀ on days 24–27, ↓ feed consumption and body weight gains, black renal cortex and enlarged pancreatic lymph nodes	CIIT, 1977 Taken from MAK, 2010
8 weeks, diet Rat, F344 130 ♂/130 ♀	0%, 0.01%, 0.03%, 0.3%, 1% in the diet aniline hydrochloride (about 5, 15, 151, 504 mg/kg bw/d aniline);	NOAEL = 15 mg/kg bw/d ≥151 mg/kg bw/d: spleen black, granular, enlarged 504 mg/kg bw/day. ↓ Body weight gain	NCI, 1978

Study type / Species (Comments)	Dose levels	Findings*	References
	calculation based on relative feed consumption of 7% of body weight) range-finding study		
103 weeks, diet Rat, F344 50 ♂/50 ♀ (25 ♂/25 ♀ as controls)	0%, 0.3%, 0.6% in diet aniline hydrochloride (aniline doses of about 174.4, 350.5 mg/kg bw7d; calculation based on relative feed consumption of 7% of bw 4-week observation	No haematology ≥174.4 mg/kg bw/d: spleen: capsular and trabecular fibrosis, fatty metamorphosis, papillary hyperplasia of the capsule; kidney: tubular haemosiderosis 350.5 mg/kg bw/d: Kupffer's cell haemosiderosis; ↓ body weight gain Neoplastic lesions spleen(incidences given for 0-174.4 and 350.5 mg/kg bw/d dose groups) ***Males:*** Sarcoma, not specified: 0/25-4/50-2/46 Fibroma: 0/25-7/50-6/46 Fibrosarcoma: 0/25-3/50-7/46 Haemangiosarcoma: 0/25-19/50-20/46 ***Females:*** Sarcoma, Not specified: 0/23-0/50-3/50 Lipoma: 0/23-0/50-1/50 Haemangioma: 0/23-0/50-1/50 Haemangiosarcoma: 0/23-1/50-2/50	NCI, 1978
8 week, diet Mouse, B6C3F1 5 ♂/5 ♀	0%, 0.01%, 0.03%, 0.3%, 1% in the diet aniline hydrochloride (aniline doses of about 10.8, 32.4, 324, 1080 mg/kg bw/d day; calculation based on relative feed consumption of 15% of body weight) range-finding study	No haematology, no histopathology NOAEL = 32.4 mg/kg bw/d ≥324 mg/kg bw/d: black, granular, enlarged spleen	NCI, 1978
103 weeks, diet Mouse, B6C3F1 50 ♂/49–50 ♀	0%, 0.6%, 1.2% in the diet (aniline hydrochloride; aniline doses of about 733–737, 1510–1560 mg/kg bw/d); 4-week observation	No haematology ≥ 733 mg/kg bw/d (♂): bile duct inflammation 1510 mg7kg bw/d: ↓ body weight gain No neoplastic lesions	NCI, 1978

Study type / Species (Comments)	Dose levels	Findings*	References
Oral, gavage Rat, SD, males	Oral gavage 1 mmol/kg ^{14}C-aniline-HCl 1 x ; 1/day	After a single dose of 1 mmol/kg bw splenic iron content was in the normal range.	Khan et al., 1995a
3-d, oral gavage Rat, SD, males	Oral gavage 1 mmol/kg ^{14}C-aniline-HCl 3 x ; 1/day	After 3 doses of 1 mmol/kg there were 112%, 79%, and 67% increases in the radioactivity in the whole blood, RBCs, and hemolysate, respectively, 14C-aniline derived radioactivity increased 256% over dose after 3 doses; indicate accumulation in spleen; no accumulation in liver After 3 doses ca 85% increase of iron content in spleen. In liver no change of iron content	
14-day, oral gavage Rat, SD, males (Sacrifice timepoints day 1, 7 and 28 after last dose)	0.7 mmol/kg bw/d for 14 days Sacrifice timepoints day 1, 7 and 28 after last dose	↑ abs. spleen weight 78%, 42% and 23% on days 1, 7 and 28 ↑ rel. spleen weight 91%, and 33% on days 1, and 7 ↑ splenic iron content: 474, 491 and 58% on day 1, 7 and 28 ↑ Met-Hb (+100% (day 1) (! Sampling 24 h after exposure!) ↑ WBC (day 1) ↓ RBC (-80%) (day 1); -10% (day 7); normal at day 28 ↓ Hb ↓HCT Day 1: severe vascular congestion and heavy iron de-position localized to red pulp. Iron appeared to be predominantly intracellular, i.e., localized within macrophages of the red pulp sinusoids. Day 7: decreased vascular congestion Day 28: congestion had disappeared, iron deposition still high Focal increases in splenic fibrous tissue, esp. in capsule IgA increase (sign on day 1) AST & ALT: ↑ (sign on day 7)	Khan et al., 1995b
7-day oral gavage Rat, SD, males	1 mmol/kg bw/day for 7 days	↑ splenic iron content ↑ free iron ↑oxidative DNA damage (↑ 8-OHdG levels)	Wu et al., 2005
30-days, oral, drinking water Rat, SD, male	0.5 mmol/kg bw/day for 30 days	↑ 8-OHdG levels (oxidative DNA damage)	Ma et al., 2008

Study type / Species (Comments)	Dose levels	Findings*	References
4-days, oral gavage Rat, SD, male	0-0.25-0.5-1-2 mmol/ kg bw/day 0-23-46-93-186 mg/ kg bw/day 4 days	NOAEL = 23 mg/kg bw/d ≥46 mg/kg bw/d: ↑ iron in the spleen (72%, 172%, 325%; redox status not determined); ↑ lipid peroxidation (24%, 32%, 44%); ↑protein oxidation, ↑iron content ↑ expansion of the splenic red pulp ≥93 mg/kg bw/d: ↑ relative and absolute spleen weights; ↓Hb, ↓ Hct; spleen: splenic red pulp cellularity increased, cellular fragmentation, congestion, iron deposits (Fe3+) in phagocytes, erythrophagocytosis 186 mg/kg bw/d: RBC↓, WBC ↑ Dose-related expansion of red pulps due to increased vascular congestion, Increased iron deposition	Khan et al., 1997a
Oral gavage Rat, SD, male (Sacrifice at 0-0.25-0.5-1-3-6-12-24 and 48 h after application)	2 mmol/kg bw/d single dose	↑ Met-Hb-levels ↑ spleen weights ↑ lipid peroxidation ↑ MDA-protein adducts Congestion of splenic blood vessels	Khan et al., 1997b
4-day, oral gavage Rat, SD, male (Sacrifice 24 h after last dose)	0.025-0.05-0.1-0.2 mmol/kg bw/d for 4 days *N*-phenylhydroxylamine	At ≥ 0.025: slightly decreased RBC, Hb, HCT, dose-related ↑ of splenic iron content, Dose-related ↑ of lipid-peroxidation At ≥ 0.5 mmol/kg ↑ protein oxidation, ↑ spleen weight (dose-related) Dose-related ↑ of the severity of spleen lesions => sinusoidal congestion; hemosiderin accumulation in red pulps at ≥ 0.1 mmol/kg: Dose-dependent ↑ of Met-Hb-levels, ↓ Body weight gain, At 0.2 mmol/kg: increased WBC The results obtained in this study with *N*-phenylhydroxylamine are in line with the findings observed in earlier studies with aniline exposure => metabolite *N*-phenylhydroxylamine is responsible for the toxicity of aniline	Khan et al., 1998
1, 2 or 3 month, oral, drinking water Rat, SD, male	65 mg/kg bw/d aniline for 1, 2 or 3 months	Time-related ↓ of RBC and HB Time-related ↑ of lipid-peroxidation, splenic iron and spleen weights, MDA-protein adducts Time-related ↑ of protein oxidation at ≥0 2 month Marked red-pulp expansion due to dilated splenic sinusoids and fibroblasts and vascular congestion Increased iron accumulation Capsular hyperplasia, fibrosis => iron accumulation correlated with increased lipid peroxidation (i.e. oxidative stress)	Khan et al., 1999b

Study type / Species (Comments)	Dose levels	Findings*	References
1 and 4-week, oral, diet Rat, F344, male	0-10-30-100 mg/kg bw/day aniline hydrochloride for 1 or 4 weeks (actual: 6-17-57 mg/kg bw/d)	1-week results: At ≥ 30 mg/kg bw/d: ↓Hb; ↑MCHC ↑Heinz bodies ↑Hb-adducts ↑spleen weights At ≥ 100 mg/kg bw/d: ↓RBC, ↓ HCT, ↑Reti, ↑ Transferrin ↑ total iron binding capacity, ↑ focal perisplenitis and vascular congestion 4-w results: At ≥30 mg/kg bw/d: ↓Hb; ↓RBC, ↑MCH ↑MCV ↑Heinz bodies ↑Hb-adducts ↑spleen weights ↑Reti At ≥ 100 mg/kg bw/d: ↓ HCT ↑MCHC, ↑ Transferrin, ↑ total iron binding capacity, ↑ focal perisplenitis and vascular congestion	Mellert et al., 2004
7-day, oral gavage Rat, SD, male	1 mmol/kg bw/d	↑ spleen weights, ↑ splenocyte population, ↑ MDA-protein adducts ↑ TGF-β1 => MDA-protein adduct formation and overexpression of TGF-β1 could together promote splenic injury & fibrogenesis	Khan et al., 2003
30-day, oral, drinking	65 mg/kg bw/d	Vascular congestion and ↑ red pulp cellularity	Khan 2003a
water Rat, SD, male	Aniline hydrochloride (ca 46.8 mg/kg bw/d aniline)	Marked ↑ of iron deposition in red pulp (iron appeared to be localized in sinusoidal macrophages) MDA-protein adducts predominantly located in sinusoidal macrophages in the red pulps an area where intense staining for iron was also observed. The co-localization of these adducts with iron provides strong evidence for iron-catalyzed lipid peroxidation leading to formation of MDA–protein adducts	
30, 60 or 90-days, oral drinking water Rat, SD male	600 mg/L aniline hydrochloride (ca 43 mg/kg bw/d Aniline)	↑ Met-Hb (high control levels possibly due to auto-oxidation, since measurement was done 24 h after blood sampling) ↓ Hb, RBC, HCT ↑MCV, MCH ↑ iron content in red pulp /time-related) Macrophages ↑ WBC (only after 30 d) Marked red pulp expansion due to increased splenic sinusoids, fibroblasts and macrophages Congestion of sinusoidal blood vessel (time-dependent: more pronounced after 90 days) Focal pericapsular fibrosis	Khan et al., 1993
Single dose, i.p. Rat, SD male (Sacrifice 2 h after application)	3 mmol/kg bw	↑ Met-Hb, ↑ free iron in erythrocytes	Ciccoli et al., 1999
30, 60 or 90-day oral drinking water Rat, SD male	50-60 mg/kg bw/d	≥ 30 days: ↑ Met-Hb, ↑ free iron (RBC & spleen, liver), ↑ spleen weights, ↑ free iron in erythrocytes	

Study type / Species (Comments)	**Dose levels**	**Findings***	**References**
2-week inhalation (6h/day, 5d/week) Rat, Wistar, males (2-week post-exposure period)	10, 30, 90, and 270mg/m³ Actual: 0, 9.2, 32.6, 96.7, 274.9 mg/m³ (2.4, 8.5, 25.1, 71.4 ml/m³)	NOAEL = 9.2 mg/m³ ≥ 32.6 mg/m³: dose-related extramedullary haematopoiesis and haemosiderosis (at 8.5 ml/m³ marginal) ≥ 96.7 mg/m³: cyanosis, ↓ body weights, ↑ spleen weights, ↑Met-Hb, ↑Heinz bodies, ↓RBC, ↑Reti, ↑ iron in spleen, reticuloendothelial system: hypertrophy, hemosiderosis 274.9 mg/m³: tachypnea, labored breathing, ↑ salivation, ungroomed hair-coat; liver hemosiderosis	Pauluhn, 2004
30-day oral drinking water Rat, SD, males	0.5 mmol/kg bw aniline	↑ 8-OHdG-levels in spleen (= oxidative DNA-damage) ↑ DNA-repair activity (BER) ↑ immunoreactivity for 8-OHdG and OGG1 predominantly in splenic red pulp areas	Ma et al., 2008

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6. Listing of key events identified for the proposed MoA

The following table lists the sequence of events (adverse outcome pathway (AOP) associated with the proposed MoA for tumor formation in rats after aniline exposure.

For a better overview the key events together with associated events are presented in Figure 1.

Table 6-1: Proposed adverse outcome pathway for aniline induced tumor formation

Molecular initiating event (MIE)	Met-Hb-formation Oxidation of Hb-Fe^{2+} (hemoglobin) to Hb-Fe^{3+} (Met-hemoglobin (Met-Hb)) due to the metabolite *N*-phenylhydroxylamine (increased Met-Hb-levels, Heinz bodies)
Key events (KE)	
Key Event 1	Increased splenic removal of damaged RBCs Due to Met-Hb-formation flexibility and function of the erythrocytes are reduced (due to impairment of membrane integrity and reduced cell deformability). The damaged RBCs are removed by the spleen (reduced RBC levels)
Key Event 2	Iron accumulation in the spleen Iron accumulation in spleen due to increased removal and degradation of damaged RBCs (hemosiderin deposits)
Associated (AE) events	AE related to key events 1 & 2
AE1	Anemia / hypoxemia (↓ Hb, ↓ RBC, ↓ HCT, ↓ MCHC, ↑MCV, ↑ MCH)
AE2	Induction of extramedullary erythropoiesis (spleen) Induction of medullary erythropoiesis in bone marrow ↑ RETI Secondary to anemia stimulation of erythropoiesis in bone marrow and extramedullary in spleen Increased RBC production (activation of iron cycle and cell proliferation; ROS formation possible => oxidative stress possibly resulting in oxidative DNA / protein damage, if repair mechanisms fail
AE3	Micronuclei formation Micronuclei formation in RBCs due to increased erythropoiesis in the spleen as well as in the bone marrow
Key Event 3	Formation of reactive oxygen species (ROS), oxidative stress Due to iron accumulation and / or increased RBC production (activation of iron cycle and cell proliferation; ROS formation possible => oxidative stress; (see KE 4 below)
Key Event 4	Oxidative damage of macromolecules due to oxidative stress Lipid peroxidation, Protein oxidation and oxidative DNA-damage
Key Event 5	Inflammation Inflammation due to phagocytosis of cell debris (e.g. cytokine signaling),
Key Event 6	Cytotoxicity
Key Event 7	Cell proliferation in spleen Regenerative cell proliferation (hyperplastic and fibrotic changes of splenic stroma) The combination of initiating events (4) and promoting events (6) finally lead to tumor formation in the spleen

CARBOFURAN and CARBOSULFAN

TOXICOLOGY

The Meeting identified concerns regarding the genotoxicity of carbofuran that required additional information.

In addition, the Meeting became aware that there was more information available on the genotoxicity testing of carbofuran which was not submitted in the dossier. As carbofuran is a major and toxic metabolite of carbosulfan and the dossier for carbofuran was incomplete, the Meeting was unable to proceed with the evaluation of either of the two compounds. The evaluations of carbofuran and carbosulfan were therefore postponed until additional information on genotoxicity for both compounds is provided.

CLETHODIM

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Explanation

Clethodim is the ISO-approved common name for (5*RS*)-2-{(1*EZ*)-1-[(2*E*)-3-chloroallyloxyimino] propyl}-5-[(2*RS*)-2-(ethylthio)propyl]-3-hydroxycyclohex-2-en-1-one (IUPAC), with the Chemical Abstracts Service number 99129-21-2. Clethodim is a selective cyclohexanedione herbicide and exhibits its pesticidal activity in plants by inhibiting acetyl coenzyme A carboxylase, an enzyme common to the pathways of fatty acid biosynthesis.

Clethodim was evaluated by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) in 1994 and 1999. An acceptable daily intake (ADI) of 0–0.01 mg/kg body wieight (bw) was established in 1994 on the basis of a no-observed-adverse-effect level (NOAEL) of 1 mg/kg bw per day in a one-year study in dogs, and the establishment of an acute reference dose (ARfD) was considered unnecessary. In 1999 the toxicological monograph prepared in 1994 was reviewed and the previous conclusions were reaffirmed.

Clethodim was evaluated by the present Meeting within the periodic review program of Codex Committee on Pesticide Residues (CCPR). The present Meeting reviewed all previous studies, and new data comprising neurotoxicity (acute and short-term), immunotoxicity studies, and studies on metabolites (acute oral, repeated dose and genotoxicity) are included in this monograph.

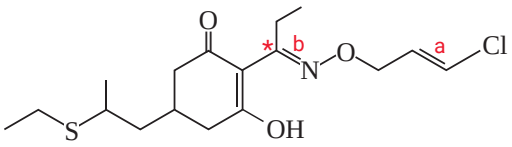
All studies were conducted to internationally recognized guidelines, generally Organisation for Economic Co-operation and Development (OECD) and good laboratory practice (GLP), or were otherwise quality audited, except where indicated. A literature search did not identify any further useful toxicological information for the current assessment.

Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and elimination (ADME) of clethodim was investigated in rats following a single oral low dose, a single oral high dose, and a single oral low dose daily dose repeated for 14 days followed by a radioactive dose. The chemical structure of [propyl-1 ^{14}C]-clethodim (see below) contains two double bonds; at C 1 of the oxyiminopropyl group (b) and at C 2 of the chloropropenyl group (a) (see Fig. 1).

Figure 1. Structure of clethodim



* denotes radiolabel position

The isomers associated with the ISO common name clethodim are the *E* and *Z* isomers at C 1 of the oxyiminopropyl group and the *E* isomer at C 2 of the chloropropenyl group. Thus "technical clethodim" as manufactured, and as used in the environment, can be considered a mixture of the *E* isomers at the oxyiminopropyl and chloropropyl groups (*E*, *E*), and the *Z* isomer at the oxyiminopropyl with the *E* isomer at the chloropropenyl group (*Z*, *E*).

When tested in any environmental system, the exact isomer ratio (*E*,*E* : *Z*,*E*) of clethodim to which the system is exposed is variable and dependent on various factors including delivery vehicle, temperature, pH etc. It is not possible to evaluate the effects of either isomer alone since isolation of either form would only result in a re-established equilibrium when introduced into any test system. The test results obtained will be appropriate for the compartment investigated and relevant for the assessment of the properties of clethodim, because test conditions are representative of the in-use exposure. Therefore, analysis of any samples taken from the studies will allow separation of the (*E*, *E*) and (*Z*, *E*) isomers of clethodim but the results so generated may not indicate the ratio which existed during the test itself.

1.1 Absorption, distribution and excretion

(a) Oral route

In an absorption, distribution and excretion study, five male and five female Crl:CD(SD)BR rats were given a single oral low dose of [propyl-1 ^{14}C]-clethodim (purity 96.6%) at 4.4 mg/kg bw or a single oral high dose of [propyl-1 ^{14}C]-clethodim (purity 96.9%) at 468 mg/kg bw, or 14 daily doses of 4.5 mg/kg bw per day (unlabelled clethodim) followed by one administration of radiolabelled material. An intravenous administration group was not included due to the low solubility of the test substance in aqueous or saline media. Urine and faeces were collected at various intervals up to seven days, (except for carbon dioxide in expired air, which was collected for 48 h) at which point time animals were sacrificed. The homogeneity and stability of the dosing suspensions were checked. Cage wash was collected at the end of the study. At sacrifice, blood was collected and the following tissues and organs were taken from the animals of all groups: brain (with brain stem), heart, liver, kidneys, lung, gonads, uterus, spinal cord, fat (peritoneal), spleen, bone (sternum), muscle, adrenals, salivary glands and the remaining carcass. Radioactivity in urine, CO_2-trapping liquid and cage wash was measured by liquid scintillation counting (LSC). Radioactivity in samples of blood and tissues, faeces and carcass was determined by combustion/LSC.

Following a single low or high dose, or 14 daily single doses, the majority of radioactivity was excreted in urine, accounting for 87–93% of the administered dose (AD). Faecal radioactivity accounted for 9–17% of the ADs. There did not appear to be any significant gender differences in the routes and rates of excretion. In rats given a single low or high dose, more than 90% of the radioactivity in urine was excreted within 24 or 48 h respectively. Radioactivity excreted with expired air ranged from 0.5 to 1% of AD, and radioactivity recovered in cage wash and tissues (including carcass) was less than 1% of AD. At seven days post dosing the total radioactivity recovered was 103–110% of AD. Considering the radioactivity recovered in urine, expired air, tissues, cage wash and residual carcass, the total absorption was 88–95% of AD.

Radioactivity was equally distributed among all sampled tissues of both females and males, and in all dose groups, reaching 0.2–0.7% (including carcass) of the AD . The highest residues were seen in the adrenals, liver and kidneys. There did not appear to be gender differences in tissue distribution. Based on residues in tissues in the low-dose group and repeated low-dose group, no accumulation was observed (Rose & Griffis, 1988).

Table 1. Excretion and retention of radioactivity (% of administered dose) in rats after single or repeated oral exposure to [propyl-1- ^{14}C]-clethodim at 24 and 168 h after dosing.

Matrix	Time point (hours)	Low dose 4.4 mg/kg bw		Repeated dose 4.8 mg/kg bw		High dose 468 mg/kg bw	
		Females	Males	Females	Males	Females	Males
Urine	0–24	84	86	80	83	53	59
	0–48	90	92	86	89	88	84
	0–168	91	93	87	91	93	88
Faeces	0–24	8.5	11	14	12	3.2	4.9
	0–48	11	15	16	15	7.7	11
	0–168	11	15	17	15	9.3	13
Carbon dioxide	0–24	0.9	1.0	0.5	0.6	0.6	0.6
	0–168	0.9	1.0	0.5	0.6	0.7	0.7
Cage wash	168	0.2	0.2	0.2	0.1	0.4	0.6
Total excreted	0–24	93	98	94	95	57	65
	0–168	104	110	105	107	103	102
Total tissues (incl. carcass)	168	0.2	0.3	0.2	0.2	0.7	0.7
Total absorbed	168	93	95	88	92	95	90
Total recovered	168	104	110	105	107	104	103

Table 2. Radioactivity in tissues (mg clethodim equivalents/kg) of rats after single or repeated oral exposure to [propyl-1- ^{14}C]-clethodim.

	Low dose 4.4 mg/kg bw		Repeated dose 4.8 mg/kg bw		High dose 468 mg/kg bw	
Tissue	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
Adrenals	0.079	0.068	0.22	0.10	13	5.4
Bone (sternum)	< 0.009	0.008	0.009	0.008	< 1.6	1.1
Brain + stem	< 0.005	< 0.005	< 0.005	< 0.003	< 0.46	0.47
Peritoneal fat	< 0.007	< 0.009	< 0.010	0.006	0.78	0.71
Gonads	< 0.023	0.003	< 0.021	0.004	< 2.4	0.44
Heart	< 0.010	< 0.008	< 0.010	0.009	1.2	1.3
Kidney	0.021	0.030	0.020	0.036	2.3	2.6
Liver	0.057	0.063	0.048	0.058	3.8	5.2
Lung	0.009	< 0.008	0.010	0.012	1.5	1.6
Muscle	< 0.006	< 0.005	< 0.005	< 0.005	0.85	1.2
Salivary glands	< 0.010	< 0.008	< 0.007	0.008	< 0.89	0.83
Spinal cord	< 0.011	0.012	< 0.011	< 0.010	1.1	1.4
Spleen	< 0.010	< 0.008	< 0.009	0.017	2.8	1.5
Uterus	< 0.010	NA	< 0.009	NA	1.1	n.a.

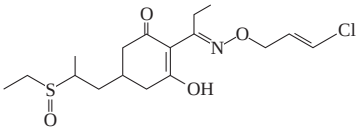
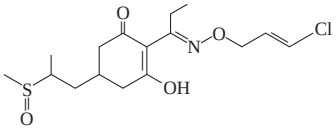
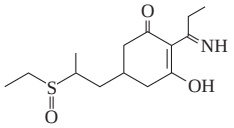
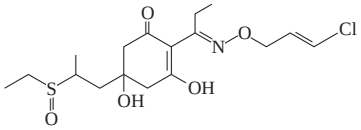
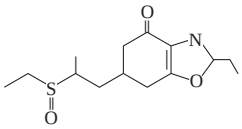
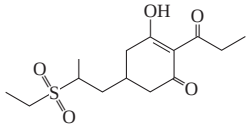
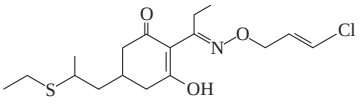
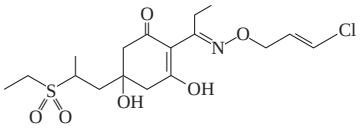
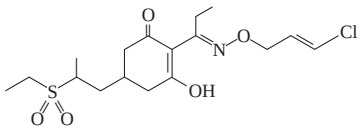
NA Not applicable

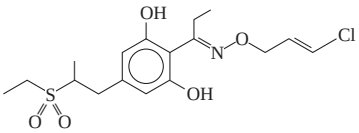
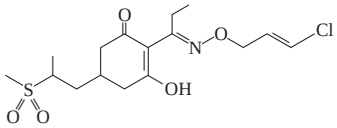
1.2 Biotransformation

The metabolism of clethodim was investigated in five male and five female Crl:CD(SD)BR rats given a single oral low doses of [propyl-1 ^{14}C]-clethodim (purity 96.6%) at 4.4 mg/kg bw or a single oral high dose of [propyl-1 ^{14}C]-clethodim (purity 96.9%) at 468 mg/kg bw, or a 14 daily doses of 4.5 mg/kg bw/day (using non-labelled clethodim) followed by one administration of radiolabelled material. An additional group of ten males was given [propyl-1 ^{14}C]-clethodim (purity 96.9%) as a single oral high dose of ca 450 mg/kg bw to generate sufficiently large quantities of urinary metabolites for identification. Metabolites were identified by thin layer chromatography (TLC) and/or high-performance liquid chromatography (HPLC) with references, and by HPLC-mass spectroscopy. Metabolites were determined in urine by pooling samples for each dose group by time interval (up to 48 h) and sex. In faeces, metabolite identification was performed pooling samples for each dose group and sex, combining various time points up to 24 h and then from 36 h and 48 h.

Clethodim was extensively metabolized in all dosed groups. Unchanged parent compound was detected in urine (0.4% of AD) of females of the high-dose group only, in faeces of females and males of the repeated low-dose and high-dose groups (0.3–1% of AD). The major metabolite in urine and faeces of females and males was clethodim sulfoxide. This metabolite represented 46–61% and 2–5% of the AD in urine and faeces, respectively. The second most abundant metabolite in females and males was *S*-methyl sulfoxide, accounting for 6–11% and 0.4–1% of the administered dose in urine and faeces, respectively. Imine sulfoxide was 6–9% of AD in urine, and 1–2% in faeces in both females and males. Other identified compounds (≤ 5% of the administered dose) identified in urine and faeces of females and males were 5-OH sulfoxide (3–5%), oxazole sulfoxide (2–3%), trione sulfoxide (~1%), 5-OH sulfone (0.3–1%), clethodim sulfone (0.1–1%), aromatic sulfone (0.2–0.7%) and *S*-methyl sulfone (0.0–0.4%). In addition, oxazole sulfone was detected in the urine of males of the high-dose group, but was not quantified. Throughout all dose groups, no significant differences in the pattern of metabolites between males and females were noted.

Table 3. Urinary and faecal metabolite identification (% of administered dose). Identification by TLC and/or HPLC with references and by HPLC-MS.

Common name and chemical structure	Repeated dose 4.8 mg/kg bw				High dose 468 mg/kg bw			
	Females		Males		Females		Males	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
	6-48h	0-48h	12-48h	0-48h	6-48h	0-48h	6-48h	0-48h
Clethodim sulfoxide* 	56	5	52	3	61	2	46	2
S-Methyl sulfoxide* 	11	1	9	1	8	0.4	6	0.4
Imine sulfoxide* 	6	2	9	1	6	1	7	1
5-OH sulfoxide 	3	0.4	5	0.8	2	0.1	4	0.3
Oxazole sulfoxide 	2	0.2	2	0.4	3	0	3	0
Trione sulfoxide 	1	0.2	1	0.4	1	0.1	1	0.3
Clethodim 	0	0.3	0	1	0.4	0.8	0	0.8
5-OH sulfone* 	1	0.1	1	0.1	0.3	0.1	0.3	0.1
Clethodim sulfone* 	0	0.1	0	0.5	1	0.1	0.7	0.4

Common name and chemical structure	Repeated dose 4.8 mg/kg bw				High dose 468 mg/kg bw			
	Females		Males		Females		Males	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
	6-48h	0-48h	12-48h	0-48h	6-48h	0-48h	6-48h	0-48h
Aromatic sulfone 	0	0.2	0	0.4	0.5	0.2	0	0.4
S-Methyl sulfone 	0	0	0	0.4	0	0.1	0	0.2
Unidentified	7	3	9	3	5	1	5	1.6

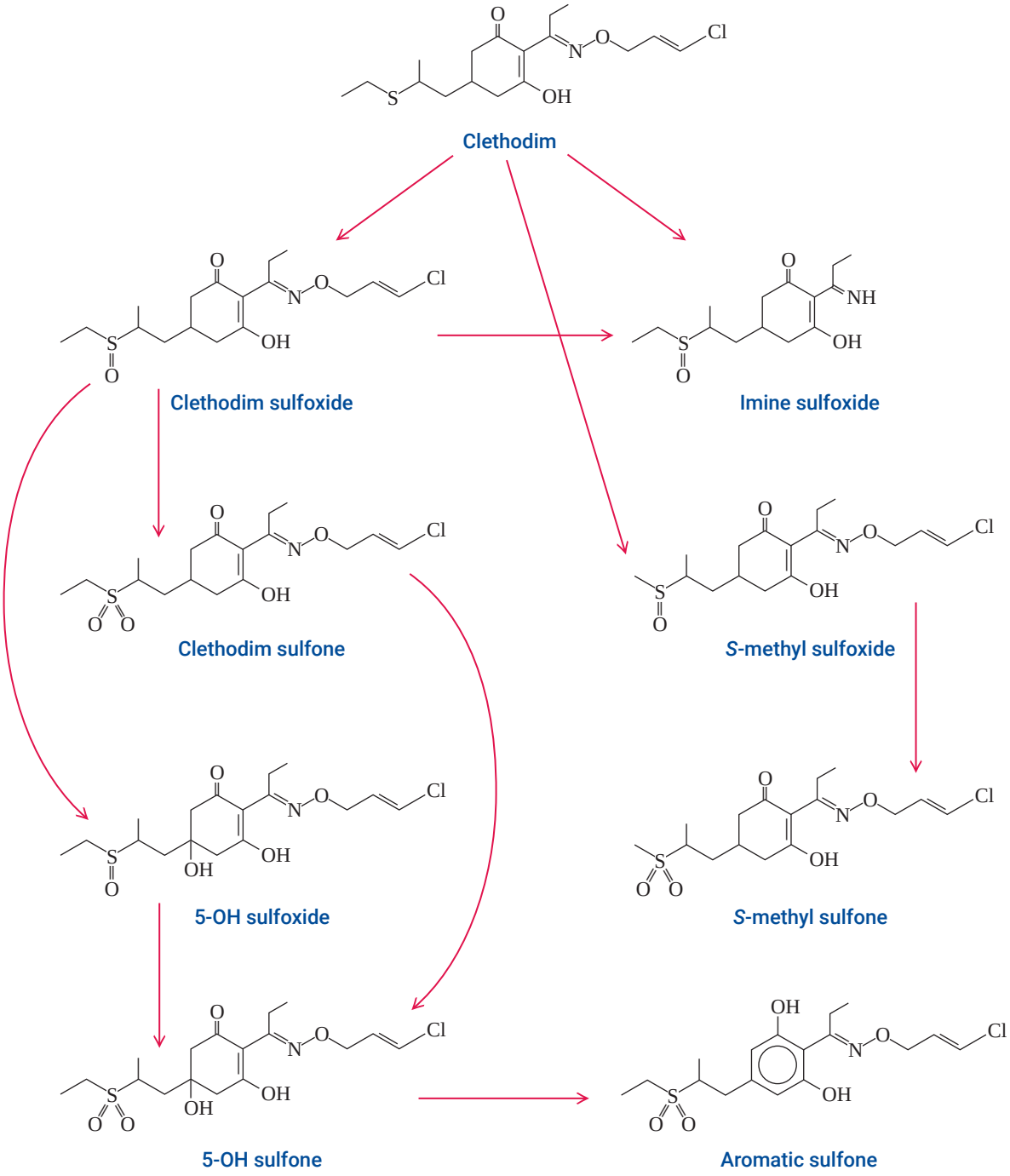
* Identity confirmed by mass spectrometry (MS) of isolated urine fraction

The proposed metabolic pathway is presented in Fig. 2. Once absorbed, clethodim can be:

- oxidized to clethodim sulfoxide (dominant process) and further to clethodim sulfone,
- converted to *S*-methyl sulfoxide via a sulfonium cation intermediate,
- converted to imine sulfoxide or
- hydrolyzed at the 5 position.

The proposed *S*-methyl path would follow the dominant metabolic process and form *S*-methyl sulfoxide and smaller amounts of *S*-methyl sulfone. Once the imine was formed, it would undergo rapid sulfur oxidation to give imine sulfoxide and imine sulfone. Similarly, the 5-OH (not detected) would oxidize to give 5-OH sulfoxide and further 5-OH sulfone (Rose & Griffis, 1988).

Figure 2. Proposed metabolic pathway of clethodim in rats.



2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with clethodim, along with the results of dermal and eye irritation, and skin sensitization studies, are summarized in Table 4.

Table 4. Acute toxicity of clethodim

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Mice	Charles River CD-1	Male & Female	Oral	83.3	LD_{50} 1787 mg/kg bw (M) LD_{50} 1688 mg/kg bw (F)	Cox, 1986a
Rat	Sprague Dawley	Male & Female	Oral	83.3	LD_{50} 1358 mg/kg bw (M) LD_{50} 1133 mg/kg bw (F)	Cushman, 1986a
Rabbit	New Zealand White	Male & Female	Dermal	83.3	LD_{50} > 4167 mg/kg bw	Cushman, 1986b
Rat	Sprague Dawley	Male & Female	Inhalation	83.3	LC_{50} > 3.25 mg/L air/4 h (whole body) (max attainable concentration)	Griffis, 1986
Rabbit	New Zealand White	Female	Skin irritation	83.2	No conclusion can be drawn	Cushman, 1986c
Rabbit	New Zealand White	Male	Skin irritation	93.4	Mildly irritating	Pelcot, 2005
Rabbit	New Zealand White	Male	Eye irritation	83.3	Mildly irritating	Cushman, 1986d
Guinea pig	Hartley	Male	Skin sensitization	93.0	No conclusion can be drawn	Coleman, 1998
Guinea pig	Hartley	Male	Skin sensitization	92.4	Skin sensitizing (Magnusson & Kligman)	Arcelin, 2006

Clethodim has low acute toxicity to rats when administered dermally or via inhalation. It is not irritating to skin or eyes of rabbits. Clethodim is moderately toxic when administered orally and is a skin sensitizer.

(a) Lethal dose

Oral

Mouse

Clethodim (purity 83.3%) was administered by gavage to five male and female Charles River CD-1 (ICR derived) mice, at 0, 1500 (males only), 2000, 2500, 3000 and 3500 mg/kg bw (females only) in 0.1% carboxymethyl cellulose and 0.7% Tween 80 in distilled water (dose volume of 20 mL/kg bw). Mortality and clinical signs were recorded at 1 and 3 h post dose and once daily for 14 days. Body weights were recorded prior to dosing and on days 3, 8, and 15, or when found dead. Pupillary response was measured on days 2, 8, and 15.

Three of five males that received 3000 and 2500 mg/kg and one of five males that received 2000 mg/kg were found dead within 1 and 2 days of treatment, respectively. Two of five females that received 2000 mg/kg, one of five females that received 2500 mg/kg, all females that received 3000 mg/kg and four of five females that received 3500 mg/kg died between observations on day 1 and day 2 after treatment. Clinical signs appeared from one hour after administration and included hypoactivity, rough coat, hunched appearance, ataxia, tremors, salivation, laboured respiration, and soft faeces. No abnormal clinical signs were observed for males and females after day 2. Exceptions for females included urine stains at and above 2000 mg/kg bw. Body weight was not affected and no gross abnormalities at necropsy were found. Slightly dark red lungs and compound-like material in the stomach and intestine was observed at each does level during necropsy. The acute oral median lethal dose (LD_{50}) of clethodim in mice was calculated to be 2460 mg/kg bw (2570 mg/kg bw for males, 2430 mg/kg bw for females). After correction for the purity this is equal to an LD_{50} of 1787 mg /kg bw for males and 1688 mg/kg bw for females (Cox, 1986a).

Rat

Clethodim (purity 83.3%) was administered by gavage to five male and female Sprague Dawley Crl:CD (SD)BR rats, at 0, 800 (female only), 1050, 1450, 1860 (male only), 2000 (female only) and 2500 mg/kg bw (male only) in 0.7% carboxymethyl cellulose and 1.0 % Tween 80 in distilled water (dose volume of 10 mL/kg bw). Mortality and clinical signs were recorded frequently on the day of dosing and at least once for 14 days. Body weights were determined immediately prior to dosing (day 0), on day 7 and day 14.

All males receiving 2500 mg/kg bw, four males receiving 1860 mg/kg bw and one male that received 1450 mg/kg, were found dead within one day of treatment. All females receiving 2000 mg/kg bw were found dead within one day of treatment. Three females that received 1450 mg/kg bw were found dead within three days of treatment. Clinical signs appeared from 30 minutes after administration. They included salivation, decreased motor activity, unsteady gait, hyperactivity, lacrimation, clonic convulsions, red nasal discharge, ocular discharge and collapse, observations of which occurred in almost all dose groups. Reduced food consumption and yellow anogenital stains were noticed in the surviving treated animals. On day 6 all surviving treated animals were normal. No treatment-related findings were noted in the control group. Body weight of females dosed at 1450 mg/kg were significantly lower than in the control group, but the body weights of males were not affected and no gross abnormalities at necropsy were found in animals dosed at 800 or 1050 mg/kg bw. Dark red gelatinous material beneath the meninges was observed in animals found dead. Reddened, darkened and/or mottled lungs with foam in the trachea were observed at the highest two doses. The acute oral LD_{50} of clethodim for rats was calculated to be 1630 mg/kg bw for males and 1360 mg/kg bw for females. After correction for purity, this is equal to an oral LD_{50} of 1350 mg/kg bw for males and 1133 mg/kg bw for females (Cushman, 1986a).

Dermal

Rabbit

Five male and five female New Zealand White rabbits were treated dermally with 5000 mg/kg bw of clethodim neat (purity 83.3%), spread on the trunk (ca 25% of body surface) of each animal. An additional group of five male rabbits was treated with 2000 mg/kg bw clethodim. The test material was applied to the shaved skin, covered with an plastic sheet, covered with a wrapping paper and held in place with porous adhesive tape for 24 h, after which it was removed. Mortality and clinical signs were recorded on the day of treatment and then daily for 14 days. Body weights were recorded on the day of dosing, and again at 2, 7 and 14 days post dosing.

Following the death of one male treated with 5000 mg/kg bw, five additional males were treated with 2000 mg/kg. No further mortality was observed. In the male given 5000 mg/kg bw, reduced food intake, decreased motor activity, decreased body temperature, unkempt appearance, diarrhoea, no faeces and collapse were observed. Twenty-four hours after dosing, treated and control animals showed comparable slight to severe erythema, accompanied by moderate to severe oedema in treated animals and ill-defined oedema in controls. By day 7, controls showed no erythema or oedema while treated animals showed no to severe erythema, and no to well-defined oedema. Erythema persisted until day 14 in one female rabbit dosed at 5000 mg/kg bw. Body weights of the rabbits were unaffected. Macroscopic postmortem examination of the animals at termination showed flaky, dry and/or reddened skin. Microscopic examination of the skin revealed trace to mild hyperkeratosis among the treated animals. The acute dermal LD_{50} of clethodim in rabbits was greater than 5000 mg/kg for both sexes. After correction for the purity, this is equal to a dermal LD_{50} of greater than 4167 mg/kg bw (Cushman, 1986b).

Inhalation

Rat

Five male and five female Sprague Dawley rats were exposed to a 4 h whole-body aerosol atmosphere of clethodim (purity 83.3%) at a concentration of 3.9 mg/L (maximum attainable concentration, analytically and gravimetrically determined). Clethodim was diluted in acetone to reduce the viscosity and facilitate aerosol production: 540 mL of clethodim was diluted to 600 mL of acetone. Five additional rats of each sex were exposed, under the same conditions, to an average acetone vapour concentration of 9.0 mg/L and served as vehicle controls. The mass median aerodynamic diameter (MMAD) was 2.8 μm and the geometric standard deviation (GSD) was 1.9. Clinical signs were recorded frequently during the

exposure and then twice daily following exposure (except at weekends when they were observed once daily) for 14 days following dosing. Body weights were recorded on days 0 (before exposure), 2, 7, and 14 during the observation period.

No mortality was noted. During exposure, squinted or closed eye and salivation were noted in all animals. Clinical signs included salivation and colorless eye discharge were observed immediately after exposure. Reddish nasal mucus, laboured breathing, mydriasis, decreased faeces, unkempt appearance and yellow–red ano-genital discharge were observed. All animals appeared normal within eight days of exposure. Therefore, the acute median lethal concentration (LC_{50}) for inhalation of clethodim in rats was greater than 3.9 mg/L for both sexes (Griffis, 1986).

(b) Dermal irritation

In a dermal irritation study, 0.5 mL of clethodim (neat; purity 83.2%) was applied with an occlusive patch (gauze patches were covered with a plastic sheet) four four hours to two abraded and two intact areas on the backs of two groups of six New Zealand White female rabbits. Irritation was scored 1, 24, 48 and 72 h after removal of the test material and at 7 and 14 days. Dermal reaction was evaluated according to the Draize scale.

Results from the first set of six animals showed slight erythema in two animals at one hour after dosing. Slight to moderate oedema in two animals at 24 h after dosing. Slight to severe erythema and slight to moderate oedema were observed in three animals at 48 and 72 hours after dosing. Most animals showed slight to well defined erythema on day 7, which had cleared by day 14. One animal displayed at the abraded site severe erythema along with moderate oedema on day 7; this persisted to day 14. Histopathological examination of the skin of this animal showed necrosis of the epithelium and inflammation of the connective tissue. In the second set of animals, irritation was less severe, probably due to better removal of the test material or the use of animals from a different supplier (Cushman, 1986).

Given the absence of information as to the size of the area treated on which the test material was applied, no reliable conclusion can be drawn on the dermal irritation properties of clethodim.

In a second dermal irritation study, a single dose of 0.5 mL of the undiluted clethodim (purity 93.4%) was applied to the closely clipped skin (ca 6 cm^2) of one flank of male New Zealand White rabbits. The test item was held in contact with the skin by means of a semi-occlusive dressing. Clethodim was first applied to one male for three minutes, one hour and four hours. Successively this treatment was carried out for 4 h on two further animals. Dermal reactions were observed approximately 1, 24, 48 and 72 h after removal of the dressing, and then daily until reversal of cutaneous reactions (day 9 at the latest). Dermal reaction was evaluated according to the Draize scale.

After a three mintue exposure, the animal showed very slight erythema (grade 1) at 24 h after dosing. After a 1 h exposure period the animal showed very slight erythema (grade 1) and dryness of the skin from 24 h up to day 7 after dosing. After 4 h of exposure a very slight or well-defined erythema was noted in all animals from day 1 up to day 3 (one animal), day 5 (one animal) and day 8 (one animal). A very slight or slight oedema (grade 1 or 2) was observed in 2/3 animals from day 2 until day 3 (one animal) or day 4 (one animal). Dryness of the skin was recorded in 1/3 animals from day 5 to day 7. Yellow coloration of the skin was noted in 2/3 animals on day 1 only.

It was concluded that clethodim is mildly irritant to the skin of rabbits (Pelcot, 2005).

(c) Ocular irritation

Undiluted clethodim (purity 83.3%) was tested on nine male albino New Zealand White rabbits for eye irritation. A volume of 0.1 mL was instilled into the conjunctival sac of one eye of each animal. After 30 seconds the treated eyes of three animals was washed for one minute with distilled water to remove the test material, while the treated eyes of six animals was not washed. Observations for ocular reactions were made 1, 24, 48 and 72 h after instillation. Ocular lesions were evaluated according to the Draize scale. Animals were observed for clinical signs twice daily.

No clinical signs were noted in any of the animals during the experimental period. In the unwashed group, clethodim elicited neither corneal opacity nor iritis. One hour after dosing, conjunctival irritation ranged from moderate to severe. At 24 to 48 h after dosing, conjunctival irritation ranged from slight

to moderate. All eyes were clear of irritation 72 h after dosing. In the washed group, clethodim elicited neither corneal opacity nor iritis. One hour after dosing, conjunctival irritation was moderate. At 24 h after dosing, slight conjunctival redness was observed. All eyes were clear of irritation at the 48 h assessment. Clethodim technical caused only mild transient ocular irritation (Cushman, 1986d).

(d) Dermal sensitization

In a first Magnusson & Kligman maximization dermal sensitization study, 25% clethodim (purity 93%) in Alembicol D was injected intradermally into ten Dunkin/Hartley guinea pigs, with and without Freund's complete adjuvent (FCA). One week later, a topical induction with undiluted clethodim was performed for 48 h. Two weeks after the topical application the animals were challenged with 75% and 35% clethodim in Alembicol D for 24 h. The application site was assessed 24 and 48 h after patch removal. No dermal response was observed in any animal in the treated group (sensitization rate: 0%). In the control group no skin reactions were noted in any of the five animals, whereas in the positive control group (2-mercaptobenzothiazole, MBT) a positive response was recorded.

Under the experimental conditions clethodim did not show skin sensitization potential in guinea pigs (Coleman, 1998). However, since in the preliminary irritation study, the undiluted test item did not induce irritation, the site of treatment should have been treated with SDS before topical induction to induce some irritation. Therefore, the results from the main study are not considered reliable to investigate the potential skin sensitizing properties of clethodim.

In a second Magnusson & Kligman maximization dermal sensitization study, 50% clethodim technical (purity 92.4%) in polyethylene glycol (PEG300) and in an emulsion of FCA/physiological saline, was injected intradermally into ten male Hartley guinea pigs. One week later, a topical induction of 75% clethodim in PEG300 was performed for 48 h. Fourteen days after the topical induction, the animals were challenged by topical application of 50% clethodim in PEG300 for 24 h. The application site was assessed 24 and 48 h after patch removal. As the positive control α-hexylcinnamaldehyde (HCA) was used. Nine (at the 24 h reading) and eight (at the 48 h reading) of ten test animals showed discrete/patchy to moderate/confluent erythema after the challenge treatment with clethodim technical at 50 % (w/w) in PEG300. In the control group no skin reactions were noted in any of the five animals, whereas in the positive group (α-hexylcinnamaldehyde) a positive response was recorded (Arcelin, 2006).

Under the experimental conditions clethodim has skin sensitization potential in guinea pigs.

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Clethodim (purity 83.3%) was administered for 28 days to six groups of CD-1 ICR-derived mice (ten mice/sex per group; four weeks of age) at nominal dietary concentrations of 0, 100, 250, 625, 1500 and 4000 ppm (equivalent to 0, 11.9, 29.7, 74.4, 178.5 and 476 mg/kg bw per day for males and females, corrected for purity of the test item). Achieved concentrations and homogeneity were verified by analysis. All animals were observed for viability/mortality twice daily. Clinical signs were recorded once daily during the treatment and animals were given a detailed physical examination twice weekly. Food consumption and body weights were recorded weekly. Blood samples were taken at termination of the study for haematology (no clinical biochemistry). All animals were subjected to detailed postmortem gross examination. Weights were recorded of adrenals, brain (inc. brainstem), kidneys, liver with gall bladder, uterus and ovaries, testes and epididymides, and lung with main-stem bronchi. Histopathology investigations were performed for all mice on liver, kidney and lung, and any grossly abnormal tissues.

No mortality and no clinical signs were noted during the study. No treatment-related effects on body weight or food consumption were observed.

Red blood cell (RBC) counts were significantly decreased in males given 1500 and 4000 ppm, and in females given 1500 ppm. Haemoglobin (Hb) was significantly decreased in males at 625, 1500 and 4000 ppm and in females at 1500 ppm. Haematocrit (Ht) was significantly decreased in males given 4000 ppm. The observed changes in red blood cell parameters in males and females were only slight (92–96% of control values) and therefore were considered to be of no toxicological significance.

Absolute and relative liver (with gall bladder) weights were significantly increased in males at 1500 ppm (113% absolute and relative liver weight) and in males (142% absolute and relative liver weight) and females (116% and 123% absolute and relative liver weight, respectively) at 4000 ppm. Histopathological evaluations demonstrated an increased incidence and severity of hypertrophy of centrilobular hepatocytes in males (slight to moderate) and females (minimal to slight) given 4000 ppm, which correlated to the higher liver weights in this group. Focal coagulative necrosis in liver was noted in males at 4000 ppm.

Table 5. Key findings of the four-week dietary toxicity study in mice

	Males						Females						dr
Dose (ppm)	**0**	**100**	**250**	**625**	**1500**	**4000**	**0**	**100**	**250**	**625**	**1500**	**4000**	
Bodyweight gain													
Weeks 0–4 (g)	5.3	5.5	4.6	4.9	5.3	5.1	5.1	5.1	4.7	5.6	5.6	4.4	-
Food consumption													
Mean weeks 0–4 (g)	196.1	192.8	189.9	192.3	196.7	207.4	215.9	225.2	214.6	245.4	251.9	219.7	
Haematology													
RBC (MI/µL)	9.89	9.89	9.68	9.54	9.46*	9.05*	9.80	9.59	9.83	9.59	9.21*	9.49	M/F
Hb (g/dL)	16.6	16.5	16.2	15.9*	15.9*	15.3*	16.6	16.2	16.7	16.2	15.6*	15.9	M/F
Ht (%)	50.1	52.3	49.2	48.6	48.7	46.2*	50.6	49.4	51.9	49.2	48.7	48.5	M
Organ weights													
Absolute liver weight incl. gall bladder (g)	1.29	1.34	1.30	1.34	1.46*	1.83*	1.05	1.01	0.98	1.10	1.12	1.22*	M
Relative liver weight ratio (%)	4.339	4.463	4.463	4.580	4.931*	6.161*	4.634	4.451	4.517	4.802	4.928	5.694*	M
Histopahtology													
Liver													
No. examined	10	10	10	10	10	10	10	10	10	10	10	10	-
Centrilobular hypertrophy	4	0	3	2	4	10	0	0	0	0	1	8	M/F
Focal coagulative necrosis	1	1	1	1	2	4	2	1	0	0	0	0	-

dr Statistically significant dose response
* $p < 0.05$ ** $p < 0.01$ (Anova + Dunnetts test two-sided)

The NOAEL was 1500 ppm (equivalent to 178.5 mg/kg bw per day) based on increased liver weights, increased liver hypertrophy in both sexes and increased liver focal coagulative necrosis in males at the 4000 ppm dose level (equivalent to 476 mg/kg bw per day) (Cox R.H. 1986b).

Rat

Clethodim (purity 83.4%) was administered for 35 days to five groups of Wistar rats (ten rats/sex per group; seven weeks of age) at dietary concentrations of 0, 5, 200, 1000, 4000 and 8000 ppm (equal to 0, 0.26, 12.5, 65.6. 216 and 515 mg/kg bw per day for males, 0, 0.29, 13.9, 70.6, 291 and 554 mg/kg bw per day for females). Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cageside observations. Detailed clinical observations

were performed once weekly during the study. Pupillary examination was performed on all animals on days 0 and 35. Body weights were measured weekly during the study and food consumption was noted twice weekly. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine was collected from each animal during the overnight fast prior to termination. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (brain, liver, adrenals, kidneys, testes and ovaries) were recorded. Histological evaluation was performed on an extensive list of organs and tissues (except bone marrow) from the control and 8000 ppm groups, and kidneys, livers, lungs and on all gross lesions from the remaining groups.

The diet formulation at the 5 ppm level was not homogenous and not considered to be stable.

No mortality was noted during the study. Reduced stool was seen in two females in the first few days at the highest dose group. Body weight was significantly reduced from day 7 to 35 (89–86% of control) in males dosed at 8000 ppm, and in females at 4000 ppm from day 21 to 35 (92–91% of control), and at 8000 ppm from day 7 to 35 (90–84% of control). Body weight gain was significantly reduced in males given 4000 and 8000 ppm (89% and 72% respectively compared to controls) and in females given 4000 and 8000 ppm (75% and 56% respectively compared to controls). Food consumption was significantly reduced in males and females at 4000 and 8000 ppm, probably due to palatability problems.

Haematology results showed significantly increased platelet counts in males only, when given 200 ppm or higher, with no clear dose relationship. Erythrocytes were significantly decreased in females at 5, 1000 and 8000 ppm. Haemoglobin was significantly decreased in males given 1000, 4000 and 8000 ppm and females given 5, 1000 and 8000 ppm. Haematocrit was significantly decreased in males given 4000 and 8000 ppm. No significant changes in erythrocyte morphology was noted in males or females. Changes in red blood cell parameters were only slight (93–96%) when compared to control values, therefore were considered of no toxicological significance. The significant decreases in the hematology parameters of females at 5 and 1000 ppm were due to three abnormal animals; one at 5 ppm and two at 1000 ppm. Clinical chemistry showed significantly increased uric acid values in females given 4000 and 8000 ppm and significantly increased cholesterol values in males given 8000 ppm (168% when compared to controls). No treatment-related effects were observed in urine analysis.

Absolute liver weight was significantly increased in males at 1000, 4000 and 8000 ppm (112–115% of control) with a dose-related trend, and at 8000 ppm (113% of control) in females. Relative liver weight was significantly increased in males and females given 4000 and 8000 ppm (118–133% of control), with a dose-related trend. Liver weight relative to brain weight was significantly increased in males at 1000, 4000 and 8000 ppm (113–116% of control). Relative brain, testis and kidney weights in animals at 8000 ppm were significantly increased to 114%, 114% and 113% of control values, respectively. Since the absolute weights of these organs, or their corresponding weight relative to brain weight, were not increased, these changes are attributed to decreased terminal body weights.

Microscopic evaluation revealed compound-related centrilobular hypertrophy (mild) of the liver in males given 1000, 4000 and 8000 ppm, and in females at 4000 and 8000 ppm, correlating with the observed liver weight changes.

Key findings of the five-week feeding study in rat are shown below in Table 6.

Table 6. Key findings of the five-week feeding study in rats.

Dose	Males						Females						
(ppm)	0	5	200	1000	4000	8000	0	5	200	1000	4000	8000	dr
Clinical signs													
Reduced stool	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	2/10	

Dose (ppm)	Males						Females						
	0	5	200	1000	4000	8000	0	5	200	1000	4000	8000	dr
Body weight (g)													
Day 0	229	226	225	226	225	225	160	161	159	160	160	158	
Day 7	279	278	292	279	267	246**	187	189	186	184	178	166**	
Day 14	323	325	335	330	312	290**	206	210	210	207	197	187**	
Day 21	353	358	366	367	342	315**	222	219	222	220	205*	193**	
Day 28	380	392	399	396	367	336**	231	236	233	230	213*	201**	
Day 35	406	415	428	419	394	353**	245	246	248	244	225**	206**	
Body weight gain (g)													
Week 0–5	178	188	200*	193	159*	128**	85	86	88	84	64**	48**	M/F
Food consumption													
Day 3	26.5	29.6	27.0	26.2	21.3	14.9**	18.4	18.7	18.3	17.7	15.1**	9.6**	M/F
Day 14	27.4	27.4	29.3	28.4	26.9	26.5	20.0	20.5	20.4	18.0	18.4	17.5**	
Day 21	26.5	27.5	28.4	29.4	26.7	22.6	20.2	19.3	19.1	19.9	18.8	17.1**	
Day 28	26.4	27.4	28.4	29.5	25.4	24.1*	19.8	19.5	19.4	18.8	17.0**	16.0**	
Day 35	29.5	28.0	28.8	28.9	25.5**	25.0**	21.1	20.7	20.5	20.0	18.1**	16.0**	
Haematology													
Platelet count (10^3/mm^3)	813	1009	1052*	1109**	1163**	1029*	914	1035	1088	995	1090	1115	M
RBC (10^6/mm^3)	7.18	7.05	7.02	6.95	6.86	6.85	6.98	6.58*	6.73	6.53**	6.73	6.60*	
Hb (g/dL)	14.8	14.5	14.4	14.2*	14.0**	13.9**	14.7	14.0*	14.3	13.9**	14.1	13.8**	M/F
Ht (%)	40.1	39.8	39.4	38.9	38.4*	37.8**	40.0	38.6	39.3	35.4	38.9	38.4	M
Clinical chemistry													
Cholesterol (mg/dL)	47	52	59	55	63	79**	73	60	62	65	79	90	
Uric acid (IU/L)	1.4	1.3	1.3	1.4	1.4	1.6	1.3	1.6	1.5	1.7	1.9**	1.9**	
ALP (IU/L)	131	129	138	132	114	108	81	92	90	83	74	76	
Urinalysis													
Urine volume	26.2	29.3	26.6	34.0	39.7**	37.8	13.4	19.3	19.8	18.9	20.1	21.3	
Organ weight													
Liver: abs. (g)	12.66	12.87	13.04	14.19*	14.32**	14.51*	7.42	7.44	7.24	7.52	8.00	8.35**	M
Liver: rel. (g)	3.30	3.26	3.22	3.57	3.94**	4.36**	3.23	3.21	3.14	3.31	3.82**	4.28**	M
Liver/brain (g/g)	6.05	6.17	6.24	6.86*	7.02**	7.02**	3.88	3.92	3.83	4.43	4.35	4.36	
Brain: rel. (g)	0.546	0.531	0.518	0.521	0.563	0.623**	0.835	0.826	0.820	0.793	0.853	0.987**	

Dose	Males						Females						
(ppm)	0	5	200	1000	4000	8000	0	5	200	1000	4000	8000	dr
Kidney rel. (g)	0.884	0.878	0.832	0.878	0.939	0.996**	0.866	0.842	0.837	0.807	0.933	0.972**	
Testes rel. (g)	0.817	0.801	0.757	0.812	0.876	0.931**	-	-	-	-	-	-	
Histopathology													
Liver: centrilobular hypertrophy													
(mild)	0/10	0/10	0/10	3/10	6/10	8/10	0/10	0/10	0/10	0/10	1/10	4/10	M/F

ALP Alkaline phosphatase

dr Statistically significant dose response

* $p < 0.05$ ** $p < 0.01$ (Anova + Dunnetts test two-sided)

The NOAEL was 1000 ppm (equal to 65.6 mg/kg bw per day) based decreased body weight and food consumption in both sexes at 4000 ppm (equal to 216 mg/kg bw per day) (Eisenlord, 1986).

Clethodim (purity 83.3%) was administered to five groups of Wistar rats (12 rats/sex per group; seven weeks of age) at dietary concentrations of 0, 50, 500, 2500 and 5000 ppm (equal to 0, 2.3, 25, 134 and 279 mg/kg bw per day for males, 0, 2.8, 30, 159 and 341 mg/kg bw per day for females) for 90 days. After this period 12 animals of each dose group were sacrificed. In the case of just the 0, 2500 and 5000 ppm dose groups 12 extra animals were kept for a recovery period lasting six weeks. Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cageside observation. Detailed clinical observations were performed once weekly during the study. Ophthalmologic examination was performed on all animals prior to study initiation and just prior to the week 13 sacrifice. Body weights were measured weekly during the study and food consumption was noted twice a week for the first nine weeks, and three times a week thereafter. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine was collected from each animal during the overnight fast prior to termination. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (brain, adrenals, kidneys, liver, ovaries and testes) were recorded. The weights of epididymis, thymus, spleen, heart and uterus were not determined. Histological evaluation was performed on an extensive list of organs and tissues in the control and the 5000 ppm groups, and on the lung, liver and kidneys of all animals. In all animals during the recovery phase, histopathological investigation was limited to liver and kidneys.

No treatment-related mortality or clinical signs were noted during the study.

Body weight was significantly reduced in males given 2500 ppm (up to 93% of control) from day 21 to 91, and in both sexes at 5000 ppm from the second week to the end of the treatment period (89% of control for males, 88% for females). Body weight gain was significantly reduced in males at 2500 and 5000 ppm to 90% and 82% of the control respectively, and in females at 5000 ppm to 76% of control.

At the end of the recovery phase, males' body weights were 94% of control for animals given 2500 ppm, and 97% of controls for animals given 5000 ppm. However, these changes did not achieve statistical significance. Body weight in females at 5000 ppm remained significantly decreased at about 93% of controls. During the recovery phase, significantly higher weight gains were recorded for males given 5000 ppm. Food consumption was significantly reduced in males and females during treatment at 5000 ppm. No effects on food consumption were seen in the recovery phase.

Reticulocyte count was significantly increased in males given 2500 ppm (139% of control). A increase in reticulocyte count, not statistically significant, was noted at 50, 500 and 5000 ppm (126–135% when compared to the control). Reticulocyte increase showed no clear dose response. No historical control data were available in the study report, however, an increase of approximately 30% is, in general, not considered toxicologically significant. No treatment-related changes in urinalysis parameters were noted.

Clinical chemistry showed significantly higher cholesterol, total protein and globulin values at 5000 ppm in males (131%, 165% and 109% of control values, respectively), which could be related to perturbations in liver function. The reversibility of these changes was not assessed.

No treatment-related effects were observed in erythrocytes morphology, urine analysis and ophthalmoscopy investigation.

Absolute liver weight was significantly increased for females given 5000 ppm (114% of control), relative liver weight was significantly increased in both sexes given 2500 ppm and 5000 ppm, with a dose-related trend (range from 112% to 128%). The significantly increased relative kidney and brain weights in both sexes given 5000 ppm were attributed to significantly reduced body weight. Following the recovery phase, absolute liver weights were significantly higher in females given 2500 ppm, and relative liver weights were significantly higher in females given 2500 and 5000 ppm, 110% and 113% of control, respectively.

An increased incidence of renal focal regeneration was seen in males treated at 2500 and 5000 ppm. The pathologist did not consider this finding to be related to treatment; rather, these lesions were regarded as typical of the earliest minimal stage of spontaneous chronic progressive nephropathy. Increased incidence of centrilobular hypertrophy of the liver in males and females at 2500 and 5000 ppm was the microscopic correlate of the liver weight changes. Following the recovery period there was no evidence of liver hypertrophy in any of the animals.

Table 7. Key findings of the 90-day feeding study in rats.

	Males						Females					
Dose (ppm)	**0**	**50**	**500**	**2500**	**5000**	**dr**	**0**	**50**	**500**	**2500**	**5000**	**dr**
Body weight (g)												
Week 0	226	224	222	221	222		160	161	163	159	158	
Week 13	536	543	511	499*	477**		280	285	284	275	250**	
Body weight gain (g)												
Week 0–13	310	319	290	278*	254**	dr	121	123	121	116	92**	
Food consumption												
	–	–	–	–	DC		–	–	–	–	DC	
Haematology												
Reticulocytes (% ± SD)	2.3 ± 0.6	3.1 ±1.0	2.9 ±1.1	3.2 ±0.5*	3.1 ± 0.7		2.8 ±1.3	2.5 ±1.3	2.6 ±1.1	2.9 ±1.0	3.1 ±1.2	
Clinical chemistry												
Cholesterol (g/dL)	70	67	65	76	92*		82	87	79	85	91	
Total protein (g/dL)	6.4	6.4	6.4	6.6	6.7*		6.6	6.9	6.5	6.7	6.8	
Globulin (g/dL)	3.3	3.3	3.2	3.4	3.6*		3.3	3.4	3.3	3.3	3.3	
Organ weights												
Brain: relative (g)	0.424	0.426	0.443	0.446	0.491**		0.766	0.765	0.746	0.789	0.867**	
Liver: absolute (g)	14.33	14.76	13.58	15.16	15.61		7.04	7.47	7.11	7.69	8.00**	
Liver: relative (g)	2.80	2.87	2.79	3.13*	3.52**	dr	2.68	2.82	2.67	2.99**	3.42**	dr
Kidney: relative (g)	0.700	0.702	0.707	0.733	0.768*		0.751	0.776	0.733	0.806	0.856**	

	Males						Females					
Dose (ppm)	**0**	**50**	**500**	**2500**	**5000**	**dr**	**0**	**50**	**500**	**2500**	**5000**	**dr**
Histopathology												
Liver: centrilobular hypertrophy	0/12	0/12	0/12	8/12	10/12	dr	0/12	0/12	0/12	2/12	7/12	dr
Kidney: focal regeneration	2/12	5/12	3/12	7/12	8/12		0/12	1/12	1/12	1/12	1/12	

dr Statistically significant dose response

DC Statistically significantly decreased

* $p < 0.05$ ** $p < 0.01$ (Anova + Dunnetts test two-sided)

Table 8. Key findings of the 90-day feeding study in rats after the six-week recovery period.

	Males						Females					
Dose (ppm)	**0**	**50**	**500**	**2500**	**5000**	**dr**	**0**	**50**	**500**	**2500**	**5000**	**dr**
Body weight (g)												
Week 0	226	–	–	221	222		160	–	-–	159	158	
Week 19	573	–	–	540	554		295	–	–	302	273*	
Body weight gain (g)												
Week 13–19	39	–	–	51	67**		17	–	–	26	25	
Organ weights												
Liver: absolute (g)	15.89	–	–	14.94	16.14		7.59	–	–	8.58*	7.90	
Liver: relative (g)	2.89	–	–	2.90	3.04		2.74	–	–	3.03*	3.10**	dr

SD Standard Deviation

dr Statistically significant dose response

* $p < 0.05$ ** $p < 0.01$ (Anova + Dunnetts test two-sided)

The NOAEL was 500 ppm (equal to 25 mg/kg bw per day) based on changes in body weights in males at 2500 ppm (equal to 134 mg/kg bw per day) (Dougherty, 1986).

Dog

Study 1

Clethodim (purity 83.3%) was administered to five groups of four male and four female Beagle dogs (aged 5–6 months) by gelatin capsules at dose levels of 0, 1, 25, 75 and 125 mg/kg bw per day (equal to 0, 0.83, 21, 62 and 104 mg/kg bw per day after correction for purity) for 90 days. Observations for mortality and gross signs of toxicity were performed at least twice daily during the study. Detailed clinical observations and food consumption were recorded daily, whereas body weight was recorded weekly. Ophthalmoscopy was performed once before the test and at the end of the treatment period. Blood collection after fasting was performed on all animals before the start of treatment and at weeks 5, 8, and 13 of the administration period. Urinalysis was performed on all animals before the start of treatment and at weeks 4, 8, and 12 of the administration period. Detailed necropsy was performed on all animals after scheduled sacrifice and the following organs were weighed: adrenals, brain (with brainstem), heart, kidneys, lungs, pituitary, testes (without epididymides), thyroids (with parathyroids), liver and ovaries. An extensive list of tissues was examined histologically.

No mortality and no clinical signs were noted during the study. No effects were noted on body weight, food consumption, ophthalmoscopy and urinalysis.

White blood cells were significantly increased in males given 75 and 125 mg/kg bw per day at day 35 of exposure. Activated partial thromboplastin time was significantly decreased in females given 75 and 125 mg/kg bw per day at day 35 of exposure. Since all these haematological deviations were only seen at one point in time, they were not regarded as of toxicological significance. Mean corpuscular haemoglobin was significantly increased in females given 125 mg/kg bw per day at day 91 of exposure.

In the course of the study, mean alkaline phosphatase (ALP) activity progressively increased in males and females given 125 mg/kg bw per day, whereas it progressively decreased in respective control animals (in females given 125 mg/kg bw per day these differences from control were statistically significant throughout the study). Similarly, mean cholesterol levels in females given 125 mg/kg bw per day progressively increased, whereas they remained unchanged in control females; these differences from control were statistically significant after one and two months. Females given 75 mg/kg bw per day showed increased cholesterol levels when compared to controls, achieving statistical significance at one and two months. Since these values were very similar to those at pretest, and since the statistical significance was probably driven by low levels of cholesterol in the controls at the corresponding time points, these changes were not considered treatment-related. Globulin was significantly increased in males given 125 mg/kg bw per day at day 91 of exposure and correspondingly the albumin : globulin ratio was significantly decreased at day 91 of exposure in males given 125 mg/kg bw/day. Chloride was statistically significantly decreased compared to controls in females given 75 and 125 mg/kg bw per day at day 35 of the study. Since chloride change was only seen at one time point and was very slight (97–98% of controls), it was considered of no toxicological relevance. The deviations in ALP, cholesterol and globulin in animals given 125 mg/kg bw per day suggest perturbations in liver function.

Absolute liver weight was increased in animals given 75 and 125 mg/kg bw per day (in males 116 and 134% of controls and in females 115 and 130% of controls), with the differences from controls being statistically significant only at 125 mg/kg per day. Relative liver weight was increased at 75 and 125 mg/kg bw per day in both males (112 and 127% of controls) and females (106 and 119%); these deviations were not statistically significant.

No macroscopic effects were observed. Histopathology investigation showed vesiculation/vacuolation in the cytoplasm of centrilobular hepatocytes in all males at all levels including the control group and in all treated females and three control females. However, an increase in severity was noted in animals given 125 mg/kg bw per day, which is considered to be treatment-related.

Table 9. Key results of the 13-week study in dogs.

Dose	Males					Females				
(mg/kg bw/day)	0	1	25	75	125	0	1	25	75	125
Haematology										
White bood cell count (10^3/µL) ± SD										
Pre-test	11.8 ± 4.7	14.1 ± 4.3	12.5 ± 1.7	13.2 ± 1.9	15.2 ± 1.4	13.3 ± 0.4	11.0 ± 2.7	11.6 ± 2.3	11.1 ± 3.6	10.4 ± 2.2
Day 35	9.7 ± 1.5	11.3 ± 1.6	11.5 ± 1.7	15.6 ± 3.1**	14.8 ± 2.8**	11.1 ± 1.3	11.6 ± 4.1	11.4 ± 2.8	12.0 ± 2.3	11.1 ± 2.8
Day 55	10.4 ± 2.1	11.5 ± 2.4	10.7 ± 0.5	13.4 ± 3.2	14.0 ± 2.7	12.5 ± 0.3	10.0 ± 2.8	10.9 ± 2.8	11.7 ± 2.6	11.0 ± 2.5
Day 91	9.3 ± 1.2	11.5 ± 1.3	11.0 ± 1.4	13.3 ± 3.2	13.6 ± 2.4	12.7 ± 4.4	8.7 ± 1.9	8.7 ± 2.3	10.3 ± 1.5	12.8 ± 2.9
MCH (µg)										
Pre-test	24.8	24.8	24.8	24.6	25.0	24.6	25.4	23.7	23.6	25.9
Day 91	24.1	24.0	23.9	24.5	24.0	25.8	26.2	25.6	26.0	28.4*

Dose	Males					Females				
(mg/kg bw/day)	0	1	25	75	125	0	1	25	75	125
APTT (s) ± SD										
Pre-test	9.0 ± 0.2	9.2 ± 0.2	9.4 ± 0.2	9.2 ± 0.4	9.8 ± 0.8	9.7 ± 0.6	9.3 ± 0.1	9.9 ± 0.4	9.2 ± 0.3	9.9 ± 0.5
Day 35	9.4 ± 0.3	9.3 ± 0.3	9.6 ± 0.7	9.2 ± 0.5	9.3 ± 0.3	9.9 ± 0.2	9.3 ± 0.1	9.9 ± 0.6	9.1 ± 0.1*	9.2 ± 0.3*
Day 55	9.1 ± 0.1	9.3 ± 0.2	9.3 ± 0.7	9.1 ± 0.4	9.3 ± 0.4	9.7 ± 0.4	9.2 ± 0.1	9.6 ± 0.7	9.0 ± 0.3	9.4 ± 0.3
Day 91	9.3 ± 0.4	9.8 ± 0.8	9.2 ± 0.7	9.3 ± 0.4	9.2 ± 0.7	9.7 ± 0.9	9.2 ± 0.1	9.9 ± 0.9	9.2 ± 0.4	9.1 ± 0.4
Clinical chemistry										
ALP (IU/L)										
Pre-test	93	99	92	83	95	86	88	87	102	95
Day 35	95	95	106	94	120	79	71	80	100	134*
Day 55	88	83	95	84	126	80	63	72	98	132*
Day 91	79	68	83	85	132	75	56	54	93	141*
Cholesterol (mg/dL) ± SD										
Pre-test	156 ± 10	181 ± 37	172 ± 12	181 ± 34	140 ± 16	138 ± 30	161 ± 22	152 ± 28	171 ± 14	174 ± 12
Day 35	130 ± 18	140 ± 36	147 ± 13	173 ± 37	138 ± 20	120 ± 18	147 ± 15	150 ± 43	170 ± 19*	190 ± 23*
Day 55	137 ± 26	149 ± 41	160 ± 13	172 ± 52	149 ± 19	129 ± 29	147 ± 15	157 ± 38	179 ± 16*	181 ± 26*
Day 91	144 ± 21	159 ± 30	168 ± 20	183 ± 21	181 ± 24	136 ± 35	174 ± 51	174 ± 42	179 ± 24	214 ± 27
Globulin (g/dL)										
Day 91	2.3	2.5	2.5	2.2	2.8*	2.3	2.1	2.5	2.5	2.6
Albumin/globulin ratio										
Day 91	1.4	1.3	1.3	1.5	1.1*	1.4	1.6	1.4	1.3	1.3
Chloride (m equiv./L) ± SD										
Pre-test	105 ± 2	103 ± 6	103 ± 2	102 ± 4	105 ± 1	104 ± 2	103 ± 1	105 ± 1	102 ± 3	104 ± 1
Day 35	106 ± 1	106 ± 1	104 ± 1	106 ± 2	106 ± 1	106 ± 1	105 ± 1	104 ± 1	103 ± 1*	104 ± 1*
Day 55	105 ± 1	106 ± 1	105 ± 2	104 ± 2	105 ± 1	106 ± 2	105 ± 2	104 ± 1	103 ± 1	104 ± 1
Day 91	106 ± 2	107 ± 1	107 ± 2	106 ± 1	106 ± 1	106 ± 1	105 ± 1	104 ± 2	105 ± 2	105 ± 1
Sodium (m equiv./L) ± SD										
Pre-test	148 ± 1	147 ± 1	146 ± 1	148 ± 2	148 ± 2	147 ± 1	147 ± 2	147 ± 1	148 ± 2	146 ± 2
Day 35	148 ± 2	148 ± 2	147 ± 1	148 ± 1	148 ± 0	148 ± 1	148 ± 2	147 ± 3	147 ± 2	147 ± 2
Day 55	147 ± 1	147 ± 1	146 ± 1	147 ± 1	146 ± 2	147 ± 3	147 ± 1	146 ± 2	145 ± 1	145 ± 1
Day 91	149 ± 1	149 ± 2	148 ± 2	150 ± 2	150 ± 1	147 ± 1	148 ± 3	148 ± 1	150 ± 4	149 ± 2

Dose	Males					Females				
(mg/kg bw/day)	0	1	25	75	125	0	1	25	75	125
Organ weights										
Liver: absolute (g)	293.6	287.3	304.3	339.8	393.3**	254.5	239.8	269.8	293.0	330.7**
Liver: relative (g)	2.67	2.56	2.84	3.00	3.38	2.83	2.62	2.86	2.99	3.36
Histopathology										
Liver:										
Number examined	4	4	4	4	4	4	4	4	4	4
Centrilobular vesicules/vacuoles	4	4	4	4	4	3	4	4	4	4
minimal	1	1	0	0	0	1	1	1	1	0
slight	3	2	3	3	1	2	1	1	3	1
moderate	0	1	1	1	1	0	2	2	0	2
moderately severe	0	0	0	0	2	0	0	0	0	1

SD Standard Deviation

MCH Mean corpuscular haemoglobin

APTT Activated partial thromboplastin time

* $p < 0.05$ ** $p < 0.01$ (Anova and Dunnett's test)

The NOAEL was 75 mg/kg bw per day (equal to 62 mg/kg bw per day) based on elevated liver weights and liver histopathology lesions (centrilobular vacuoles) in both sexes, and increased serum cholesterol and ALP at 125 mg/kg bw per day (equal to 104 mg/kg bw per day) (Daly, 1987).

Study 2

Clethodim (purity 83.3%) was administered for 52 weeks by gelatin capsule to Beagle dogs (4–6 months old) distributed into five groups of six dogs/sex per group at dose levels of 0, 1, 75 and 300 mg/kg bw per day (equal to 0, 0.83, 62 and 250 mg/kg bw per day after correction for purity). In the absence of any signs of overt toxicity, the high dose of 200 mg/kg bw per day was increased after seven weeks of treatment to 300 mg/kg bw per day. Achieved concentrations and homogeneity were verified by analysis. All animals were observed twice daily with detailed examinations performed once each week. Food consumption was monitored daily and body weight measurements were taken weekly. Ophthalmological examination was performed pre-test and on days 180 and 363. A battery of behavioural tests and observations was performed on all animals before initiation of treatment and once weekly thereafter. Blood collection after fasting was undertaken on all animals before the start of treatment and on days 31, 90, 180, 270 and 360 of the administration period. Urinalysis was performed on all animals before the start of treatment and on days 180 and 360 of the administration period. Bone marrow smears were prepared for all animals and examined, based on findings evident during haematology. At necropsy, all animals were subjected to thorough gross examination and the following organs were weighed: liver (with drained gallbladder), kidneys, brain (with brainstem), testes, heart, thyroids (with parathyroids), pituitary, lung (with mainstem bronchi), ovaries, adrenals and spleen. Histopathology was performed on an extensive list of tissues.

No mortality and no clinical signs were noted during the study. No effects were noted on body weight or food consumption,nor on the results of ophthalmoscopy and urinalysis. Erythrocyte count was statistically significantly decreased in males given 300 mg/kg bw per day on days 270 and 360, and in females given 300 mg/kg bw per day on days 180, 270 and 360. However, erythrocyte changes in males and females at 300 mg/kg bw per day were minor in males, and if compared to the pre-test values, changes in females showed an increase. Haemoglobin and Ht were statistically significantly decreased in females given 300 mg/kg bw per day on days 180, 270 and 360. These changes were considered of doubtful toxicological relevance since changes appear to be opposite in direction if compared to pre-test

values. Platelet counts were statistically significantly increased during the whole exposure period in both sexes given 300 mg/kg bw per day. In males and females at 75 mg/kg bw per day platelets progressively increased, achieving statistical significance in females on days 180 and 360. However, the increase in platelets in males at 75 mg/kg bw per day was considered of doubtful toxicological relevance, since the change was minor. Prothrombin time was statistically significantly decreased in all treated males at day 90. However, this decrease was considered of no toxicological significance since the change was observed only at one time point and not subsequently.

The white blood cell count was statistically significantly increased in females given 300 mg/kg bw per day on days 90, 180, and 360. In females given 75 mg/kg bw per day, white blood cells tended to increase when compared to pre-test values; statistical significance compared to control was achieved on day 90. An increase in segmented neutrophils was observed in both sexes at 75 and 300 mg/kg bw per day when compared to controls or pre-test values, with females dosed at 300 mg/kg bw per day achieving statistical significance on days 31, 90 and 270. However, changes in white blood cells and segmented neutrophils were considered of doubtful toxicological relevance since only minor.

The albumin : globulin ratio was decreased in females given 300 mg/kg bw per day at days 270 and 360. The glucose level was significantly decreased in males given 300 mg/kg bw/day on day 270; in females given 300 mg/kg bw/day on days 180 and 360, and in females given 75 mg/kg bw/day at day 360. The decrease in glucose was considered of doubtful toxicological relevance, since the changes were minor and similar to pre-test values. Alkaline phosphatase was significantly increased in both sexes from day 90 onwards at 300 mg/kg bw/day. Alanine transaminase (ALT) was statistically significantly increased at 300 mg/kg bw per day in both sexes from day 180 onwards. Cholesterol was statistically significantly increased in males given 300 mg/kg bw/day at day 360 only, but in females throughout the study. Triglycerides were significantly increased at day 360 in both sexes given 300 mg/kg bw per day. Urinalysis showed no treatment-related findings.

Both absolute and relative thyroid + parathyroid weight were significantly increased in males given 300 mg/kg bw per day (183 and 200% of controls, respectively), but there was no microscopic correlate. Absolute and relative liver weights was significantly increased in both sexes given 300 mg/kg bw per day (156 and 160% of controls for males, respectively, 170 and 168% of controls for females, respectively), and in females given 75 mg/kg bw/day (134 and 158% of controls, respectively). In males given 75 mg/kg bw per day absolute liver weight was increased (127%) and relative liver weight was statistically significantly increased (116%). Macroscopic examinations revealed a dark liver in four males and four females given 300 mg/kg bw per day, and an enlarged liver in two males and two females given 300 mg/kg bw/day.

Histopathology showed centrilobular to midzonal hepatocellular hypertrophy in five out of six males and four out of six females given 300 mg/kg bw/day. Increased pigmentation of the liver was observed in one male given 75 mg/kg bw per day and all animals given 300 mg/kg bw per day. The pigment was negative for bile, iron and lipofuscin and its nature was not identified. Hyperplasia of the sternum marrow was observed in one male and one female given 75 mg/kg bw per day and in all animals at 300 mg/kg bw/day, probably as a response to anaemia and an explanation for the increases in peripheral leukocytes and platelets at 300 mg/kg bw per day. There was an increased incidence of spleen pigmentation in males and females at 75 and 300 mg/kg bw per day. The incidence of histopathological findings in liver, sternum marrow and spleen in males and females at 75 mg/kg bw per day were considered marginal.

Table 10. Key results of the one-year feeding study in dogs.

	Males					Females				
Dose (mg/kg bw/day)	**0**	**1**	**75**	**300**	**dr**	**0**	**1**	**75**	**300**	**dr**
Haematology										
RBC (MI/μL) ± SD										
Pre-test	6.83 ± 0.59	6.18 ± 0.42	6.87 ± 0.22	6.85 ± 0.17		6.19 ± 0.44	6.82 ± 0.56	6.89 ± 0.23	6.12 ± 0.35	
Day 180	6.56 ± 0.52	6.85 ± 0.48	6.55 ± 0.28	6.11 ± 0.48		6.97 ± 0.41	6.95 ± 0.60	6.81 ± 0.62	5.58 ± 0.67*	

		Males					Females				
Dose (mg/kg bw/day)		**0**	**1**	**75**	**300**	**dr**	**0**	**1**	**75**	**300**	**dr**
	Day 270	6.82 ± 0.53	7.03 ± 0.54	6.57 ± 0.18	6.17 ± 0.24*		7.11 ± 0.31	6.91 ± 0.65	7.23 ± 0.44	5.93 ± 0.61*	
	Day 360	7.25 ± 0.51	7.76 ± 0.67	7.06 ± 0.29	6.58 ± 0.44*		7.59 ± 0.46	7.16 ± 0.78	7.30 ± 0.51	6.20 ± 0.45*	
Haemoglobin (g/dL) ± SD											
	Pre-test	13.7 ± 1.2	13.8 ± 1.1	13.5 ± 0.5	13.3 ± 0.5		14.0 ± 1.0	13.6 ± 1.2	13.8 ± 0.6	13.9 ± 0.9	
	Day 180	15.0 ± 1.4	15.4 ± 0.8	15.0 ± 1.0	14.2 ± 1.8		15.8 ± 0.9	16.0 ± 0.9	16.2 ± 1.7	13.5 ± 1.8*	
	Day 270	15.6 ± 1.3	16.0 ± 1.3	15.1 ± 0.5	14.5 ± 0.3		16.4 ± 0.8	16.1 ± 1.3	17.6 ± 1.2	14.2 ± 1.7*	
	Day 360	16.7 ± 1.2	17.7 ± 1.3	16.3 ± 0.7	15.3 ± 0.7		17.2 ± 1.5	16.6 ± 1.8	17.4 ± 1.2	14.8 ± 1.4*	
Haematocrit (%) ± SD											
	Pre-test	40.8 ± 3.4	41.3 ± 3.2	40.2 ± 1.4	39.6 ± 1.3		41.5 ± 2.8	40.4 ± 3.0	41.0 ± 1.7	41.0 ± 2.5	
	Day 180	43.8 ± 4.2	45.1 ± 3.2	43.8 ± 2.8	41.4 ± 3.3		46.2 ± 2.7	46.4 ± 2.5	46.9 ± 5.3	39.2 ± 5.3*	
	Day 270	45.8 ± 3.7	46.4 ± 3.1	44.1 ± 1.6	42.8 ± 1.1		47.6 ± 1.6	46.4 ± 4.1	50.0 ± 3.1	41.5 ± 4.8*	
	Day 360	47.5 ± 3.1	50.4 ± 3.8	46.7 ± 2.1	43.7 ± 1.7		49.0 ± 4.0	47.5 ± 4.9	49.7 ± 3.5	42.3 ± 4.1*	
MCH ± SD (pg)	Day 180	22.8 ± 0.8	22.5 ± 0.9	22.8 ± 0.9	23.2 ± 0.9		22.7 ± 1.2	23.1 ± 1.1	23.7 ± 0.5	24.2 ± 1.0*	
Platelet count											
	Pre-test	464	461	381	517		428	428	350	477	
	Day 31	319	319	343	484*		297	297	326	452*	
	Day 90	317	314	381	516*		268	266	334	466*	
	Day 180	310	319	395	530*		264	271	367*	513*	
	Day 270	324	294	393	532*		308	284	396	572*	
	Day 360	332	341	399	562*		294	304	410*	599*	
PT ± SD (s)	Day 90	6.3 ± 0.1	6.0 ± 0.2*	6.0 ± 0.2*	6.0 ± 0.2*		6.3 ± 0.3	5.9 ± 0.3	6.2 ± 0.2	6.0 ± 0.1	
	Day 270	6.0 ± 0.1	5.8 ± 0.2	5.8 ± 0.2	5.9 ± 0.2		5.8 ± 0.2	5.8 ± 0.1	6.0 ± 0.2	5.8 ± 0.1	
	Day 360	6.1 ± 0.3	5.8 ± 0.1	5.9 ± 0.3	6.0 ± 0.1		5.9 ± 0.5	5.9 ± 0.3	6.0 ± 0.3	6.0 ± 0.1	
WBC (TH/µL) (±SD)											
	Pre-test	12.8 ± 1.5	12.8 ± 1.7	11.0 ± 4.9	12.4 ± 1.9		14.5 ± 6.9	11.6 ± 2.0	10.9 ± 1.4	14.4 ± 2.8	
	Day 90	11.4 ± 1.8	13.3 ± 2.0	11.8 ± 2.9	14.0 ± 1.8		10.5 ± 1.0	10.5 ± 1.4	13.3 ± 1.5*	14.9 ± 2.4*	dr
	Day 180	11.8 ± 1.8	13.8 ± 1.3	12.1 ± 2.0	15.1 ± 2.1*		12.1 ± 2.5	9.9 ± 0.8	14.8 ± 3.4	15.5 ± 1.7*	dr
	Day 360	11.3 ± 2.5	11.5 ± 2.2	11.1 ± 2.3	15.8 ± 3.5		9.9 ± 1.7	9.7 ± 3.8	14.0 ± 4.0	15.8 ± 2.9*	dr

	Males					Females				
Dose (mg/kg bw/day)	**0**	**1**	**75**	**300**	**dr**	**0**	**1**	**75**	**300**	**dr**
Segmented neutrophils ± SD (TH/μL)										
Pre-test	7.5 ± 1.3	7.1 ± 1.3	6.2 ± 4.1	6.9 ± 1.6		9.8 ± 4.6	6.4 ± 0.6	6.1 ± 1.4	8.8 ± 1.9	
Day 31	7.5 ± 1.6	7.7 ± 2.2	9.2 ± 2.7	10.7 ± 2.4		8.6 ± 3.9	6.4 ± 1.0	8.3 ± 1.5	12.6 ± 4.9*	
Day 90	6.4 ± 1.6	7.5 ± 1.1	6.5 ± 2.8	8.3 ± 2.8		6.8 ± 0.9	5.3 ± 1.2	7.9 ± 0.9	9.7 ± 2.9*	
Day 270	7.3 ± 2.5	8.3 ± 1.5	9.5 ± 2.8	10.0 ± 2.2		8.2 ± 1.2	6.4 ± 1.7	8.0 ± 1.5	12.8 ± 4.7*	
Day 360	7.8 ± 2.5	7.8 ± 2.1	8.8 ± 2.1	11.0 ± 3.4		6.6 ± 1.5	6.2 ± 3.8	9.8 ± 3.9	10.7 ± 2.9	
Clinical chemistry										
Albumin/globulin ratio										
Day 360	1.14	1.15	1.10	0.98		1.29	1.22	1.18	0.96*	dr
Glucose (mg/L)										
Pre-test	108	106	107	105		101	98	99	97	
Day 180	105	108	103	98		104	103	94	92*	dr
Day 270	111	111	103	98*		105	104	104	98*	dr
Day 360	108	105	99	95		108	103	98*	93*	dr
Alkaline phosphatase (U/L)										
Day 90	68	70	78	116*		58	72	79	121*	
Day 360	30	32	44	112*		34	43	55	150*	
Cholesterol (mg/dL)										
Pre-test	187	179	191	162		194	191	169	189	
Day 31	191	170	198	181		170	171	169	224*	
Day 90	181	161	197	207		157	169	171	256*	
Day 180	155	155	194	184		151	174	201	247*	
Day 270	149	141	175	183		151	182	163	236*	
Day 360	153	159	188	202*		168	171	218	270*	
Triglycerides (mmol/L)										
Day 360	34	39	44	56*		32	34	45	59*	
ALT (U/L) Day 180	27	25	23	75*		25	27	19*	47*	
Day 270	32	26	24	74*		28	25	23	36*	
Day 360	36	31	29	96*		27	32	22	66*	
Organ weights										
Thyroid/parathyroid: absolute (g)	0.93	1.13	1.35	1.78*		1.0	1.03	0.99	1.04	
Thyroid/parathyroid: relative (g)	0.009	0.012	0.012	0.018*		0.013	0.012	0.011	0.012	
Liver: absolute (g)	250	259	317	390*	dr	208	209	279*	354*	dr
Liver: relative (g)	2.5	2.7	2.9*	4.0*	dr	2.4	2.4	3.0*	4.2*	dr

	Males					Females				
Dose (mg/kg bw/day)	**0**	**1**	**75**	**300**	**dr**	**0**	**1**	**75**	**300**	**dr**
Macroscopy										
Liver, dark	0/6	0/6	0/6	4/6		0/6	0/6	0/6	4/6	
Liver, enlarged	0/6	0/6	0/6	2/6		0/6	0/6	0/6	2/6	
Histopathology										
Liver:										
number examined	6	6	6	6		6	6	6	6	
hepatocyte hypertrophy	0	0	0	5		0	0	0	4	
hepatocyte pigment	0	0	1	6	dr	0	0	0	6	
Sternum marrow:										
hyperplasia	0	0	1	6	dr	0	0	1	6	dr
Spleen:										
increased pigment	1	0	2	2		0	0	2	3	

SD Standard deviation RBC Red blood cells MCH Mean corpuscular haemoglobin
PT Prothrombin time WBC White blood cells dr Statistically significant dose response

* $p < 0.05$ ** $p < 0.01$ (Anova and Dunnett's test)

The NOAEL was 75 mg/kg bw per day (equal to 62 mg/kg bw per day), based on increased platelets, liver weights and associated increase in biochemical parameters (ALP and cholesterol) and histopathological effects (hypertrophy and pigment), and hyperplasia in sternum marrow and pigments in the spleen in both sexes at 300 mg/kg bw per day (equal to 250 mg/kg bw per day) (Cox, 1988a).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

Clethodim (purity 83%) was administered to five groups of 60 male and 60 female Crl:CD-1(ICR)BR mice (ca six weeks old) at dietary concentrations of 0, 20, 200, 1000 and 3000 ppm (equivalent to 0, 2.4, 24, 119 and 238/357 mg/kg bw per day, after correction for purity) for 18 months. From week 15, the maximum dose was increased from 2000 ppm to 3000 ppm. Ten animals/sex per dose group were randomly selected for sacrifice after 52 weeks of treatment. The other 50 animals/sex per group remained under treatment until week 79. Dose levels, homogeneity, and stability in the diet were confirmed by analysis. Animals were checked for viability/mortality and clinical signs at least once daily. Detailed clinical observations (including palpation of tissue masses) were performed weekly. Body weight and food consumption were determined for weeks 1–14, again at week 16 and then once every four weeks thereafter. Blood samples were collected for haematological evaluation from five random mice/sex for pre-test screening, from ten mice/sex in control groups and also the groups receiving 1000 or 3000 ppm during weeks 15 and 27, and from ten mice/sex in all groups during weeks 53 and 79 of treatment. A detailed gross necropsy was performed at termination, and for all mice found dead or killed in extremis. The following organ weights were measured for all animals: adrenals, brain (with brain stem), kidneys, liver (with gallbladder), lung (with mainstem bronchi), testes (with epididymides) and uterus (with ovaries). An extensive list of tissues (including bone marrow) was examined histologically including all gross lesions and tissue masses.

Treatment for 78 weeks with the test substance at 2000→3000 ppm significantly reduced the survival rate in both sexes (from week 56 for males and from week 60 for females). The predominant cause of death at this dose was an increased incidence and severity of systemic amyloidosis. No treatment-related clinical signs were observed. No statistically significant changes in body weights or food consumption were noted; in high-dose females a decrease in body weight gain (71% of controls; not statistically significant) was noted after the dose was changed from 2000 ppm to 3000 ppm (from week 16 to 78). No relevant biological changes in the efficiency of food utilization were observed in either sex.

Red blood cell count was statistically significantly decreased in males given 2000→3000 ppm in weeks 27 and 79, and in females given 2000→3000 ppm in week 27. Haemoglobin and Ht were statistically significantly decreased in males given 2000→3000 ppm in week 27. These RBC deviations are consistent with a mild normochromic normocytic anemia, as noted in mice and other species in subacute and subchronic toxicity studies; they were considered to be related to treatment, but given their marginal nature they were considered of doubtful toxicological significance.

After 52 weeks of treatment, increased liver weights and centrilobular hypertrophy of the liver were observed in males given 1000 ppm and in males and females given 3000 ppm. Increased pigment, described as morphologically compatible with haemosiderin and bile, was noted in males given 3000 ppm.

After 78 weeks of treatment, liver weights were increased in males and females given 3000 ppm (112–114% of controls).

Histopathology investigations of unscheduled deaths and animals killed at termination showed a treatment-related increase in systemic amyloidosis in males and females given 3000 ppm. A compound-related increase of liver centrilobular hypertrophy was observed in both males and females given 1000 and 3000 ppm. An appreciable increase in bile duct hyperplasia was observed in males at 1000 and 3000 ppm, graded minimal to moderate. Two males at 200 ppm had bile duct hyperplasia graded minimal to slight. Bile duct hyperplasia is not an uncommon age-related finding in rodents, therefore this slight increase in males at 200 ppm, graded minimal to slight, was considered unrelated to treatment. Increased pigment, graded minimal to moderate, was observed in males and females 1000 and 3000 ppm; such increase was more pronounced in males.

An increased incidence of multifocal, amphophilic alveolar lung macrophages was also observed in animals of each sex given 1000 and 3000 ppm. This change was graded minimal to slight and consisted of large macrophages present in the alveolar space with no evidence of inflammation or necrosis. The macrophages were positive with periodic acid–Schiff stain (PAS) and negative for iron (except one animal) and lipofuscin. This finding was also present in one male at 200 ppm (graded minimal), but given such low incidence it was not considered treatment-related.

A relatively high incidence of alveolar/bronchiolar adenomas and carcinomas were observed in control and treated females and in treated males. In both sexes, the incidences of these tumours were not dose-related. In addition, no preneoplastic lesions were evidenced.

Hepatocellular adenomas were evidenced in control and treated males, but with no dose response. Hepatocellular adenomas were observed in 4/60 females at 3000 ppm compared with 0/60 in controls. Nevertheless it is pointed out that no treatment-related preneoplastic lesions were observed, and that the dose level of 3000 ppm exceeded the maximum tolerated dose.

Table 11. Key results of the carcinogenicity study in mice.

	Males					Females					dr
Dose (ppm)	**0**	**20**	**200**	**1000**	**2000/ 3000**	**0**	**20**	**200**	**1000**	**2000/ 3000**	
Survived animals	29/50	33/50	30/50	25/50	16/50*	33/50	42/50	40/50	29/50	24/50*	M/F
Survival (%)	58	66	60	50	32	66	84	80	58	48	
Body weight gain											
Week 0–78	10.9	11.7	11.1	11.7	10.6	13.0	13.0	13.5	12.4	11.9	
Week 16–78	30.	3.4	2.8	3.3	3.0	5.6	5.3	5.2	4.6	4.0	
Haematology											
RBC (mi/μL)											
Week 27	9.40	-	-	9.23	9.15	9.37	-	-	9.31	8.92*	
Week 79	9.61	8.69	8.44*	9.23	8.23*	8.63	8.34	8.07	8.91	8.58	

Haemoglobin (g/dL)											
Week 27	16.0	-	-	15.7	14.9*	16.5	-	-	16.1	16.1	
Haematocrit (%)											
Week 27	48.7	-	-	46.6	44.7*	49.0	-	-	48.8	48.3	
Organ weights											
Interim sacrifice											
Liver: absolute (g)	1.53	1.50	1.58	1.71	1.78*	1.47	1.27	1.35	1.47	1.70	
Liver: relative (%)	4.220	4.152	4.319	4.932*	5.350*	4.779	4.490	4.613	5.045	6.117*	
Terminal sacrifice											
Liver: absolute (g)	1.77	1.77	1.81	1.89	1.98	1.64	1.55	1.71	1.64	1.84	
Liver: relative (%)	5.054	5.004	5.187	5.276	5.695	5.404	5.144	5.450	5.482	6.255*	
Histopathology – non-neoplastic lesions											
Interim sacrifice											
Liver:											
centrilobular hypertrophy	0/10	1/10	1/10	8/10	10/10	1/10	2/10	2/10	8/10	9/10	M
increased pigment	0/10	0/10	0/10	0/10	5/10	0/10	0/10	0/10	0/10	0/10	
Terminal sacrifice + unscheduled											
Systemic amyloidosis	14/50	15/50	14/50	16/50	21/50	11/50	3/50	5/50	11/50	18/50	
Liver:											
Centrilobular hypertrophy	3/49	2/50	3/50	32/50	46/47	3/49	1/50	2/50	10/49	28/50	M/F
Hyperplasia bile duct	0/49	0/50	2/50	7/50	12/47	1/49	0/50	0/50	0/49	2/50	M
Increased pigment	0/49	0/50	2/50	12/50	25/50	3/49	2/50	4/50	6/49	16/50	M/F
Lung:											
Multiple foci of amphophilic alveolar macrophages	0/50	0/50	1/50	10/50	14/50	0/50	0/50	0/50	5/50	22/50	M/F
Pneumonitis	6/50	7/50	4/50	8/50	11/50	11/50	9/50	12/50	16/50	13/50	
Inflammation, granulomatous	0/50	1/50	1/50	1/50	1/50	0/50	0/50	0/50	1/50	0/50	
Broncopneumonia	0/50	0/50	1/50	1/50	0/50	0/50	0/50	0/50	0/50	0/50	
Bronchioles, chronic inflammation	0/50	0/50	1/50	0/50	0/50	0/50	0/50	0/50	0/50	0/50	
Alveolar/ bronchiolar hyperplasia	2/50	6/50	2/50	3/50	2/50	0/50	3/50	3/50	3/50	3/50	
Perivascular/ peribronchial lymphoid infiltrates	17/50	25/50	19/50	24/50	12/50	35/50	33/50	33/50	25/50	30/50	

Histopathology – neoplasms										
Lung:										
Alveolar/ bronchiolar adenoma	5/60	10/60	12/60	11/60	10/60	9/60	10/60	11/60	10/60	8/60
Alveolar/ bronchiolar adenoma multiple	0/60	0/60	0/60	0/60	2/60	0/60	2/60	0/60	1/60	2/60
Alveolar/ bronchiolar carcinoma	0/60	0/60	2/60	2/60	0/60	1/60	3/60	1/60	0/60	0/60
Combined	5/60	10/60	14/60*	12/60	12/60	10/60	15/60	12/60	11/60	10/60
Liver:										
Hepatocellular adenoma	2/59	2/60	6/60	3/60	5/57	0/59	0/60	0/60	0/59	4/60
Hepatocellular adenoma, multiple	0/59	2/60	1/60	2/60	0/57	0/59	0/60	1/60	0/59	0/60
Hepatocellular carcinoma	2/59	0/60	3/60	2/60	0/57	1/59	1/60	0/60	0/59	0/60

dr Statistically significant dose response

* $p < 0.05$ ** $p < 0.01$ (Anova and Dunnett's test, two-sided)

The NOAEL for systemic toxicity was 200 ppm (equivalent 24 mg/kg bw per day) based on hepatic changes, notably centrilobular hypertrophy, increased pigmentation and bile duct hyperplasia, and an increased incidence of multiple foci of amphophilic alveolar macrophages in the lungs of mice given 1000 ppm (equivalent to 119 mg/kg bw per day). No evidence for carcinogenic potential of clethodim in mice was observed (Cox, 1988b).

Rat

Clethodim (purity 83.3%) was administered for 104 weeks to five groups of 65 male and 65 female Sprague Dawley Crl:CDBR rats (six weeks old) at dietary concentrations of 0, 5, 20, 500 and 2500 ppm (equal to 0, 0.15, 0.57, 16 and 86 mg/kg bw per day for males, 0, 0.20, 0.72, 21 and 113 mg/kg bw per day for females). Ten animals/sex per dose group were randomly selected for sacrifice after 52 weeks of treatment. Dose levels, homogeneity, and stability in the diet were confirmed by analysis. Animals were checked daily for mortality and clinical signs. A detailed examination for clinical signs and palpable masses was made once a week. Body weights were recorded weekly for the first 13 weeks and every four weeks after week 16. Food consumption was measured continuously for the first 13 weeks of the study, at the beginning of week 16, and every four weeks thereafter. Ophthalmoscopic examinations were performed on all animals before treatment, after one year, and just before the two-year sacrifice. Haematology was performed, on 20 animals before the study commenced, at three and six months, and then at one year and two years. Serum chemistry and urinalysis was performed at the outset of the study on 20 animals and then on 10 animals at six months, one and two years. An additional group of 10 animals was used for urinalysis at three months. All animals either found dead or sacrificed, were subject to detailed necropsy and collection of tissues. The following organs were weighed: brain, adrenals, liver, ovaries, testes and kidneys. An extensive list of tissues was examined histologically from the control and 2500 ppm groups, in animals that died or from unscheduled sacrifices, including gross lesions and tissue masses. In addition, the lung, liver and kidneys in all animals from all groups were examined histologically.

There were 15 “accidental deaths” during the study, due to accidental injury or ether overdose. Excluding these accidental deaths the mortality for 0, 5, 20, 500 and 2500 ppm was 52.8%, 50.9%, 53.8%, 50.0% and 67.3% for males, and 45.3%, 42.3, 49.1, 60.0 and 50.9% for females, respectively.

The high mortality rate noted in males at 2500 ppm (mean day of death, 559) and in females at 500 ppm (mean day of death, 581) were not statistically significantly different from controls, but suspected to be treatment-related in males at 2500 ppm.

Clinical signs most frequently reported were decreased motor activity, malocclusion, weakness, anogenital discharge, reduced stools, exophthalmia, ocular red discharge, nasal discharge, broken teeth, alopecia, malocclusion, stained fur and scabs. The incidence of these clinical signs, which are not uncommon in chronic rat studies, did not distinguish treated animals from controls. No treatment-related changes in the incidence of ocular findings were observed.

Males and females at 2500 ppm weighed significantly less than controls for most of the study. No significant body weight differences were observed in the first year of the study for the other dosed groups. During the second year of treatment, males and females at 500 ppm showed significant reduction in body weight during weeks 68–84 and week 92–96, respectively. At 5 ppm animals also had significant body weight decrease. No body weight reduction was observed in animals at 200 ppm during the second year of treatment. During the first year of treatment, a significant reduction in food consumption with a corresponding increase in relative food consumption, were observed in males at 2500 ppm, and similarly in females, although less consistently so. No treatment-related food consumption changes were observed in the other dosed groups. Food efficiency, for the first three months was significantly decreased in males at 2500 ppm. During the second year of treatment, fluctuations in absolute and relative food intake were observed in all treatment groups but no consistent pattern was evident, except for increased relative food consumption for females at 2500 ppm.

There were no treatment-related deviations in clinical chemistry, haematology or urinalysis parameters.

At interim sacrifice after one year, increased liver weights and slight to mild centrilobular hypertrophy were noted in animals of each sex given 2500 ppm. Increased liver weights seen in females given 500 ppm were not correlated with any microscopically discernible change. At terminal sacrifice, liver weights of females given 2500 ppm were increased; males had no significant increase in liver weight. In animals killed at termination or suffering unscheduled deaths, a slight increase of liver hypertrophy was noted in males and females at 2500 ppm. Females offered 2500 ppm had a slightly greater (12%) incidence of binucleated cells in the liver than the controls (2%), but the effect was of uncertain toxicological significance. A statistically significant increase in chronic pancreatitis (trace to mild) was observed in females at 2500 ppm when compared to controls. However, it is noted that the incidence of this change was lower than that in control males.

A statistically significant increase in benign granulosa cell tumours was observed in 2/63 females (one unilateral and one bilateral) at 2500 ppm compared with 0/64 in the controls. No historical control data (HCD) were provided in the report and since this finding was not ascribable to treatment by the study's pathologist, no statistical analysis was performed. Nevertheless it is pointed out that no treatment-related preneoplastic lesions were observed in this study nor in any repeated-dose studies in rodents.

Table 12. Key results of the combined chronic toxicity/carcinogenicity study in rats.

	Males					Females				
Dose (ppm)	**0**	**5**	**20**	**500**	**2500**	**0**	**5**	**20**	**500**	**2500**
Mortality[§]	28/53	28/55	28/52	27/54	37/55	24/53	22/52	26/53	33/55	27/53
Accidental deaths	2	0	3	1	0	2	3	2	0	2
Animals alive at terminal sacrifice (survival %)	25 (47)	27 (49)	24 (46)	27 (50)	18 (33)	29 (55)	30 (58)	27 (51)	22 (40)	26 (49)

	Males					Females				
Dose (ppm)	**0**	**5**	**20**	**500**	**2500**	**0**	**5**	**20**	**500**	**2500**
Body weight (g)										
Day 0	200	200	200	200	200	151	152	150	150	149
Day 360	678	651	659	645	633**	401	379	404	393	368*
Day 724	698	641	634	663	642	496	458	488	444	433
Body weight gain (g)										
Day 0–91	308	305	302	308	273**	129	123	130	129	113**
Food consumption (g/animal per week)										
Year 0–2	-	-	-	-	dc	-	-	-	-	dc
Organ weights										
Interim sacrifice										
Liver: absolute (g)	16.58	18.01	16.71	17.52	19.08	8.76	9.22	9.18	10.57	10.85*
Liver: relative (g/100 g bw)	2.64	2.86	2.60	2.85	3.21**	2.58	2.65	2.67	3.04	3.05
Liver/brain: (g/g)	7.38	8.09	7.56	7.75	8.54	4.22	4.66	4.42	5.23*	5.21*
Terminal sacrifice										
Liver: absolute (g)	19.06	16.59	17.75	17.63	18.70	11.86	11.10	11.60	11.68	12.51
Liver: relative (%)	2.93	2.82	2.89	2.78	3.06	2.54	2.62	2.58	2.84	3.07*
Pathology – non-neoplastic lesions										
Interim sacrifice										
Liver centrilobular hypertrophy	0/10	0/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10	3/10
Terminal sacrifice + unscheduled deaths										
Liver centrilobular hypertrophy	0/54	0/53	0/53	0/53	1/50	1/51	0/49	0/52	0/53	2/52
Chronic pancreatitis	22/54	2/29	8/30	7/24	13/50	4/51	3/22	3/25	3/31	15/52**
Pathology – neoplastic findings										
Granulosa cell tumour, benign	-	-	-	-	-	0/64	0/29	0/33	0/39	2/63*

dc Statistically significant decrease

* $p < 0.05$ ** $p < 0.01$ (Fischer's exact and Cochrane–Armitage tests for histopathology findings)

§ Number of animals that died prior to termination/number of animals in group
(The former number excludes accidental deaths and animals for interim sacrifice)

The NOAEL was 500 ppm (equal to 16 mg/kg bw per day), based on decreased body weight gain, decreased food intake in both sexes, marginally increased mortality in males and increased chronic pancreatitis in females at 2500 ppm (equal to 86 mg/kg bw per day).

The NOAEL for carcinogenicity was 500 ppm (equal to 21 mg/kg bw per day), based on increased incidence of benign granulosa cell tumours in the ovary (2/63) at 2500 ppm (equal to 113 mg/kg bw per day) (Dougherty, 1988a).

2.4 Genotoxicity

Clethodim was found to be negative in the in vitro mutagenicity tests (reverse and forward mutation). In both in vitro chromosome aberration tests performed in Chinese Hamster ovary cells, clethodim technical (without metabolic activation) was found positive at the two highest doses tested (0.91 and 1.1 mg/mL). However the in vivo chromosome aberration test in rats was negative using clethodim technical. Also negative was the unscheduled DNA synthesis (UDS) test in hepatocytes of male mice. Considering the results in all the genotoxicity studies, clethodim technical raises no concern as to its genotoxicity.

Table 13. Results of genotoxicity studies performed with clethodim

Type of study	Organism/cells	Dose range tested	Purity (%)	Result	Reference
In vitro					
Gene mutation (Ames test)	*Salmonella typhimurium* (TA98, TA100, TA1535, TA1537)	−S9: 0.1–10 mg/plate +S9: 0.1–10 mg/plate	83	Negative (−/+S9)	Machado, 1986a
	Salmonella typhimurium (TA98, TA100, TA1535, TA1537) and *Escherichia coli* (WP2*uvr*A)	−S9: 0.1–10 mg/plate +S9: 0.1–10 mg/plate	83.3	Negative (−/+S9)	Machado, 1986b
HGPRT forward mutation assays	Chinese Hamster Ovarian (CHO) Cells	−S9: 100–500 µg/mL +S9: 100–500 µg/mL	92.7	Negative (−/+S9)	Lehn, 1990
Chromosome aberration assay	Chinese Hamster ovarian (CHO) cells	−S9: 30–910 µg/mL +S9: 30–910 µg/mL	83.3	Positive (−S9) Negative (+S9)	Putman, 1986a
	Chinese Hamster ovarian (CHO) cells	−S9: 30–910 µg/mL +S9: 30–910 µg/mL	96.1	Negative (−/+S9)	Putman, 1986b
In vivo					
Chromosome aberration assay	Bone marrow cells of Sprague Dawley rat (gavage)	150–1500 mg/kg	83.3	Negative	Putman, 1987
Unscheduled DNA synthesis test (UDS)	Hepatocytes of male B6C3F1 mice	150–1500 mg/kg	83.3	Negative	Steinmetz & Mirsalis, 1986

(a) In vitro studies

Clethodim (purity 83%) was tested in two separate experiments for its mutagenic potential on *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and once on *E. coli* strain WP2uvrA using the plate incorporation method at concentrations up to 10 mg/plate (0.1–10 mg/plate) in the absence or presence of S9. Clethodim was soluble with dimethylsulfoxide (DMSO) but was not completely soluble with the top agar at ≥ 3.3 mg/plate. Cytotoxicity in TA100 and TA1537 strains was seen at 10 mg/plate with and without S9 mix. No cytotoxicity was observed for WP2uvrA, TA98 and TA1535 with or without S9 mix. There was no significant dose-related increase in the number of revertant colonies with or without S9 mix. A slight increase in number of revertants/plate (a two-fold increase) was observed in strain TA98 at 10 mg/mL. This increase was not statistically significant and was not reproducible, therefore was not considered biologically relevant. Positive controls showed a marked increase in the number of revertant colonies. Clethodim was not mutagenic under the test conditions (Machado, 1986a, b).

Clethodim (purity 92.7%) was tested in a mammalian gene mutation (HGPRT locus) assay in Chinese hamster ovary cells (CHO). Clethodim was tested from 100 µg/mL to 500 µg/mL. Based on the solubility limit in DMSO, the highest concentration was set at 500 µg/mL in the medium. Precipitates were seen in the medium at 500 µg/mL. Two independent experiments were performed with metabolic

activation and three without such activation. Cytotoxicity was observed only at the highest dose level of 500 μg/mL in the absence of metabolic activation.

In the absence of metabolic activation, a statistically significant increase in mutant frequency was observed at 100 μg/mL (duplicate), 200 μg/mL (duplicate) and 500 μg/mL (single). This was due to the low mutant frequencies of the vehicle controls, so even one negative control provided a statistically significant increase. The increases were not dose-related and did not exceed the range, which is typical of vehicle control variations. So only one increase of one duplicate culture in the highest dose group was evaluated as biologically significant. Two additional experiments were performed, in which no biologically relevant increases were observed.

In the presence of metabolic activation, two cultures showed statistically significant increases in mutant frequency over the concurrent negative controls at doses of 100 μg/mL (single) and 450 μg/mL (single) in one experiment; one increase occurred in the other experiment at a test article concentration of 500 μg/mL. These increases were not dose-related, and were not confirmed by the duplicate treatment of the other trial. In addition, they were in the normal range of vehicle control values. The positive control article (7,12-dimethylbenz(a)anthracene, DMBA) was clearly positive in both trials. Clethodim did not induce chromosomal aberrations under the test conditions (Lehn, 1990).

Clethodim (purity 83.3%) was tested for its clastogenic potential in CHO cells. Based on the solubility limit in DMSO, the highest concentration was set at 1100 μg/mL in the medium. Precipitates were seen in the medium at and above 550 μg/mL. No cytotoxicity was demonstrated in the toxicity test up to and including 910 μg/mL. There were two independent experiments. A significant increase in the frequency of structural chromosome aberrations per cell was observed at 910 μg/mL in the absence of metabolic activation. No increase was seen in either structural aberrations per cell in the activated test system, or in numerical aberrations for either test system. In the confirmatory assay a significant increase in the frequency of structural chromosome aberrations per cell was observed at 910 and 1100 μg/mL in the non-activated system. Significant increases were seen in neither the structural aberrations per cell in the activated system, nor in the number of numerical aberrations for the S9-activated and unactivated test systems. Technical clethodim was found to induce chromosomal aberrations in CHO cells in the absence of metabolic activation, whereas no chromosomal aberrations were induced in the presence of metabolic activation (Putman, 1986a).

A second chromosome aberration test was performed to assess the clastogenic potential of purified clethodim (purity 96.1%) in CHO cells. Based on the solubility limit in DMSO, the highest concentration was set at 1100 μg/mL in the medium. Precipitates were seen in the medium at and above 270 μg/mL. No cytotoxicity was observed in the toxicity test up to and including 910 μg/mL. There were two independent experiments. A significant increase in the frequency of structural chromosome aberrations per cell was only observed in the first experiment at 0.1 μg/mL in the presence of metabolic activation when compared to the solvent control, but not when compared to the untreated control. The statistical significance was due to an unusually low background rate and was not considered biologically significant. Clethodim did not induce chromosomal aberrations in CHO cells under the test conditions (Putman, 1986b).

(b) In vivo studies

Clethodim (purity 83.3%) was tested for its clastogenic potential in a chromosome aberration assay in rat (Sprague Dawley) bone marrow. Single oral (gavage) doses of 150, 500 and 1500 mg/kg bw were given to five rats/sex per group. The test material was suspended in a carboxymethyl cellulose (CMC)/Tween-80 mixture, and administered at a volume of 10 mL/kg bw. Animals were sacrificed at 12, 24 and 48 h after dosing. In a preliminary acute toxicity study the test item was lethal to some animals at levels of 1200 mg/kg bw and above. In the high-dose group three females and five males died prior to their scheduled sacrifice. A reduction in body weight gain compared to the control was observed in this group. Clinical signs observed in the highest dose group included prostration, lethargy, hunching, tremors, lacrimation, excessive salivation, crusty eyes and crusty nose. In animals that received 500 mg/kg bw, lethargy and excessive salivation were observed, and in animals that received 150 mg/kg bw only lethargy was observed. The negative and positive controls fulfilled the requirements for a valid test. Clethodim did not induce chromosomal aberrations in male or female rat bone marrow cells at the doses tested (highest dose, 1500 mg/kg bw) (Putman, 1987).

Clethodim (purity 83.3%) was tested for its potential to induce DNA repair in an UDS assay in male mouse (B6C3F1) hepatocytes. A single oral (gavage) dose of 100, 1000 and 5000 mg/kg bw was administered to three males per dose group. Each dose group was composed of two subgroups of three mice each; one subgroup sacrificed 2 h and the other 16 h after treatment. In animals that received 5000 mg/kg bw, three of the eight treated mice died before their scheduled time of sacrifice. The UDS data from the remaining two mice were pooled. No abnormal clinical signs were observed in the remaining mice. Exposure of the animals to the vehicle control and the test substance resulted in a mean net grains/nucleus count ranging from –4.1 to –7.4. In the positive control group, the net grains/nucleus count was 11.2. Mice receiving clethodim or the vehicle presented less than 2% of their cells in repair, compared to 56% in the mice of the positive control group. In all treatment groups, and at the two different time points examined, clethodim technical failed to induce any increase in UDS. Clethodim technical did not induce UDS in male B6C3F1 mice under the experimental conditions (Steinmetz & Mirsalis, 1986).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a preliminary dose range-finding study, groups of eight male and eight female Crl:COBS/CD (SD) rats were offered clethodim (purity 83.3%) at dietary concentrations of 0, 500, 2000 and 5000 ppm. Rats received the test material for one week before mating. Adult males were sacrificed after mating was complete. Offspring were exposed to the test material in utero and while nursing until they were sacrificed and necropsied on day 7 of lactation. Adult females were sacrificed at day 7 of lactation.

No mortality and no clinical signs were observed. A significant reduction in body weight was observed in parents receiving 5000 ppm. Food consumption was consistently lower for treated males and females in all dose groups. However, the difference was statistically significant in high-dose males only. Mating performance and fertility appeared unaffected at all dose levels. There were no effects observed in pup litter size, survival or sex ratio. There was a significant decrease in combined pup weight (male and female) at day 7, and a decrease in combined pup weight gain between days 0 and 7 for all three dose levels. At necropsy, no abnormal changes attributable to the test substance were noted in either sex. On the basis of results in this study, a high dose of 2500 ppm was considered appropriate for the main study (MacKenzie, 1986).

The main two-generation rat reproduction study was conducted with clethodim (purity 83.3%) at dietary dose levels of 0, 5, 20, 500 and 2500 ppm (equal to 0, 0.5, 1.2, 32.2 and 163.3 mg/kg bw per day for males, 0, 0.5, 1.5, 37.4 and 181.3 mg/kg bw per day for females, when corrected for purity). Groups of 30 male and 30 female Crl:COBS/CD (SD) rats were allocated to treatment groups (F0 generation) and exposed to treated diets ten weeks prior to mating, and then during gestation, and lactation, until scheduled sacrifice. At the end of the lactation period (24 days after mating), 30 male and female pups were selected to become the F1a parental animals. Following 84 days of treatment, each F1a male was paired with one F1a female from the same treatment group. Mating of siblings was avoided and females were allowed to rear their litters to form the F2a generation. The F2 animals were monitored until weaning and the study then terminated. Exposure to the treated diet continued throughout the study including the premating, mating, gestation and lactation periods of F0 and F1 generations. The F2 pups were indirectly exposed to test substance until weaning (days 21–23 postpartum).

Mortality and clinical signs were checked at least once daily on all animals. Body weights were measured weekly for all groups. Food consumption was measured at each diet change: daily for animals offered 5 and 20 ppm and three times a week for all other groups. For females, body weight and food consumption were recorded weekly during the premating periods, on days 0, 7, 14 and 21 postcoitum and on days 0, 4, 7, 14 and 21 postpartum. A record of mating of females was made throughout the pairing period by daily examination of vaginal smears for spermatozoa and/or appearance of a vaginal plug. The day on which evidence of mating was observed was designated day 0 postcoitum. Females without litters 24 days after the end of the cohabitation period were killed and necropsied. Day 0 of lactation was the day on which a female had delivered all its pups. All F0 and F1 animals were sacrificed at the end of the lactation period. From the F1 litters produced, 30 pups/sex per treatment group were randomly selected to generate the F2 litters. Once the F1 pups were selected to produce the F2 litters,

all unselected pups were sacrificed on days 21–24 of lactation and were subjected to necropsy. Organ weights of testis, epididymis, prostate, seminal vesicles, uterus and ovaries were recorded from all F0 and F1 parent animals. Full histopathological examination was carried on an extensive list of tissues, including gross lesions. Further, the following organs/tissues of all males and females in the control and 2500 ppm groups where subject to a full histopathological examination: cervix and vagina, epididymides, ovary, prostate, seminal vesicles, testes and uterus.

In parental animals, no mortality and no clinical signs were noted during the study. A decrease in body weights and food consumption was recorded among males and females of the F0 and F1 generations receiving the highest dose level (2500 ppm). Effects on body weights were particularly noted in males of both generations, while females' body weights were also reduced, but only in the F1 generation. In the high-dose group decreased food consumption values were noted among males and females of the F0 and F1 generations. For F1 generation males this was seen during many intervals while for females it was only seen for a few days during gestation. There were no changes detected between parental animals of the treated and control groups mating indices, pregnancy rates, male fertility, estrus cycle, macroscopic findings, microscopic findings or organ weights.

No treatment-related changes were detected in litter size, pup weights, sex ratio or litter survival of F0 and F1 offspring. A statistically significant increase in stillborn pups was noted in F1 litters (14 stillborn pups in seven litters) at 2500 ppm. However, this difference was most probably due to a low control value (two stillborn pups in two litters) because in the control group of the F2 generation the number of stillborn pups was seven in five litters, and in a previous two-generation reproduction study the number of stillborn pups was nine in six litters. Additionally, it is noted that the number of pups delivered at 2500 ppm was higher than those in the concurrent control.

Necropsy observations did not reveal any treatment-related adverse effects in F0 or F1a adults or in F1a or F2a pups. Nor did histopathological examination of tissues preserved at necropsy reveal any treatment-related findings. No treatment-related changes in reproductive organ weights were observed in either generation.

Clethodim showed no evidence of an effect on fertility or reproductive function.

Table 14. Summary of key results of the two-generation reproduction study in rats.

	Males					Females				
Dose level (ppm)	**0**	**5**	**20**	**500**	**2500**	**0**	**5**	**20**	**500**	**2500**
					Parental animals (F0)					
Clethodim intake (mg/kg bw per day)	0	0.5	1.2	32.2	163.3	0	0.5	1.5	37.4	181.3
Body weight (g)										
Pre-mating (Week 9, ending day 63)	495	490	495	474	460**[dc]	265	270	265	270	262
Gestation day 21	556	543	557	541	516**	396	387	389	393	385
Lactation day 21	577	358	573	548	523*	319	311	312	317	312
Food consumption (g/animal per day)										
Over treatment period					dc[#]					dc
Pre-mating (Week 1, days 5–7)	51	50	50	50	49	36	36	34	36	34*
Pre-mating (Week 6, days 44–47)	82	81	83	81	77*	-	-	-	-	-
Pre-mating (Week 8, days 56–58)	55	54	54	53	50*	-	-	-	-	-
Lactating (Week 2, day 12–14)	-	-	-	-	-	131	113	121	131	119*

	Males					Females				
Dose level (ppm)	**0**	**5**	**20**	**500**	**2500**	**0**	**5**	**20**	**500**	**2500**
					F1 pup data					
					Male and female pups					
Females mated (*N*)	22		25		23		24		28	
(%)	78.6		83.3		76.7		82.8		96.6	
Pups delivered (*N*)	298		308		286		285		367	
Stillborn (*N*)	2		5		5		7		14	
(%)	0.7		1.6		1.7		2.5		3.8**§	
					F1 animals					
Clethodim intake										
(mg/kg per day)	0	0.5	1.8	47.8	251	0	0.5	1.9	51.6	269
Body weight (g)										
Pre-mating day 196	492	491	467	489	438**	279	269	269	279	256**
Gestation day 21 (day 224)	539	536	534	526	485**	402	379	397	398	378*
Lactation day 21 (day 245)	572	566	563	566	511*	316	312	321	320	314
Body weight gain (g)										
Over treatment period					dc					dc
Lactation (days 14–21)	-	-	-	-	-	−14	−15	−16	−5	3*
Lactation (days 0–21)	-	-	-	-	-	6	14	16	13	20*
Food consumption (g/animal/day)										
Over treatment period					dc					dc
Premating (days 154–156)	61	61	58	60	57*	44	42	30*	47	43
Premating (days 194–196)	58	57	56	58	54*	43	39	38	41	41
					F2 pup data					
	Males					**Females**				
Diet concentration (ppm)	0	5	20	500	2500	0	5	20	500	2500
					Male and female pups					
Pups delivered (*N*)	259		250		357		335		304	
Stillborn (*N* [%])	7 [2.7]		6 [2.4]		7 [2.0]		11 [3.3]		10 [3.2]	

dc Statistically significantly decreased/increased compared to the controls

§ Within historical control values; no similar effects noted in F2 generation

Occasionally, not during the same period when reduced body weights occurred

* $p < 0.05$ ** $p < 0.01$ (Dunnett's test)

Analysis of sperm parameters, developmental and functional observations of pups, weighing of the adrenals, brain, kidney, liver, pituitary gland, spleen and thyroids, and histopathology of the vagina were not performed. However, results from reproductive indices did not suggest any adverse effects.

The NOAEL for parental toxicity was 500 ppm (equal to 32.2 mg/kg bw per day), based on body weight changes and reduced food consumption in the F0 and F1 generations at 2500 ppm (equal to 163.3 mg/kg bw per day).

The NOAEL for offspring toxicity was 2500 ppm (equal to 163.3 mg/kg bw per day).

The NOAEL for reproductive toxicity was 2500 ppm (equal to 163.3 mg/kg bw per day) (Tellone, 1987).

(b) Developmental toxicity

Rat

In a preliminary developmental study in rats, clethodim (purity 83.3%) was administered by gavage at dose levels of 0, 50, 150, 300 and 500 mg/kg bw per day to ten animals per group during days 6–15 of gestation. The material was suspended in a CMC/Tween/distilled water mixture (the vehicle). All females were observed twice daily for mortality and toxicity signs. Body weights were recorded on days 0, 3, 6–15 and 20. Food consumption was also recorded during the gestation period. Additionally during gestation, females were periodically given a detailed physical evaluation. On day 20 of gestation (GD20), all dams were sacrificed, given a gross postmortem examination and corpora lutea/uterine implantation data recorded. Recovered fetuses were weighed, sexed externally and given a gross examination for malformations/variations of the external form.

Several females at 300 and 500 mg/kg bw per day showed excessive salivation. In dams at 500 mg/kg bw per day, body weight at GD 20 was reduced by about 10% when compared to controls. Body weight gain was also reduced in dams at 500 mg/kg bw per day during GD 6–15 and GD 15–20 by 27% and 39%, respectively. No effects on food consumption were noted.

No treatment-related changes were observed in the mean number of corpora lutea. Decreases in the mean number of uterine implantations and an increase in the mean pre-implantation loss ratio were noted at 500 mg/kg bw per day. Mean fetal weights (both sexes combined) were reduced at 300 and 500 mg/kg bw per day. No adverse effect of treatment was evident from fetal external observations (Schroeder, 1986).

In the main developmental study, clethodim (purity 83.3%) was administered by gavage to groups of 25 presumed pregnant Crl:COBS/CD (SD) rats at dose levels of 0, 10, 100, 350 and 700 mg/kg bw per day (equal to 0, 8.3, 83.3, 291.6 and 583.1 mg/kg bw per day after correction for purity) during GD 6–15. The material was suspended in a CMC/Tween/distilled water mixture (vehicle). All females were observed twice daily for mortality and toxicity signs. Food consumption was recorded for the following periods: days 0–3, 3–6, 6–15 and days 15–20. Body weights were recorded on days 0, 3, 6–15 and 20. Additionally during gestation, females were periodically given a detailed physical evaluation. On day 20, all dams were sacrificed. Postmortem examination, including gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed and the data recorded. All organs or tissues showing grossly visible abnormalities were preserved. The uteri (and contents) and placentae of all females with live fetuses were weighed at necropsy. Fetuses were removed from the uterus, sexed, weighed individually, and examined for gross external abnormalities. One half of the fetuses in each litter was evaluated for visceral malformation and the remaining half eviscerated and processed for staining of the skeletal structures and subsequent evaluation.

At 700 mg/kg bw per day maternal toxicity was manifest as indicated by a 20% increased mortality (GD11–16). A statistically significant decreased body weight (up to 10%) was noted at 700 mg/kg bw per day during GDs 10, 11, 14, 15 and 20, and at 350 mg/kg bw per day on GD 20. Body weight gain was reduced during the treatment period (GD 6–15) in animals at 350 and 700 mg/kg bw per day, by 15% and 40%, respectively; the reduction at the high dose achieved statistical significance. Body weight gain was found to be significantly reduced (by 17%) after the treatment period (GD 15–20) at 350 and 700 mg/kg bw per day. Gravid uterus weight was noted as decreased by 10% and 26% at 350 and 700 mg/kg bw per day respectively; statistical significance was achieved at the high dose. When body weight on day 20 is corrected for uterus weight a statistically significant decrease is noted only at 350 mg/kg bw per day; this change was ascribable to a single dam that was in poor condition at necropsy. When body weight gain for GD 6–20 is calculated using body weight on day 20, excluding

uterus weight, a decrease is observed at 350 and 700 mg/kg bw per day of ca 19% . Food consumption was significantly reduced in animals given 700 mg/kg bw per day from GD 7 to 11.

A higher incidence of clinical signs (excessive salivation, excessive lacrimation, poor condition, red/mucoid nasal discharge, alopecia, chromodiacryorrhea and staining of the anogenital area) was observed in animals given 700 mg/kg bw per day. With the exception of excessive lacrimation and poor condition, similar effects, but less severe, were noted at 350 mg/kg bw per day. None of the treated groups revealed any adverse effects in pathology. The slightly increased incidence of excessive lacrimation at 100 mg/kg bw/day was without dose relation.

Analysis of uteri showed an increase, not statistically significant, in resorption by females at 700 mg/kg bw per day. This finding was in part due to a single female that showed 15 resorption out of 16 implants. Excluding this female, the mean litter incidence of resorptions was 1.1, which was slightly higher than control value of 0.8. Dose levels of 350 and 700 mg/kg bw/day were considered to be fetotoxic, based on decreased fetal weights and an associated retardation of ossification.

Fetal weights were significantly decreased at 350 and 700 mg/kg bw per day, both when combined or as separate sexes.

Fetal external examination evidenced a statistically significant increase in malformations, both on a fetal and litter basis, at 700 mg/kg bw per day. Seven fetuses in seven litters had tail malformations (absence of tail, short tail or filamentous tail). Some of these fetuses also had an imperforated anus. Among fetuses not having tail malformations exencephaly was noted in one fetus.

Visceral malformations (exencephaly, defects of the lung, aortic arch and large intestine, absence of kidneys, bladder or ureter) were observed in four fetuses in three litters of dams exposed to 700 mg/kg bw per day, but not in concurrent or in historical controls (5032 fetuses from 806 litters in 38 studies). Three of the four fetuses with visceral malformations also had external malformations.

A number of skeletal malformations were reported only at 700 mg/kg bw per day. They were mainly localized in the lumbar, sacral and caudal region. Among these fetuses, two had more than one malformation, and two had a tail defect observed at external examination.

Skeletal variations, consisting of incomplete ossification was particularly seen in sacral and caudal vertebral elements and in the 5th and 6th sternebrae among fetuses in the 350 and 700 mg/kg bw /day dose groups.

It is noted that the external, visceral, skeletal malformations at 700 mg/kg bw per day, and increased skeletal variations at 300 and 700 mg/kg bw per day were observed in the presence of severe maternal toxicity.

Table 15. Key findings of the teratogenicity study in rats.

Dose (mg/kg bw per day)	**0**	**10**	**100**	**350**	**700**	**HC (mean; range)/dr**
Number of females mated	25	25	25	25	25	
Mortality	0/25	0/25	0/25	0/25	5/25	
Clinical signs						
Excessive salivation				+	++	
Excessive lacrimation			+		+	
Poor condition					+	
Red/mucoid nasal discharged				+	++	
Alopecia				+	++	
Staining anogenital area				+	++	
Chromodiacryorrhea					+	

Dose (mg/kg bw per day)	0	10	100	350	700	HC (mean; range)/dr
Pregnant animals	25	25	24	25	24	
Body weight (g)						
GD 10	263	263	260	251	248*	
GD 11	268	270	264	258	250**	
GD 14	284	284	280	273	266*	
GD 15	293	292	288	279	271**	
GD 20	362	362	357	337*	332**	
GD 20 (corrected for uterus weight)	280	282	281	264*	272	
Body weight gain (g)						
Days 6–15	47	47	45	40	28**	dr
Days 15–20	70	69	69	58*	58**	
Days 6–20 (corrected for uterus weight)	34.7	36.9	37.2	24.5	27.8	
Gravid uterus weight	81.7	79.8	76.4	73.6	60.4**	dr
Food consumption						
Gestation day 7–8	100	100	96	86**	76**	dr
Gestation day 8–9	100	99	95	100	75**	
Gestation day 9–10	102	103	101	97	70**	
Gestation day 10–11	102	105	99	99	71**	
Litter response						
Live fetuses	353	351	329	350	221	
Litter size	14.1	14.0	13.7	14.0	12.3	
Fetal weight (g)	3.65	3.61	3.48	3.26**	2.75**	dr
Resorptions						
Total	20	16	13	14	34	
Mean per litter	0.8	0.6	0.5	0.6	1.9	0.7; 0.2–1.8
Examination of the fetuses; fetal incidence (litter incidence)						
External examination						
Exencephaly	0(0)	0(0)	0(0)	0(0)	1(1)	
Tail, absent	0(0)	0(0)	0(0)	0(0)	3(3)	
Tail, filamentous	0(0)	0(0)	0(0)	0(0)	2(2)	
Tail, short	0(0)	0(0)	0(0)	0(0)	2(2)	
Imperforated anus	0(0)	0(0)	0(0)	0(0)	2(2)	
Total external malformations	0(0)	0(0)	0(0)	0(0)	8*(6)*	

Dose (mg/kg bw per day)	0	10	100	350	700	HC (mean; range)/dr
Visceral examination						
Litters evaluated	25	25	24	25	18	
Fetuses evaluated	185	181	172	161	118	
Aortic arch defect	0(0)	0(0)	0(0)	0(0)	2(2)	
Pulmonary arch defect	0(0)	0(0)	0(0)	0(0)	1(1)	
Absence of kidney	0(0)	0(0)	0(0)	0(0)	1(1)	
Defect in large intestine	0(0)	0(0)	0(0)	0(0)	1(1)	
Absence of bladder	0(0)	0(0)	0(0)	0(0)	1(1)	
Absence of ureter	0(0)	0(0)	0(0)	0(0)	1(1)	
Total visceral malformations	0(0)	1(1)	1(1)	0(0)	4(3)	
Skeletal findings						
Malformations						
Litters evaluated	25	25	24	25	18	
Fetuses evaluated	168	170	158	169	110	
Misshapen ossification posterior to lumbar vertebrae	0(0)	0(0)	0(0)	0(0)	2(2)	
Lumbar centra fused	0(0)	0(0)	0(0)	0(0)	1(1)	
Lumbar transverse process(es) – fused	0(0)	0(0)	0(0)	0(0)	1(1)	
Lumbar transverse process(es) – additional	0(0)	0(0)	0(0)	0(0)		
Lumbar vertebra absent	0(0)	0(0)	0(0)	0(0)	0(0)	
Sacral vertebra absent	0(0)	0(0)	0(0)	0(0)	0(0)	
Caudal vertebra absent	0(0)	0(0)	0(0)	0(0)	1(1)	
Ribs fused	0(0)	0(0)	0(0)	0(0)	0(0)	
Additional pelvic ossification	0(0)	0(0)	0(0)	0(0)	0(0)	
Variations						
Ossification variation – incidence of fetuses[§] (%)	122 (72.6%)	103 (60.6%)	126 (79.7%)	150** (88.8%)	106** (96.4%)	
Ossification variation – incidence of litter (%)	24 (96.4%)	25 (100%)	24 (100%)	25 (100%)	18 (100%)	

HC Historical control generated between 1976 and 1985 including 809 control pregnant females

dr Dose-related

§ Incidence of fetuses with at least one ossification variation

* $p < 0.05$ ** $p < 0.01$ (Dunnett's or Dunn's rank sum)

The NOAEL for maternal toxicity was 83.3 mg/kg bw per day based on reduced in body weight gain, reduced food consumption and clinical signs (excessive salivation, red/mucoid nasal discharge, alopecia, stained anogenital area) at 291.6 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 83.3 mg/kg bw per day, based decreased fetal weight and delayed ossification at 291.6 mg/kg bw per day (Schroeder, 1987).

Rabbit

In a range-finding developmental study, clethodim (purity 83.3%) was administered via gavage to presumed-pregnant female New Zealand White rabbits (eight/group) at doses of 0, 50, 150, 300 or 500 mg/kg bw per day from days 7 to 19 of gestation. The test material was administered as a suspension in a 0.5% Tween 80 and 0.7% sodium CMC solution, at a dose volume of 5 mL/kg bw.

Observations were recorded at least once daily during the dosage period. Body weights were measured immediately prior to insemination and daily on days 7 to 29. Food consumption was recorded daily to GD 29. All females that died during the study were examined for gross morphological abnormalities and possible cause of death. Gross lesions observed at necropsy were sampled and preserved. Any doe with clinical evidence of abortion or pressure delivery of a litter was sacrificed on the day, necropsied and examined for gross lesions and uterine content. Aborted or delivered specimens were subjected to gross examination to the extent that was possible. Surviving females were sacrificed on GD 29 and a gross examination performed. Gravid uterine weight was measured. Fetuses were removed and the number of corpora lutea, number of implantations, number of live fetuses, intrauterine position number and time of embryonic/fetal deaths, were all recorded.

Signs of maternal toxicity were noted in females treated at 50 to 500 mg/kg bw per day and comprised clinical signs (dried faeces) and decreased body weight and food consumption. Alopecia was significantly increased in does at 150 mg/kg bw per day and above.

Increased liver weights and gastrointestinal lesions (hairball and/or gastric ulceration) were observed at 300 and 500 mg/kg bw per day. In the groups given 300 and 500 mg/kg bw per day, two of seven pregnant rabbits died, apparently due to concomitant weight loss, decreased food intake, gastrointestinal lesions and/or abortion.

Adverse effects on development were observed only for litters of from the 300 and 500 mg/kg bw/day groups. Such effects were characterized by decreased fetal body weights and a possible increase in resorption incidence that was observed. However, the possible increased resorption incidence could not be appropriately evaluated in animals given 500 mg/kg bw per day because only one doe survived to day 29. No other treatment-related effects were observed in either the parents or the fetuses. A top dose of 300 mg/kg bw per day was selected for the main developmental study (Dearlove, 1986).

In the main developmental study in rabbits, clethodim (purity 83.3%) was administered via gavage to 20 presumed-pregnant female New Zealand White rabbits/group, at doses of 0, 25, 100 and 300 mg/kg bw per day (equal to 0, 20.8, 83.3 and 250 mg/kg bw per day after correction for purity) from GD 7 until GD 19. Clethodim was suspended in a 0.5% aqueous polyoxyethlenesorbitan monoleate and 0.7% sodium CMC solution at a dose volume of 5 mL/kg bw. Observations were recorded at least twice daily during the dosage period and at least once daily during the predosage and postdosage periods. Body weight and food consumption were recorded daily to GD 29. Females were sacrificed on GD 29 and a gross examination performed. Gravid uterine weight was measured and fetuses were removed by caesarean section. The number of corpora lutea, number of implantations, number of live fetuses, number and intrauterine position of embryonic/fetal deaths (including time of resorptions) were recorded. Each live fetus was weighed and examined externally. Serial sections of the head were examined and preserved in 10% neutral buffered formalin (NBF). Abnormal fetal tissues considered appropriate for retention were examined and then preserved in 10% NBF. All fetuses were dissected, sexed and the ir viscera examined. The remaining skeletons were examined for skeletal abnormalities.

On GD 22 one female at 100 mg/kg bw per day died prematurely and on GD 17 one female of at 25 mg/kg bw per day aborted. These incidences were, in the absence of a dose–response, considered not to have a relationship with treatment.

Signs of maternal toxicity were noted in females treated at 100 and 300 mg/kg bw per day and comprised clinical signs (red substance in the pan and dried faeces), decreased body weight gain (to some extent also decreased body weight) and food consumption. A decrease in gravid uterus weight (ca 10% and not statistically significant) was reported at 300 mg/kg bw per day. When comparing treated does with those of the control group, no treatment-related effects were noted in the number of pregnancies, the number of live fetuses, the litter size or postimplantation loss.

Increased incidence of hyoid with angulated ala(e) was observed at 25 and 300 mg/kg bw per day, achieving statistical significance at the high dose. Based on historical controls, this finding is not uncommon, and the lack of dose–response suggests that this finding is not treatment-related.

A slight increase in small ossification sites (supernumerary spicular intramembranous ossification sites at the margin of the nasals, frontals and/or parietals), in several skull structures were observed, with no dose relation. When these irregular ossification results are calculated for the nasal structures

an apparent increase is observed at the mid- and high-dose groups. However, it is noted that similar incidences were reported in controls for other structures (for example, frontals irregular ossification). Therefore the slight increase for these findings in nasal structures were considered spontaneous because of the absence of a dose-related increase in these irregular ossifications in the skull, and also due to the absence of fetuses with multiple alterations.

Table 16. Key findings of the main teratogenicity study in rabbits.

Dose (mg/kg bw per day)	**0**	**25**	**100**	**300**	**HCD (mean;range)/dr**
Number of females inseminated	19	20	20	20	
Mortality	0/19	0/20	1/20 [a]	0/20	
Aborted	0/19	1/20 [b]	0/20	0/20	
Clinical signs					
Red substance in pan				+**	
Dried faeces			+	+**	
***Pregnant* (*N* [%])**	19, [100]	18, [90]	17, [85]	17, [85]	
Used for fetal evaluation	0	17	16	0	
Body weight gain					
Days 13–16	0.09	0.05	0.02	−0.05**	dr
Days 7–20	0.18	0.13	0.05	−0.10**	
Gravid uterus weight	440.88	404.34	443.91	395.48	
Food consumption					
Days 16–20	156.4	145.8	129.3	98.3**	dr
Days 7–20	161.8	151.8	137.8	116.9**	
Litter response					
Live fetuses (*N*)	138	114	117	111	
Litter Size (*N*)	7.3	6.8	7.3	6.5	
Pregnant (*N*)	19	18	17	17	
Died (*N*)	0	0	1	0	
Aborted (*N*)	0	0	0	0	
Rabbits pregnant and delivered by caesarean section (*N*)	19	17	16	17	
Corpora lutea (mean)	9.6	8.8	10.6	11.4	
Implantations (mean *N*)	8.3	6.9	7.8	7.1	
Litter size (mean *N*)	7.3	6.8	7.3	6.5	
Live fetuses – (*N*) [mean *N*]	138 [7.3]	114 [6.7]	117 [7.3]	111 [6.5]	
Dead fetuses – (*N*) [mean *N*]	1 [0.0]	1 [0.0]	0 [0.0]	0 [0.0]	

Dose (mg/kg bw per day)	0	25	100	300	HCD (mean;range)/dr
Fetal findings; fetal incidence (litter incidence)					
Litters evaluated	19	17	16	17	
Fetuses evaluated	140	115	117	111	
Skeletal malformations/alterations					
Hyoid angulated ala(e)					
Litter incidence	1	4	0	6**	1; 0–6
Foetal incidence	2	6	0	7**	1.1; 0–6
Skull irregular ossification					
Litter incidence	7	9	11	9	
Foetal incidence	12	14	23	17	
Nasal, irregular ossification (internasal, intranasal, irregular suture, intranasal penetrate frontal)					
Litter incidence	2	1	3	5	
Foetal incidence	3	1	5	7*	
Nasal/frontal irregular ossification (misaligned sutures)					
Litter incidence	0	2	2	4	
Foetal incidence	0	2	2	4	

a/b GD 17/GD 22

dr Dose related

* $p < 0.05$ ** $p < 0.01$ (Dunnett's or Fisher's exact)

The NOAEL for maternal toxicity was 20.8 mg/kg bw per day, based on increased clinical signs (red substance in pan, dried faeces), decreased body weights and food consumption at 300 mg/kg bw per day (equal to 250 ng/kg bw per day).

The NOAEL for embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested (Dearlove, 1987).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

Clethodim (purity 95.4%) was administered orally by gavage as a single dose to three groups of 12 Crl:CD(SD) rats per sex at dose levels of 0, 10, 100 and 1000 mg/kg values based on findings from a previous acute neurotoxicity study (Sommer, 2006). Clethodim was suspended in 0.5% CMC with 0.5% Tween 80. The dose volume was 5 mL/kg for all groups. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, except on days of functional observational battery (FOB) assessments. Individual body weights were recorded weekly. FOB and locomotor activity data were recorded for all animals prior to the initiation of dose administration, at approximately three hours following dose administration on study day 0 and on study days 7 and 14. All animals were anaesthetized on study day 15 and perfused in situ; brain weights and brain length (excluding olfactory bulbs) and width were recorded. A neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on six rats per sex in the control and 1000 mg/kg groups.

All animals survived to the scheduled euthanasia. There were no test substance-related clinical findings. Body weights were unaffected by clethodim administration. Test substance-related effects during the FOB evaluations were limited to slightly soiled fur for five of 12 females at 1000 mg/kg

during the handling observations, compared to none of the 12 control females on study day 0. At the study day 7 and 14 evaluations, fur appearance was normal for all 1000 mg/kg group females. There were no treatment-related effects on home cage, open field, sensorimotor, neuromuscular, or physiological parameters on study days 0, 7 or 14.

Mean total and ambulatory counts for the 1000 mg/kg group females were significantly ($p \leq 0.008$) lower at15.5% and 19.4% respectively, than the control group during the first subinterval (0–10 minutes) on study day 0. These transient decreases, whilst test substance-related, were not of sufficient magnitude to affect mean total and ambulatory counts for the overall session (0–60 minutes) and did not persist beyond study day 0. A similar effect was not observed in males of the 1000 mg/kg group. Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test substance administration in males and females in the 10 and 100 mg/kg groups. No remarkable shifts in the pattern of habituation occurred in any of the test substance-treated groups when the animals were evaluated on study days 0, 7 and 14.

No treatment-related effects were apparent in brain weights or brain dimensions for perfused animals. No treatment related neuropathological lesions were observed upon microscopic examination of central and peripheral nervous system tissues from six animals per sex from the 1000 mg/kg group.

The NOAEL for systemic toxicity was 100 mg/kg bw based on decreased locomotor activity in females (total and/or ambulatory counts) at 1000 mg/kg bw.

The NOAEL for neurotoxicity was 1000 mg/kg bw per day, the highest dose tested (Herberth, 2012a).

Short-term study of neurotoxicity

In a dose range-finding neurotoxicity study clethodim (purity 95.4%) was administered in the diet to three groups of five Crl:CD(SD) rats per sex at concentrations of 0, 500, 1500 and 5000 ppm (equal to 0, 45, 132, and 441 mg/kg bw per day for males, 0, 51, 155, and 475 mg/kg per day for females) for approximately 28 days. All animals were observed twice daily for mortality and moribundity. Detailed physical examinations, body weights, and food consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for all animals during the last week of test diet administration (study week 3). All animals were necropsied on study day 28. Brain and liver weights and brain length (excluding olfactory bulbs) and width were recorded, and the brain from each animal was retained for possible future histopathologic examination.

All animals survived to scheduled necropsy. There were no test substance-related clinical findings during the weekly observations. In males, body weight gain and food efficiency significantly decreased in all dose groups during the first week of treatment (days 0–7). For the reminder of the exposure period, lower mean body weight gains, food consumption, and food efficiency were noted at 5000 ppm. As a result, mean body weights for the 5000 ppm males were up to 14.8% lower than the control group during the study.

Lower mean body weight gains and correspondingly reduced mean food consumption during study days 0–7 were noted for females given 5000 ppm. While mean body weight gains and food consumption for the 5000 ppm group females were similar to the control group throughout the remainder of exposure, the initial decrements resulted in slightly lower mean body weight gain when the entire exposure period was evaluated. Mean body weights and food efficiency for the 5000 ppm group females were similar to the control group. There were no test substance-related effects on mean body weights, body weight gains, food consumption, and food efficiency in males and females from the 500 and 1500 ppm groups.

No test substance-related effects were noted on FOB parameters (including home cage, handling, open field, sensory, neuromuscular, and physiological observations) in the 500, 1500 and 5000 ppm group males and females. Locomotor activity in these groups was unaffected by test substance exposure.

There were no test substance-related alterations in organ weights, brain measurements, or macroscopic observations.

Findings for this 28-day dietary dose range-finding neurotoxicity study are shown below in Table 17.

Table 17. Key findings of the 28-day dietary dose range-finding neurotoxicity study in rats.

		Males			
Day	**Dose (ppm)**	**0**	**500**	**1500**	**5000**
−7–0	Body weight change (g)	70	66	64	68
	Food consumption (g/kg per day)	126	134**	127	128
	Food efficiency§	42.3	37.5	38.6	40.4
0–7	Body weight change	63	55*	53**	43**
	Food consumption (g/kg per day)	112	114	109	105
	Food efficiency§	31.9	27.7*	28.2*	24.1**
7–14	Body weight change (g)	63	52	56	43**
	Food consumption (g/kg per day)	97	97	94	95
	Food efficiency§	29.4	25.4	28.3	22.5*
14–21	Body weight change (g)	54	42*	44	38**
	Food consumption (g/kg per day)	85	86	82	84
	Food efficiency§	23.7	19.9	22.0	19.7
21–28	Body weight change (g)	45	40	41	34
	Food consumption (g/kg per day)	74	73	75	75
	Food efficiency§	20.5	20.2	19.5	18.1
0–28	Body weight change (g)	225	189*	194	158**
	Food consumption (g/kg per day)	90	90	88	88
	Food efficiency§	26.2	23.3	24.4	21.1**

		Females			
Day	**Dose (ppm)**	**0**	**500**	**1500**	**5000**
−7–0	Body weight change (g)	38	34	41	44
	Food consumption (g/kg per day)	133	134	133	134
	Food efficiency§	30.1	27.0	32.2	33.6
0–7	Body weight change (g)	25	20	21	13**
	Food consumption (g/kg per day)	120	121	129	102
	Food efficiency§	17.9	15.2	14.7	10.7
7–14	Body weight change (g)	21	19	21	17
	Food consumption (g/kg per day)	106	114	108	100
	Food efficiency§	14.1	12.5	15.2	13.8
14–21	Body weight change (g)	23	20	20	21
	Food consumption (g/kg per day)	95	96	98	96
	Food efficiency§	16.1	14.9	13.9	15.6
21–28	Body weight change (g)	17	18	15	17
	Food consumption (g/kg per day)	85	84	85	85
	Food efficiency§	12.5	14.1	11.2	12.9
0–28	Body weight change (g)	86	77	78	68
	Food consumption (g/kg per day)	100	102	104	95
	Food efficiency§	15.2	14.1	13.7	13.4

§ Body weight gained as a per cent of feed consumed

* $p < 0.05$ ** $p < 0.01$ (Dunnett's test).

Based on these results, exposure levels of 500, 1500 and 5000 ppm clethodim were selected for a the 90-day neurotoxicity study in rats (Herberth, 2012b).

Clethodim (purity 95.4%) was administered in the diet to three groups of 12 Crl:CD(SD) rats per sex at concentrations of 500, 1500 and 5000 ppm (equal to 0, 31, 94 and 331 mg/kg bw per day for males, 0, 38, 115 and 380 mg/kg bw per day for females) for approximately 13 weeks. All animals were observed twice daily for mortality and moribundity. Detailed physical examinations, body weights, and food consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for all animals during pretest and then during the fourth, eighth, and thirteenth weeks of test diet administration (study weeks 3, 7 and 12, respectively). During week 13 all animals were deeply anesthetized and perfused in situ; liver weights, brain weights and brain length (excluding olfactory bulbs) and width were recorded. In addition, a neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on six animals/sex from the control and 5000 ppm groups.

All animals survived to scheduled necropsy. There were no test substance-related clinical findings in the 500, 1500 or 5000 ppm groups. Lower mean body weights, body weight gains, and food consumption were noted for males and females in the 5000 ppm group primarily during the first half of the study (until study week 6–7). However, lower mean body weights (5.2% to 10.9%) persisted in both sexes, and slightly lower food consumption continued for the 5000 ppm group females through to the end of the study. No test substance-related effects on home cage, handling, open field, sensory, neuromuscular, or physiological parameters (other than body weights) were noted at any exposure level during the study week 3, 7, and 12 evaluations. Locomotor activity was also unaffected by test diet consumption; no changes in the pattern of habituation were noted. There were no test substance-related macroscopic or microscopic findings, and no effects on liver weights, brain weights, or brain dimensions.

Table 18. Key results of the 90-day dietary neurotoxicity study in rats.

		Males				Females			
Day	**Dose (ppm)**	**0**	**500**	**1500**	**5000**	**0**	**500**	**1500**	**5000**
0	Body weight (g)	211	211	215	213	144	151	150	146
0–7	Body weight change (g)	58	60	59	41**	22	26	19	11**
	Food consumption (g/kg/day)	107	107	104	99**	115	115	108	111
91	Body weight (g)	587	587	582	530*	273	302*	287	251
0–91	Body weight change (g)	376	376	367	316**	129	150	137	105*

* $p < 0.05$ ** $p < 0.01$ (Dunnett's test).

The NOAEL for systemic toxicity was 1500 ppm (equal to 94 mg/kg bw per day for males and 115 mg/kg bw/day for females) based on the treatment-related reductions in mean body weights, and food consumption in both sexes at 5000 ppm (equal to 331 mg/kg bw per day).

The NOAEL for neurotoxicity was 5000 ppm (equal to 331 mg/kg bw per day), the highest dose tested (Herberth, 2012c).

(b) Immunotoxicity

In a range finding immunotoxicity study (non-GLP) clethodim (purity 95.4%) was offered to three groups of eight female B6C3F1 mice at a dietary concentration of 0, 400, 2000 and 4000 ppm (equal to 0, 101, 551 and 958 mg/kg bw per day) for 28 days. All animals were immunized with an intravenous (i.v.) injection of sheep red blood cells (sRBC) on study day 24. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed approximately weekly. Individual body weights and food consumption were recorded twice weekly. Blood samples for possible future serum IgM antibody analysis were collected from all animals at the scheduled necropsy (study day 28). Complete necropsies were conducted on all animals and the liver, lymph nodes, Peyer's patches, spleen and thymus collected. Liver, spleen and thymus weights were recorded. Spleen cell suspensions were prepared, spleen cell counts were performed and the number of specific IgM antibody-forming cells (AFC) directed towards the sRBC was determined.

There were no test substance-related effects on survival, clinical observations, body weight, food consumption, or macroscopic findings in any of the test substance-treated groups.

Significantly higher liver weights were noted in animals at 2000 and 4000 ppm, up by 16% and 41%, respectively for both absolute and relative weights. There were no test substance-related effects on spleen and thymus weights. There were no test substance-related effects on spleen cell numbers or on the humoral immune response when evaluated as either specific activity (AFC/10^6 spleen cells) or as total activity (AFC/spleen) of splenic IgM to the T-cell-dependent antigen sRBC.

The NOAEL for the humoral immune response was therefore considered to be 4000 ppm (equalt to 958 mg/kg bw per day), the highest dietary concentration administered. Dietary concentrations of 400, 2000 and 4000 ppm were selected for the subsequent definitive immunotoxicity study (Burke, 2012a).

Clethodim (purity 95.4%), was offered ad libitum to three groups of ten female B6C3F1 mice at a dietary concentration of 0, 400, 2000 or 4000 ppm (equal 0, 136, 603 and 1312 mg/kg bw per day) for 28 consecutive days. The positive control group received cyclophosphamide monohydrate (CPS) via intraperitoneal injection (50 mg/kg bw per day) once daily for four consecutive days (study days 24–27). All animals were immunized with an i.v. injection of sRBC on study day 24. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily. Detailed physical examinations were performed approximately weekly, beginning during pretest. Individual body weights and food consumption were recorded approximately weekly, beginning during pretest. Blood samples for possible future serum IgM antibody analysis were collected from all animals at scheduled necropsy (study day 28). Complete necropsies were conducted on all animals and the lymph nodes, Peyer's patches and thymus were collected for possible future microscopic examination. Liver, spleen, and thymus weights were recorded. Spleen cell suspensions were prepared, spleen cell counts performed and the number of specific IgM AFCs directed towards the sRBC was determined.

There were no test substance-related effects on survival, clinical observations, body weight, food consumption, or macroscopic findings in any of the test substance-treated groups.

Significantly higher liver weights (absolute and relative) were noted in animals in the 2000 and 4000 ppm groups, by up to 16% and 44%, respectively . There was a slight but not statistically significant decrease of spleen weights (up to 10%), specific activity (AFC/10^6 spleen cells), total activity (AFC/spleen) of splenic IgM to the T-cell dependent antigen sRBC and spleen cell numbers at 2000 and 4000 ppm. These changes did not occur in a dose-dependent manner, and were not observed in the dose range-finding study, at comparable dose levels. No treatment related effects where observed on thymus weights.

By contrast, spleen and thymus weights, and spleen cell numbers, were all lower in the positive control group (CPS-treated) than the vehicle control group, and all to a statistically significant extent.

The NOAEL for immunotoxicity was 4000 ppm (equal to 1312 mg/kg bw per day) (Burke, 2012b).

(c) Mechanistic studies

Many of the toxicity studies carried out have been with technical clethodim with a purity of about 83%. The technical clethodim currently produced, has a purity of about 93%. To investigate whether the toxicity observed in studies performed with low purity clethodim (purity 84.3%) could be ascribed to the impurities or process neutrals, high purity clethodim (purity 96.2%) and process neutral (containing 3.3% clethodim) were administered ad libitum in the diet to ten rats/sex per group (Sprague Dawley; 44–46 days old) for five weeks. Clethodim (purity 96.2%) was administered at a concentration of 6800 ppm (equal to 597 mg/kg bw per day for males, 667 mg/kg bw per day for females) and process neutrals administered at a concentration of 1200 ppm. This was equal in males to 4.87 mg/kg bw per day pure clethodim (148 mg/kg bw per day of process neutrals), and 5.78 mg/kg bw per day pure clethodim (175 mg/kg bw per day of process neutrals) for females The dose levels were selected to approximate levels of the components of the 8000 ppm dietary level used in a five-week study on rats by Eisenlord (1986). All animals were checked for overt clinical signs by daily general cageside observations. Detailed clinical observations were performed once weekly during the study. Body weights were measured weekly during the study and food consumption noted twice weekly. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical

and haematological parameters. All animals were subjected to postmortem gross examination, and all abnormalities recorded. However, only a limited number of organs, including the target organs, were investigated macroscopically/microscopically.

No mortality was noted during the study.

Effects of high purity at 6800 ppm

Mean body weights in males and females were significantly lower (up to −15%) during week 2 until week five, leading to significantly lower total body weight gains (up to −42%) in both genders. Absolute food consumption values were decreased in males (statistically significant) in each week of the study, and in females in weeks 1, 3 and 5, but relative food consumption was significantly decreased only in males during the first week of the study, suggesting that the diet was initially not palatable to males.

Deviations in RBC parameters were observed in males (significantly reduced RBC counts, Hb and Ht) and in females (significantly reduced RBC counts and Hb).

Total protein and albumin were significantly increased in males, but since these deviations were within historical reference ranges, they did not appear to be treatment-related.

Absolute and relative liver weights were significantly increased in males (112% and 134% of male controls, respectively) and relative liver weights significantly increased in females (124% of female controls). The decreased absolute adrenal weights and increased relative kidney and brain weights in both sexes, and increased relative testes weights in males, were all attributed to lower terminal body weights. There were no treatment-related gross changes at necropsy. Histopathology revealed centrilobular hypertrophy in all males (seven trace and three mild) and eight females (trace) and kidney regeneration in seven males (trace to mild) and three females (trace).

Effects of process neutral at 1200 ppm

Mean body weights were significantly lower (–7%) in males in week 5 only and total body weight gains were also significantly lower in males (–12%), but no significant difference in mean body weight or total body weight gain was noted in females. Absolute food consumption was significantly less than controls in males during weeks 1, 3, 4 and 5, while no significant deviations in relative food consumption were noted in males. No changes in food consumption were observed in females.

No effect was evidenced from haematological investigation. A significant decrease of ALP levels was noted in females in the process neutrals group, however the change was within the historical control ranges.

Absolute liver weight was significantly increased in females (110% of controls) and relative liver weight significantly increased in males and females (107% and 110% of controls, respectively). Histopathology revealed trace centrilobular hypertrophy in six males and three females and kidney regeneration (trace) in four males and three females.

Table 19. Key dose-related findings of the five-week feeding study in rats with clethodim technical (purity 83.4%) and clethodim technical (purity 96.2%).

	Males				Females			
		Process neutrals	RE-45601	HCD Mean;		Process neutrals	RE-45601	HCD Mean;
Dose (ppm)	**0**	**1200**	**6800**	**Range**	**0**	**1200**	**6800**	**Range**
Body weight gain (g)								
Day 0–35	209	184*	139**		78	79	45**	
Food consumption								
Day 7	30.9	28.4*	26.6**		20.8	20.8	18.8*	
Day 21	33.0	29.9*	29.0**		22.7	22.5	20.1*	
Day 35	32.6	28.7**	27.4**		22.3	21.9	18.5**	

	Males				Females			
		Process neutrals	RE-45601	HCD Mean;		Process neutrals	RE-45601	HCD Mean;
Dose (ppm)	**0**	**1200**	**6800**	**Range**	**0**	**1200**	**6800**	**Range**
Haematology								
RBC (10^6/mm^3)	7.34	7.25	6.94*		7.14	6.87	6.63*	
Hb (g/dL)	15.0	14.8	14.1**		14.6	14.1	13.7*	
Ht (%)	40.6	39.5	38.0**		39.1	38.0	37.2	
Clinical chemistry								
Total protein (g/dL)	6.0	5.9	6.4**	6.0; 5.2–6.8	6.1	6.1	6.3	
Albumin (g/dL)	3.0	3.0	3.2**	3.3; 2.9–3.8	3.1	3.1	3.1	
ALP (IU/L)	148	120	124		111	84*	110	79; 40–118
Organ weights								
Liver: absolute (g)	12.17	12.24	13.66*		6.97	7.65*	7.48	
Liver: relative (g)	3.03	3.25*	4.06**		3.18	3.49*	3.95**	
Adrenals: abs. (g)	0.0618	0.0602	0.0457*		0.0664	0.0687	0.0550*	
Kidney: relative (g)	0.403	0.423	0.467**		0.845	0.863	0.925**	
Brain: relative (g)	0.515	0.552	0.612**		0.873	0.861	1.004**	
Testes: relative (g)	0.825	0.833	0.960**		-	-	-	
Pathology – microscopy								
Liver: Centrilobular hypertrophy	0/10	6/10	10/10		0/10	3/10	8/10	
Kidney: regeneration	2/10	4/10	7/10		1/10	3/10	3/10	

* $p < 0.05$ ** $p < 0.01$ (Anova and Dunnett's tests, two-sided)

The results observed with high purity clethodim (purity 96.2%) are consistent with those seen in the five-week feeding study with clethodim (purity 83.4%), indicating that the active ingredient was responsible for the changes in body weight gain, RBC parameters and liver. However, the result observed with the process neutral indicate that the impurities may also have made a minor contribution to these effects (Cushman, 1988).

In order to investigate the ability to induce liver mixed microsomal cytochrome P-450, clethodim (purity 83.8%) was administered by gavage as an aqueous suspension (2.0 mL/kg) to a group of eight male Sprague Dawley (Crl: CD BR) rats (seven weeks old) at a concentration of 125 mg/mL (w/w), equal to 250 mg/kg bw per day for 21 consecutive days. A concurrent control group was identically treated with 2.0 mL/kg of the vehicle (0.7% CMC and 0.5% Tween 80 in distilled, deionised water). In addition, a group of five male rats were dosed as described above at with test material at 250 mg/kg bw per day except on the first and last day of dosing, when they were dosed with ^{14}C-labelled test material. Following dosing of animals with ^{14}C-labelled test material the ratio of 5-hydroxy-cyclohexanone to cyclohexanone in urine from naïve rats (i.e. after the first dose) was compared to the ratio in the urine of the same rats after 21 days of treatment.

All animals survived to scheduled necropsy. There were no test substance-related clinical findings during the weekly observations. There were no significant differences in mean body weights between the eight treated and control animals.

Mean absolute and relative liver weights were significantly higher (121–123% of control) when compared to control values, and at necropsy enlarged livers were observed in two animals administered 250 mg/kg bw/day.

In treated animals the content of microsomal cytochrome P-450 expressed as nmoles/liver, and proteins expressed as mg protein/g liver, are significantly higher than control values. However, there were no significant differences in mean microsomal cytochrome P-450 concentrations expressed as nmoles/mg microsomal protein or nmoles/g liver between treated and control animals. These results may reflect increased liver weight and do not provide evidence that clethodim induces liver cytochrome P-450 (Brorby & Beatty, 1989).

Table 20. Key findings of the potential of clethodim to induce cytochrome P-450following 21-day oral administration study in rats.

	Males	
	Controls	**Clethodim**
Dose (mg/kg bw per day)	**0**	**250**
Body weight		
Day 0	269	267
Day 21	349	340
Liver weight		
Absolute (g)	10.2	12.3*
Relative (g/100 g bw)	2.94	3.61*
Cytochrome P-450 data		
P-450 (nmoles/mg protein)	0.99	0.92
P-450 (nmoles/g liver)	29.3	33.7
P-450 (nmoles/liver)	301	410*
Protein concentration (mg protein/g liver)	29.9	37.1*

* $p < 0.05$ (Student's t-test, two-sided)

(d) Toxicity studies on metabolites

Clethodim imine sulfone (RE-47719)

Metabolite clethodim imine sulfone (5-(2-(ethylsulfinyl)propyl)-3- hydroxy-2-(1-iminopropyl) cyclohex-2-en-1-one) is a metabolite of soya bean (leaves), carrot (leaves), cotton (leaves), rotational crops, and goats.

Table 21. Results of toxicity studies with the metabolite clethodim imine sulfone

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Sprague Dawley)	98.6	LD_{50} > 1400 mg/kg bw	Dougherty, 1988b
Five-week oral toxicity study	Rat (Sprague Dawley)	99.3	NOAEL 70.9 mg/kg bw per day: increased reticulocytes, liver weights and higher serum cholesterol	Bagos and Beatty, 1988a
Ames test	*S. typhimurium, E. coli*	98.6	Not mutagenic	Machado, 1988
In vitro chromosomal aberration test	Chinese hamster ovary cells (CHO)	98.6	Not clastogenic	Putnam, 1988a
Teratogenicity study	Rat (Crl: CD(SD)BR)	98.6	NOAEL maternal: 10 mg/kg bw NOAEL developmental: 100 mg/kg bw No teratogenic effects	Hoberman, 1988a

In an acute oral toxicity study (comparative) in rats a single oral dose of 1400 mg/kg bw clethodim imine sulfone (purity 98.6%), as a suspension in 0.7% CMC and Tween 80 solution in distilled water, was administered by gavage to five fasted female rats. One of the five animals was found dead the morning after dosing. The animal which died showed decreased motor activity four hours after dosing and was found to have its intestine filled with a red, gelatinous material. The remaining four animals did not show any signs of toxicity. Body weights were not affected and no gross abnormalities at necropsy were found in surviving animals.

The oral LD_{50} of clethodim imine sulfone in female rats was > 1400 mg/kg bw (Dougherty, 1988b).

Clethodim imine sulfone (purity 99.3%) was administered for five weeks to four groups of Sprague Dawley rats (ten rats/sex per group; 44 days old) at dietary concentrations of 0, 100, 1000 and 8000 ppm (equal to 0, 6.6, 70.9 and 604 mg/kg bw per day for males, 0, 7.8, 83.7 and 723 mg/kg bw per day for females) . Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cageside observations. Ophthalmoscopic examination was performed on all animals on day −7 and day 35. Body weights were measured weekly during the study and food consumption noted every Wednesday, Friday and Monday. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. All animals were subjected to detailed postmortem gross examination and all abnormalities were recorded. Organ weights (brain, liver, adrenals, kidneys, testes and ovaries) were recorded. Histological evaluation was performed on an extensive list of organs and tissues. The weight of epididymides, thymus, spleen and heart was not determined, neither was the blood clotting potential measured. Histopathology was not performed on prostate or epididymides.

The test material was not stable in diet. Mean concentrations in the diet from hopper samples were 69.4, 793 and 7810 ppm rather than the nominal 100, 1000 and 8000 ppm, respectively. Therefore, from day 13 onwards diets were offered daily instead of three times a week.

No mortality was noted during the study and no treatment-related clinical signs (including pupil response) were observed. In males given 8000 ppm a statistically significant reduction in food consumption (both in absolute and relative terms) and weekly body weight gain were seen on day 7, and significantly lower body weights persisted in this group up to day 21. These initial deviations can be attributed to poor palatability of the diet at the highest test substance concentration.

Reticulocyte count was increased (statistically significant) in males at 8000 ppm. Reticulocyte counts ranged from 0.2% to 1.0% in control males and from 0.8% to 3.8% in males given 8000 ppm; the deviation was therefore considered to be related to treatment and indicative of increased erythropoiesis.

Serum cholesterol was increased in males and females given 8000 ppm to a statistically significant extent. Significantly lower serum alanine aminotransferase (ALT) and ALP activity in males and females given 8000 ppm, respectively These slight changes were not considered to be toxicologically significant. Significantly higher serum albumin and calcium levels were recorded in males given 8000 ppm. Statistically significant decreases in serum globulin and increases of the albumin : globulin ratio were noted in males of all dose groups, but these deviations showed no dose-related trend and were probably incidental. Besides cholesterol, the other clinical parameter changes were mild and not considered to be biologically relevant.

Absolute and relative liver weights were increased to a statistically significant extent in males (114% and 119% of control) and in females (115% and 117% of control) given 8000 ppm.

At necropsy there were no treatment-related macroscopic findings. Histopathological changes did not distinguish treated animals from controls.

Key dose-related findings for this five-week study into rats fed clethodim imine sulfone are shown below in Table 22.

Table 22. Key dose-related findings of the five-week feeding study in rats with clethodim imine sulfone.

	Males				Females			
Dose (ppm)	**0**	**100**	**1000**	**8000**	**0**	**100**	**1000**	**8000**
Body weight gain (g)								
Day 7	47.4	46.8	45.6	34.6**	19.1	18.0	19.9	16.4
Food consumption (g/animal per day)								
Day 7	24.3	24.5	24.1	20.0**	16.5	16.2	17.9	17.1
Haematology								
Reticulocyte count (%)	0.5	0.8	0.9	1.6***	1.0	1.8	1.0	1.0
Clinical chemistry								
ALT (IU/L)	28	25	24	19**	27	25	23	24
Cholesterol (mg/dL)	58	60	63	91**	60	66	72	82**
Albumin (g/dL)	4.0	4.2	4.1	4.4**	4.3	4.3	4.3	4.4
Globulin (g/dL)	1.8	1.5*	1.5**	1.5**	1.4	1.4	1.4	1.4
Albumin:globulin ratio	2.3	2.8**	2.7*	2.9**	3.1	3.2	3.3	3.2
Calcium (mg/dL)	9.5	9.7	9.7	10.0**	9.6	9.7	9.8	9.8
ALP (IU/L)	93	107	105	83	90	80	103	61*
Organ weight								
Liver: absolute (g)	11.99	11.82	12.00	13.62**	7.21	6.60	6.89	8.28*
Liver: relative (g)	3.20	3.10	3.18	3.81**	3.31	3.13	3.11	3.86*

* $p < 0.05$ ** $p < 0.01$ (Anova and Dunnetts test, two-sided)

The NOAEL was 1000 ppm (70.9 mg/kg bw per day) based on increased reticulocytes, liver weights and higher serum cholesterol levels at 8000 ppm (equal to 604 mg/kg bw per day) (Bagos & Beatty 1988a).

In a reverse gene mutation assay in bacteria, clethodim imine sulfone (purity 98.6%) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation. Doses ranged from 100–10000 µg/plate. Slight toxicity was observed at 10 mg/plate in the tester strain TA100. There was no statistically dose-related increase in the number of revertant colonies, or reproducible increase of revertant colonies at one or more concentrations, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. Clethodim imine sulfone was not mutagenic under these test conditions (Machado, 1988).

The clastogenic potential of clethodim imine sulfone (purity 98.6%) was examined in an in vitro chromosomal aberration test using CHO cells with and without metabolic activation (S9 mix). Doses ranged from 50–400 µg/mL. Precipitation was noted at 400 µg/mL. No increase in chromosomally aberrant cells (structural or numerical) was observed. Metaphase cells were collected at 12 h, in the absence of metabolic activation, and at 8 h and 12 h in the presence of metabolic activation. All negative and positive control cultures gave values of chromosomal aberrations within the expected range. Clethodim imine sulfone did not induce chromosome aberrations in CHO cells under the test conditions (Putnam, 1988a).

In a screening developmental toxicity study, clethodim imine sulfone (purity 98.6%) was administered to groups of ten presumed pregnant Crl:CD(SD)BR rats by gavage at dose levels of 0, 10, 100 and 700 mg/kg bw per day from GD 6 until GD 15. The material was suspended in a 0.7% CMC/Tween/distilled water mixture (dosage volume 10 mL/kg). All females were observed twice daily for mortality and toxicity signs. Food consumption was recorded on days 0–3, 3–6 and 6–20. Body weights were recorded on days 0, 3 and 6–20. On day 20, all dams were sacrificed. Postmortem examination, including gross macroscopic examination of all internal organs (with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea) was performed, and the data recorded. All organs or tissues showing grossly visible abnormalities were preserved. The uteri (and

contents) and placentas of all females with live fetuses were weighed at necropsy. Fetuses were removed from the uterus, sexed, weighed individually, and examined for gross external abnormalities. One half of the fetuses in each litter was evaluated for visceral malformation and the remaining half eviscerated and processed for staining of the skeletal structures and subsequent evaluation.

No mortality was recorded during the study. Three animals had excess salivation at 700 mg/kg bw per day. Significantly decreased body weight gain and food consumption were observed at 700 mg/kg bw per day, during GD 6–18 (–16%) and GD 6–9; 6–12, respectively. In animals receiving 100 mg/kg bw per day, a decrease in weight gain (–18%) was the only change observed.

Pathology examinations of the dams did not reveal any microscopic findings or differences in the number of live fetuses, litter size or postimplantation loss.

Fetal weight was significantly reduced (–14%) at 700 mg/kg bw per day, compared to controls.

One fetus at 700 mg/kg bw per day had two left carotids (arising from aorta). The occurrence of this single malformation was not ascribed to treatment by the study's author. No other fetus in the study had visceral alterations.

Skeletal examination showed a significant increase of skeletal variations; consisting of increased incidence of cervical rib present (on litter and fetal basis) and a significant decrease in the average number of sternal centres ossification sites (per fetus per litter) at 700 mg/kg bw per day. However, the incidence of these skeletal findings was considered not indicative of a teratogenic effect.

Table 23. Key findings of the teratogenicity study in rats with clethodim imine sulfone

Dose (mg/kg bw per day)	**0**	**10**	**100**	**700**
Number of females mated	10	10	10	10
Mortality	0/10	0/10	0/10	0/10
Clinical signs				
Excessive salivation	0/10	0/10	0/10	3/10
Pregnant animals	10	10	9	9
Body weight gain (g)				
Days 6–18	89.6	88.1	74.0**	75.3**
Days 6–20	125.3	117.3	107.7**	111.4*
Food consumption				
Days 6–9	22.0	23.0	21.1	18.6**
Days 6–12	21.6	22.3	21.2	18.4**
Litter response				
Live fetuses	157	158	125	153
Litter size	15.7	15.8	13.9	17.0
Fetal weight (g)	3.37	3.32	3.38	2.92**
Examination of fetuses				
Skeletal findings				
Litters evaluated	10	10	9	9
Fetuses evaluated	81	82	65	78
Cervical rib present				
Litter Incidence *N,* [%]	1 [10.0]	0	0	9 [100]**
Foetal Incidence *N,* [%]	1 [1.2]	0	0	30 [38.5]**
Sternal ossification sites§	3.72	3.64	3.70	3.09**

§ Per fetus per litter

* $p < 0.05$ ** $p < 0.01$ (Dunnett's or Dunn's test)

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on a marginal reduction in body weight gain in females at 100 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, based on reduced fetal weight and increased incidence of skeletal findings at 700 mg/kg bw per day (Hoberman, 1988a).

Clethodim 5-OH sulfone

Clethodim 5-OH sulfone (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-5-(2-(ethylsulfonyl)propyl)-3,5-dihydroxycyclohex-2-en-1-one) is a metabolite of soya bean (seeds), carrot (roots), and minor rat metabolite (1% in urine).

Table 24. Results of toxicity studies with the metabolite clethodim 5-OH sulfone.

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Sprague Dawley)	98.6	LD_{50} > 1400 mg/kg bw	Dougherty, 1988b
Five-week oral toxicity study	Rat (Sprague Dawley)	94.8	NOAEL: 588 mg/kg bw per day (highest dose tested)	Bagos and Beatty, 1988b
Ames test	*S. typhimurium, E. coli*	Unknown	Not mutagenic	Machado, 1987
In vitro chromosomal aberration	Chinese hamster ovary cells (CHO)	98.68	Not clastogenic	Putnam, 1988b
Teratogenicity study	Rat (Crl: CD(SD)BR)	98.6	NOAEL maternal: 100 mg/kg bw NOAEL developmental: 100 mg/kg bw No teratogenic effects	Hoberman, 1988b

In an acute comparative oral toxicity study in rats, clethodim 5-OH sulfone (RE-51228; purity 99.9%) or RE-45601 technical (SX-1688, 83.8% purity) was administered by gavage as a suspension in of 0.7% CMC and 0.1% Tween 80 solution, to five female rats per group in a single oral dose of 1400 mg/kg bw. This dose was chosen because it was found in a previous study that it was approximately the LD_{50} of clethodim in female rats. There were no mortalities or signs of toxicity in animals that were dosed with RE-51228. By comparison, all of the animals dosed with RE-45601 technical died within the three days after dosing, after displaying signs of toxicity including displayed salivation, decreased motor activity, collapse, hyper-reactivity, tremors, reduced food consumption, diarrhoea, dehydration, and nasal, ocular, oral and anogenital discharges. Animals exposed to RE-51228 gained body weight, contrary to the animals exposed to RE-45601 which lost body weight until time of death.

After gross pathology, no gross tissue abnormalities were observed in the animals exposed to RE-51228. Exposure to RE-45601 resulted in red discoloured lungs, blood pooled beneath the cranial meninges, black discoloured spleen, black coloured areas on the gastric mucosa, and green and black gelatinous material in the small intestine and cecum. Therefore, the oral LD_{50} of clethodim 5-OH sulfone in rats was greater than 1400 mg/kg bw (Dougherty, 1988b).

Clethodim 5-OH sulfone (RE-51228; purity 94.8%) was administered to four groups of Sprague Dawley rats (ten rats/sex per group; 44 days old) at dietary concentrations of 0, 100, 1000 and 8000 ppm (equal to 0, 5.94, 67.7 and 588 mg/kg bw per day for males, 0, 6.43, 75.5 and 663 mg/kg bw per day for females) for five weeks. Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cageside observations. Ophthalmoscopic examination was performed on all animals on days –5 and 35. Body weights were measured weekly during the study and food consumption was noted every Wednesday, Friday and Monday. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. All animals were subjected to detailed postmortem gross examination and all abnormalities recorded. Organ weights (brain, liver, adrenals, kidneys, testes and ovaries) were recorded. Histological evaluation was performed on an extensive list of organs and tissues.

At the lower concentrations the test substance was not sufficiently stable in the diet when in hoppers; the overall mean dietary test article concentrations during the study were 73.2, 856 and 7290 mg/kg feed for the nominal concentrations of 100, 1000 and 8000 mg/kg feed, respectively. Diets were offered daily instead of three times a week from day 14 onwards. These deviations were not considered to have affected the validity of the study results.

No mortality was noted during the study and no treatment-related clinical signs were observed on dietary exposure of rats to 100, 1000 or 8000 ppm of RE-51228. No treatment-related findings on body weight gain or food consumption were observed. No treatment-related changes in organ weights were observed. At necropsy, there were no notable treatment-related macroscopic and histopathological findings.

Haemoglobin and Ht values were decreased (< 7%; statistically significant) in males given 1000 and 8000 ppm. These deviations were a result of low Hb and Ht values in one male at 1000 ppm and one male at 8000 ppm. The low Hb and Ht values in these animals were accompanied by decreased RBC counts and increased reticulocyte values in the absence of changes in mean corpuscular volume (MCV), mean corpuscular Hb (MCH), or mean corpuscular Hb concentration (MCHC) values; this suggests a regenerative normocytic normochromic anaemia. However, there was no histopathological evidence of an erythropoietic response in the bone marrow or spleen. Thus, minor haematology deviations were observed in two treated animals but were not considered toxicologically relevant.

Table 25. Key dose-related findings of the five-week feeding study in rats with clethodim 5-OH sulfone.

	Males				Females			
Dose ppm	**0**	**100**	**1000**	**8000**	**0**	**100**	**1000**	**8000**
Haemoglobin (g/dL)	15.2	14.9	14.1**	14.4*	13.8	14.0	14.3	14.0
Haematocrit (%)	43.5	42.1	39.8***	40.8**	38.0	38.9	39.4	38.8

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ (Dunnett's test)

The NOAEL was 8000 ppm (equal to 588 mg/kg bw per day), the highest dose tested (Bagos & Beatty 1988b).

In a reverse gene mutation assay in bacteria (Ames test), clethodim 5-OH sulfone (purity unknown) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation (S9 mix). Doses ranged from 30–5000 µg/plate. Toxicity was observed in the presence of S9 mix at 3300 µg/plate and higher in TA98 and at 1000 µg/plate and higher in TA100. There was no statistically dose-related or reproducible increase in the number of revertant colonies, with or without S9 mix. Positive controls showed an appropriate increase in the number of revertant colonies. Clethodim 5-OH sulfone was not mutagenic under these test conditions (Machado, 1987).

The clastogenic potential of clethodim 5-OH sulfone (purity 99.9%) was examined in an in vitro chromosomal aberration test using CHO cells, with and without metabolic activation (S9 mix). Doses ranged from 313–2500 µg/mL. Precipitation was noted at 625 µL/mL and above with and without S9 mix. When CHO cells were exposed to clethodim 5-OH sulfone for 6 h and 10 h with S9 mix, or for 12 h without S9 mix, no increase in chromosomally aberrant cells (structural or numerical) was observed. All negative and positive control cultures gave values of chromosomal aberrations within the expected range. Clethodim 5-OH sulfone has no potential to induce chromosomal aberrations in CHO cells under the test conditions (Putnam, 1988b).

In a screening developmental toxicity study clethodim imine sulfone (purity 99.9%) was administered to groups of 10 presumed pregnant Crl: CD(SD)BR rats by gavage at dose levels of 0, 10, 100 and 700 mg/kg bw per day, from GD6 to GD15. The material was suspended in a CMC (0.7%)/Tween/distilled water-mixture (vehicle). All females were observed daily for mortality and toxicity signs. Body weights and Food consumption was recorded on days 0 and 3 and then daily thereafter. Postmortem examination, including gross macroscopic examination of all internal organs (with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea) was performed and the data recorded. All organs or tissues showing grossly visible abnormalities were preserved. The

uterus (and contents) and placenta of all females with live fetuses were weighed at necropsy. Fetuses were removed from the uterus, sexed, weighed individually and examined for gross external abnormalities. One half of the fetuses in each litter was evaluated for visceral malformation and the remaining half eviscerated and processed for staining of the skeletal structures and subsequent evaluation.

No mortality was noted during the study. At 700 mg/kg bw per day, two animals had rales on a total of 13 days, and one animal had excessive salivation on GD 14. A small inhibition of body weight gain, without an effect on food consumption, was noted among dams receiving 700 mg/kg bw per day. However, this effect was not evident after correction for the slightly reduced gravid uterine weights in this dose group. The slight decrease noted in gravid uterine weights of top dose females was considered to reflect small decreases (2–3%) in fetal weight and number. Compared to controls, none of these differences attained a level of statistical significance. There were no pathological findings noted for treated dams.

Small non-statistically significant decreases in the number of live fetuses and fetal weights were observed in the 700 mg/kg bw per day dose group.

Fetal examinations did not reveal significantly higher incidences in malformations, variations or skeletal ossification.

Table 26. Key findings of the teratogenicity study of clethodim 5-OH sulfone in rats.

Dose (mg/kg bw/day)	**0**	**10**	**100**	**700**
Number of females mated	10	10	10	10
Clinical signs				
Rales	0/10	0/10	0/10	2/10
Excessive salivation	0/10	0/10	0/10	1/10
Pregnant animals	10	9	9	9
Body weight gain (g)				
Days 0–20	184.9	186.3	187.6	182.3
Gravid uterus weight (g)	90.0	95.6	97.3	86.0
Litter response				
Live fetuses	152	144	148	131
Litter size	15.2	16.0	16.4	14.6
Fetal weight (g)	3.77	3.80	3.72	3.68

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on clinical signs at 700 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 700 mg/kg bw per day, the highest dose tested (Hoberman, 1988b).

Clethodim sulfone (RE-47253) (free and conjugated)

Clethodim sulfone (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-5-(2-(ethylsulfonyl)propyl)-3-hydroxycyclohex-2-en-1-one) is a minor metabolite in rats (≤ 1% in urine), of spinach, carrot (outdoor: roots and leaves), soil, goats and hens. The conjugate form is a plant (soya bean) metabolite. The submitted studies were performed with the free form of the metabolite only.

Results of genotoxicity studies on clethodim sulfone are summarized on the following page in Table 27.

Table 27. Results of genotoxicity studies with the metabolite clethodim sulfone

Study	Species	Purity (%)	Results	Reference
Ames test	*S. typhimurium, E. coli*	99.2	Positive activation in TA100 & TA1535 (–S9) Negative all other strains (+/–S9)	Stevenson, 2004a
Ames test	*S. typhimurium (TA100 & TA 1535)*	99.86	Not mutagenic (−S9)	Williams, 2008
In vitro chromosomal aberration	Chinese hamster ovary cells (CHO)	99.2	Negative (−S9) Positive (+S9)	Innes, 2003
In vitro chromosomal aberration	Chinese hamster ovary cells (CHO)	99.86	Negative (+S9; with and without S9 co-factors)	Lloyd, 2009
In vitro mammalian gene mutation	Mouse lymphoma L5178Y cells	99.2	Equivocal (-S9) Positive (+S9)	Riach, 2003a
In vitro mammalian gene mutation	Mouse lymphoma L5178Y cells	99.9	Negative (+/−S9)	Stone, 2009
In vivo mouse micronucleus	Mouse, Crl:CD-1 (ICR)	99.3	Equivocal	Beevers, 2007a
In vivo unscheduled DNA synthesis	Mouse, Crl:CD-1 (ICR)	99.3	Negative	Beevers, 2007b

A bacterial mutagenicity test (Stevenson, 2004a), an in vitro chromosomal aberration test (Innes, 2005), an in vitro mammalian mutagenicity test (Riach, 2003a) an in vivo mouse micronucleus test (Beevers, 2007a) and an in vivo/in vitro unscheduled DNA synthesis study (Beevers, 2007b) were conducted on synthesized samples of clethodim sulfone which were between 99.2 and 99.3 % pure. However, because of unusual results which indicated the metabolite sample was mutagenic and clastogenic in vitro under certain test conditions, further studies were conducted on a very pure (99.9%) sample of clethodim sulfone to establish whether the results of the first tests could be attributed to possible genotoxic impurities arising from the synthetic process. These additional studies consisted of a bacterial mutagenicity test (Williams, 2008), an in vitro chromosomal aberration test (Lloyd, 2009) and an in vitro mammalian mutagenicity test (Stone, 2009).

In a reverse gene mutation assay in bacteria (Ames test), clethodim sulfone (RE-47253; purity 99.2%) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation (S9 mix). Two mutation assay were conducted; direct plate and preincubation. The test item was dissolved in DMSO, and doses ranged from 17–5000 µg/plate. No cytotoxicity and no precipitation were noted. Positive controls showed an appropriate increase in the number of revertant colonies. A dose-related and reproducible mutagenic response was observed with TA 1535 and TA 100 in the absence of S9 mix only, at both 1667 and 5000 µg/plate. The response was detected only with the preincubation method. No mutagenic activity was observed in *S. typhimurium* TA 1537 and TA 98 or with *E. coli* WP2*uvr*A (Stevenson, 2004a).

In a second reverse gene mutation assay in bacteria (Ames test), using a preincubation method, clethodim sulfone (RE-47253) (purity 99.86%) was tested on two strains of *S. typhimurium* (TA100 and TA1535) in the absence of S9, in two separate experiments. The test item was dissolved in DMSO, and doses ranged from 1.6–5000 µg/plate in the first experiment and from 156.3–5000 µg/plate. No cytotoxicity and no precipitation were noted. Positive controls showed an appropriate increase in the number of revertant colonies. There was no statistically dose-related and reproducible increase in the number of revertant colonies, without S9 mix, the only condition used in this study. Positive controls showed an appropriate increase in the number of revertant colonies (Williams, 2008).

The clastogenic potential of clethodim sulfone (purity 99.2%) was examined in an in vitro chromosomal aberration test using CHO cells, with and without metabolic activation (S9 mix), in two independent tests. The test item was dissolved in DMSO, and doses ranged from 1250–5000 µg/mL. Toxicity (reduced cell count) was observed in cultures treated with 5000 µg/mL in the absence of S9 mix. In the presence of S9 mix, an indication of clastogenicity was found at 2500 and 5000 µg/mL

following 6 h treatment. In a repeat test, clastogenic responses were found in duplicate cultures treated with 3000 and 5000 μg/mL and one of the cultures treated with 4000 μg/mL. In the absence of S9 mix, (except for one suspicious response in the repeat test at 5000 μg/mL) all cultures treated with clethodim sulfone were within the 95% confidence limits compared to historical negative control data. All negative and positive control cultures gave values of chromosomal aberrations within the expected range. Clethodim sulfone was clastogenic when tested with CHO cells in vitro in the presence of S9 mix at high concentrations, such as usually cause cytotoxicity (Innes, 2003).

In a second in vitro chromosomal aberration test using CHO cells, the clastogenic potential of clethodim sulfone (purity 99.86%) was examined in the presence of metabolic activation (S9 mix), in the presence and absence of S9 co-factors in duplicate cultures in a single experiment. Concentrations for the main experiments were selected based on the results of this cytotoxicity range-finding experiment. Since no evidence of precipitation or toxicity was observed in the range-finding experiment, up to 2500 μg/mL, the highest dose for the main study was 3920 μg/mL (equivalent to ca 10 mM). The test item was dissolved in DMSO, and doses ranged from 392.4–3920 μg/mL. No cytotoxicity was noted. Precipitation was observed at dose levels of ≥ 294 μg/mL at the start of the incubation and 3920 μg/mL after the 3 h treatment incubation period. Clethodim sulfone did not induce increased frequency of structural chromosome aberrations in CHO cells when tested up to a maximum of 10 mM in the presence of S-9, neither with nor without S9 cofactors (Lloyd, 2009).

Clethodim sulfone (purity 99.2%) was tested for mutagenic potential in mouse lymphoma cells (L5178Y cell line) in the absence and presence of a postmitochondrial supernatant fraction obtained from Aroclor 1254-induced rat livers (S9) using the microwell method. Four independent experiments were conducted: two in the absence of metabolic activation and two in the presence of metabolic activation. In all experiments exposure to cells was for 4 h. A preliminary cytotoxicity test showed a reduction of relative suspension growth to 5.8% and 60.3% respectively in the absence and presence of S9 mix, at 2500 μg/mL. When the exposure period in the absence of S9 mix was increased to 24 h, the relative suspension of growth was reduced to 12.3% at 750 μg/mL. Consequently dosages for assay 1 ranged from 62.5 to 3000 μg/mL in the absence of S9 mix. For assay 2, in the presence of S9 mix, the range was 250–5000 μg/mL. For assay 3, in the absence of S9 mix, 200–3000 μg/mL. Assay 4, in the presence of S9 mix, covered the range 1400–5000 μg/mL.

In the absence of S9 mix, in the first assay a weak mutagenic activity was evidenced at the highest concentration of 2000 μg/mL, with a relative total growth (RTG) of 10%. In the second assay, in the absence of S9 mix, a statistically significant ($p < 0.05$) increase in mutant fraction was observed at 1000 μg/mL. The mutant fraction for this group was of 55 mutants per million higher than the vehicle control. The RTG of 12% at 1000 μg/mL was close to the limit of acceptable toxicity. At higher dose levels an excess of toxicity was observed. As the increase was considered marginal and the statistical significance to be of reduced confidence, the result of this second assay was considered inconclusive.

In the presence of S9 mix, in the first assay a significant mutagenic activity was obtained at 3000 and 4000 μg/mL. The RTG at 4000 μg/mL was 30%. In the second assay in the presence of S9 mix significant mutagenic activity was obtained at dose levels ≥ 2600 μg/mL, at RTG between 30% and 13%. The test was classed as positive at high concentrations that extended into the toxic range and beyond the limit of solubility in the test system.

The ratios of small to large colonies showed a distinct tendency towards greater numbers of small-type colonies, suggesting that clethodim sulfone may be more closely associated with large scale chromosomal damage than with point mutations or small deletions. All negative and positive control cultures gave values of mutation frequency within the expected range.

To investigate the possibility that the mutagenic activity of the batch was due to an impurity, an additional test was performed with an ultrapure sample (99.96%), in the presence of metabolic activation (S9 mix). Results showed that the pure sample is markedly less toxic and less mutagenic than the original batch, but still gave mutagenic responses.

It is concluded that clethodim sulfone is considered to be mutagenic to mouse lymphoma L5178Y cells in the presence of S9 mix and there is limited evidence of weak mutagenic activity at high toxic concentrations in the absence of S9 mix, under the test conditions (Riach, 2003a).

In a second mouse lymphoma cell (L5178Y cell line) study, clethodim sulfone (purity 99.9%) was tested for mutagenic potential in the absence and presence of a postmitochondrial supernatant fraction obtained from Aroclor 1254-induced rat livers (S9). The study was composed of three independent experiments. In assay 1 cells were exposed for 3 h in the presence and absence of S9 mix at dose range 200–3920 μg/mL. In assay 2 cells were exposed for 24 h in the absence of S9 mix (dose range 31.25–1500 μg/mL) and for 3 h in the presence of S9 mix (dose range: 250–3000 μg/mL). In assay 3 cells were exposed for 24 h in the absence of S9 mix at a dose range of 400–3920 μg/mL.

No toxicity was observed. Precipitation was observed at dose levels of ≥ 400 μg/mL at the start of the incubation and ≥ 2500 μg/mL after the 3 h treatment incubation period in the first experiment. In the second experiment precipitation was observed at dose levels of ≥ 500 μg/mL at the start of incubation, and ≥ 2750 μg/mL after the 3 h treatment incubation period. In the third experiment, precipitation was observed at dose levels of ≥ 1200 μg/mL at the start of incubation, but no precipitate was observed following the 24 h treatment incubation period. The mutant frequency values of the concentrations plated were all considerably lower than the sum of the mean frequency plus the global evaluation factor (GEF), indicating a clearly negative result. Statistically significant linear trends were observed in experiments 1 and 3 in the absence of S9, and experiment 2 in the presence of S9. However, no marked increases in mutant frequency were observed at any concentration analysed in these experiments and the trends were not reproducible either in or between experiments. Therefore these observations were not considered biologically relevant. Clethodim sulfone was not considered to be mutatagenic to mouse lymphoma L5178Y cells when tested under the conditions of this study. These conditions included treatments up to precipitating or toxic concentrations in three independent experiments, in the absence and presence of a rat liver metabolic activation system (S9) (Stone, 2009).

Clethodim sulfone (purity 99.3%) was tested for its ability to induce micronuclei in the bone marrow of treated ICR mice. A group of six male mice received a single oral gavage dose of clethodim sulfone at the limit dose of 2000 mg/kg bw once daily for two consecutive days. Animals were sampled 24 h after the second administration. Two further groups of six male mice received the vehicle, 0.7% CMC with 1% (v/v) Tween, (negative control) or cyclophosphamide at 40 mg/kg bw (positive control). The choice of dose levels was based on an initial toxicity range-finding experiment in which clethodim sulfone was given to three male and three female mice at 2000 mg/kg bw. At sacrifice, bone marrow cells were collected from the femurs and cells were prepared and stained according to standard cytological procedures. Subsequently, 2000 or 4000 polychromatic erythrocytes (PCEs) were scored for the presence of micronuclei. The number of micronucleated normocytes was noted. The proportion of PCEs to total erythrocytes was also recorded. In the range-finding study no clinical signs of toxicity or weight loss were observed at 2000 mg/kg/day in either male or female mice.

No clinical signs of toxicity or weight loss were observed except for one treated animal which showed decreased activity on the day of sacrifice. The relative proportions of PCEs and normochromatic erythrocytes (NCEs) were determined from a total of at least 1000 cells (PCEs plus NCEs) and the frequencies of micronucleated PCEs from a total of 2000 PCEs per animal.

As the analysis of 2000 PCEs suggested a positive test compound-induced response, a further 2000 PCEs were scored from the reserve slides for the vehicle- and test compound-treated animals. The results obtained showed that the clethodim sulfone animal group exhibited percentage PCE values that were similar to those observed in the vehicle control group, indicating the absence of any toxicity to the bone marrow. Treatment with clethodim sulfone at 2000 mg/kg resulted in an increase in micronucleated PCEs that was statistically significant, increased 4.75-fold when compared to concurrent vehicle control values. Although no individual animal exceeded the laboratory's historical maximum frequency of five micronucleated PCEs/2000 PCEs scored, the overall distribution of micronucleated PCE frequencies within the group did exceed the historical range. Four animals out of six (67%) treated with clethodim sulfone had micronucleated PCE frequencies of eight or higher (which is greater than 4 MN PCEs/2000 PCEs scored) compared to an expected percentage of just 8% in animals from the historical data which had shown greater than 4 MN PCEs/2000 PCEs counted. The negative and positive controls fulfilled the requirements for a valid test. Overall it can be concluded that although the induction of micronucleated PCEs was not marked following oral administration of clethodim sulfone at 2000 mg/kg/day, the biological relevance of the effect cannot be excluded (Beevers, 2007a).

Clethodim sulfone (purity 99.3%) was tested for its potential to induce DNA repair in an in vivo unscheduled DNA synthesis (UDS) assay in cultured primary mouse hepatocytes. Groups of six male outbred Crl:CD-1 (ICR) mice were treated once with the vehicle 0.7% w/v CMC with 1% v/v Tween 80 in distilled water, clethodim sulfone (at 2000 mg/kg) or the required positive control, via oral gavage, at a dose volume of 10 mL/kg. The positive controls used were 200 mg/kg Fast Garnet GBC (FG-GBC) suspended in corn oil (12–14 h experiment) and 10 mg/kg dimethyl nitrosamine (DMN) dissolved in purified water (2–4 h experiment). No clinical signs were observed in any animal in the main study. Hepatocytes isolated from groups of mice treated with clethodim sulfone exhibited net nuclear grain (NNG) values well below the threshold value of 0 required for a positive response. Less than 0.7% of the cells were observed in repair at 2000 mg/kg of clethodim sulfone. All individual animal NNG counts or percentage of cells in repair (NNG ≥ 5) were within the historical control range. The positive control materials responded as expected. Under the conditions of this UDS experiment, clethodim sulfone at a dose of 2000 mg/kg did not induce UDS in mouse hepatocytes isolated ex vivo 12–14 h or 2–4 h after dosing, and had therefore no genotoxic activity detectable in this test system (Beevers, 2007b).

Clethodim oxazole sulfone (RE-47797)

Clethodim oxazole sulfone (2-ethyl-6-(2-(ethylsulfonyl)propyl)-6,7-dihydrobenzo[d]oxazol-4(5H)- one) is a soil and rotational crops metabolite.

Table 28. Results of toxicity studies with the metabolite Clethodim oxazole sulfone.

Study	Species	Purity (%)	Results	Reference
Ames test	*S. typhimurium, E. coli*	98.9	Negative (+/−S9)	Stevenson, 2004b
In vitro chromosomal aberration	Chinese hamster ovary (CHO) cells	98.9	Negative (−S9) Positive (+S9)	Hart & Stevenson, 2005
In vitro mammalian gene mutation	Mouse lymphoma L5178Y cells	98.9	Negative (+/−S9)	Riach, 2009b
In vivo mouse micronucleus	Mouse, Crl:CD-1 (ICR)	99.5	Negative	Beevers, 2007c

In a reverse gene mutation assay in bacteria (Ames test), clethodim oxazole sulfone (RE-47797; purity 98.9%) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation (S9 mix). Two mutation assays were conducted (one direct plate method and one preincubation method). Doses ranged from 17–5000 µg/plate. No precipitation was noted. Positive controls showed an appropriate increase in the number of revertant colonies. Clethodim oxazole sulfone did not induce mutagenic activity in any of the five bacterial strains used, in either activation conditions. Toxicity, seen as a reduction in the number of revertant colonies, occurred with TA 1535 in the absence of S9 only, at the highest concentration of 5000 µg/plate (Stevenson, 2004b).

The clastogenic potential of clethodim oxazole sulfone (purity 98.9%) was examined in an in vitro chromosomal aberration test using CHO cells, with and without metabolic activation (S9 mix), in one experiment with duplicate cell cultures per dose group. Doses ranged from 1250–5000 µg/mL. The choice of dose levels was based on an initial toxicity range-finding (20–5000 µg/mL, +/−S9 mix), in which toxicity (taken as a reduced cell count of > 55%) was observed in both cultures treated with 5000 µg/mL in the presence of S9 mix. From slide observations clethodim oxazole sulfone was also deemed toxic to cells at 5000 µg/mL. In the presence of S9 mix, clastogenicity was found at 5000 µg/mL in both cultures. This concentration was deemed toxic to the cells. At lower concentrations (2500 µg/mL), no toxicity to the cells was observed and no indications of clastogenicity were found. In the absence of S9 mix no aberration was observed in the cultures treated with clethodim oxazole sulfone. All negative and positive control cultures gave values for chromosomal aberrations within the expected range. Clethodim oxazole sulfone was clastogenic when tested with CHO cells in vitro in the presence of S9 mix at a cytotoxic concentration of 5000 µg/mL (Hart & Stevenson, 2005).

Clethodim oxazole sulfone (purity 98.9%) was tested for mutagenic potential in mouse lymphoma cells (L5178Y cell line) in the absence and presence of a postmitochondrial supernatant fraction obtained from Aroclor 1254-induced rat livers (S9) using the microwell method. In a preliminary toxicity study (0.3–3000 μg/mL), the test item dissolved in DMSO allowed a maximum attainable concentration of 3000 μg/mL, at which little or no toxicity was seen within 4 h of exposure. For the mutation assays the solvent was changed to the tissue culture medium. Four independent experiments were conducted: 1000–5000 μg/mL for assay 1 in the absence of S9 mix (4 h exposure); 500–5000 μg/mL for assay 2 in the presence of S9 mix (4 h exposure); 500–4000 μg/mL for assay 3 in the absence of S9 mix (24 h exposure); and 500–4000 μg/mL for assay 4 in the presence of S9 mix (4 h exposure). All treatments were performed on duplicate cell cultures. The positive controls were ethyl methanesulphonate (EMS) and methyl methanesulphonate (MMS; without S9), and 3-methyl chloranthrene (3-MC; with S9).

Clethodim oxazole sulfone was demonstrated not to be mutagenic in the absence of S9 mix, at up to and including the maximum concentration of 5000 μg/mL for the 4 h exposure and in the extended range after 24 h exposure. The test item remained soluble throughout the test period. In the first assay, in the presence of S9 mix, a statistically significant increase (pair-wise and linear trend) of mutation frequency was observed at 3000 μg/mL, at a relative total growth of 27% relative to control. In the second assay, in the presence of S9 mix, a statistically significant increase (pair-wise and linear trend) of mutation frequency was observed at 3500 μg/mL, at a relative total growth of 15% relative to control. These statistically significant increases in the number of mutations were not considered biologically relevant because they were marginal since in both assays the increases were well below the global evaluation factor (GEF). It is concluded that clethodim oxazole sulfone induces statistically significant, but biologically irrelevant increases in mutant numbers of mouse lymphoma L5178Y cells, in the presence of S9 mix. Thus, these results show that clethodim oxazole sulfone is not a mammalian cell mutagen as defined by internationally agreed criteria for interpretation of the mouse lymphoma assay (Riach, 2009b).

Clethodim oxazole sulfone (purity 99.5%) was tested for its ability to induce micronuclei in the bone marrow of treated ICR mice. In a preliminary toxicity range-finding experiment a group of three male and three female mice received a single oral gavage dose of clethodim oxazole sulfone at 2000 mg/kg bw for two consecutive days. Observations were made over a two day period following the second administration and signs of toxicity were recorded. Since mild, transient clinical signs of toxicity (lethargy) were observed, 2000 mg/kg bw was considered the maximum suitable dose. In the main micronucleus test clethodim oxazole sulfone, dissolved in 0.7% CMC with 1% (v/v) Tween, was administered to three groups of six male mice which received a single oral gavage dose of 500, 1000 or 2000 mg/kg bw. Two further groups of six male mice received the vehicle alone or cyclophosphamide at 40 mg/kg bw (positive control). Clethodim oxazole sulfone was given as two administrations, 24 h apart and animals were sampled 24 h after the second administration. Cells were therefore exposed to the clethodim oxazole sulfone over a period 24 to 48 h prior to when the sampling were examined. At times of sacrifice, bone marrow cells were collected from the femurs and cells were prepared and stained according to standard cytological procedures. Subsequently, 2000 PCEs were scored for the presence of micronuclei. The proportion of polychromatic erythrocytes to total erythrocytes was also recorded.

No clinical signs of toxicity were observed in animals treated with 500 mg/kg bw per day. At 1000 mg/kg bw per day, five animals showed lethargy on day 1, but returned to normal from day 2. At 2000 mg/kg bw per day, animals showed lethargy on day 1 and 2, but not on the day of sacrifice. Three animals showed palpebral closure on day 1. The clinical observations reported in this study indicated that animals treated with clethodim oxazole sulfone were likely systemically exposed to the test article. Groups of mice treated with clethodim oxazole sulfone exhibited percentage PCE values that were similar to vehicle controls and which fell within the laboratory's historical control range. There was no evidence of test article-induced toxicity to the bone marrow. Frequencies of micronucleated PCEs were also similar to those seen in vehicle controls and were consistent with the laboratory's historical control data. Under the conditions of this micronucleus assay, clethodim oxazole sulfone did not induce increased micronuclei in the PCE of the bone marrow of ICR mice treated up to 2000 mg/kg/day (Beevers, 2007c).

DME sulfoxide acid (M17R)

DME sulfoxide acid (M17R) (3-[(2-Ethylsulfinyl) propyl]- pentanedioic acid) is a crop metabolite (spinach, carrot roots and foliage).

Table 29. Results of toxicity studies with the metabolite DME sulfoxide acid.

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Sprague Dawley)	99.51	LD_{50} > 5000 mg/kg bw	Beerens-Heijnen, 2010a
28-day oral toxicity study	Rat (Sprague Dawley)	99.51	NOAEL > 5000 ppm (400 mg/kg bw/day)	Beerens-Heijnen, 2010b
Ames test	*S. typhimurium, E. coli*	98.9	Negative (−/+S9)	Verspeek-Rip, 2009a
In vitro chromosomal aberration	Peripheral human lymphocytes	98.9	Negative (−/+S9)	Buskens, 2010

In an acute oral toxicity study in rats, DME sulfoxide acid (purity 99.5%) was administered by gavage, as a suspension in distilled water, to six female rats (three animals per step) in a single oral dose of 2000 mg/kg bw. All animals survived the observation period. Clinical signs included hunched posture and/or piloerection noted in all animals on day 1. All animals gained weight and no pathological findings were noted during necropsy. Therefore, the oral LD_{50} of DME sulfoxide acid in rats was greater than 2000 mg/kg bw. According to the OECD 423 test guideline, the LD_{50} cut-off value was considered to exceed 5000 mg/kg bw (Beerens-Heijnen, 2010a).

DME sulfoxide acid (purity 99.51%) was administered for 28 days to four groups of Sprague Dawley rats (five rats/sex per group; six weeks old) at dietary concentrations of 0, 200, 1000 and 5000 ppm (equal to 0, 15, 80 and 396 mg/kg bw per day for males, 0, 16, 78, and 407 mg/kg bw per day for females). Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cageside observations. Body weights and food consumption were measured weekly. During the last week of treatment hearing ability, pupillary reflex, static right reflex, grip strength and motor activity were evaluated in all animals. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. All animals were subjected to detailed postmortem gross examination, and all abnormalities recorded. The following organ weights were recorded: adrenals, brain, epididymides, heart, kidneys, liver, spleen, thymus, uterus, prostate, seminal vesicles, thyroid (including parathyroid), testes and ovaries. Histological evaluation was performed on an extensive list of organs and tissues.

Dietary analyses confirmed that diets were prepared accurately and homogenously, and were stable over at least ten days. No substance-related mortality, clinical signs, body weight gain or altered food consumption were observed. No treatment-related effects were observed from the functional observations. Minor statistically significant haematology and clinical chemistry differences arising between controls and treated animals were considered to represent a change not of biological significance. These findings occurred in the absence of a dose-related distribution, and/or remained within the range considered normal for rats of this age and strain and were therefore considered not to have toxicological significance.

A statistically significant increase in the absolute and relative thymus weights (24% and 25% respectively) was noted in males at 5000 ppm, and in females at 200 ppm, (33% and 40%, respectively). In addition an increased relative heart weight was noted in females at 1000 ppm and an increased relative adrenal weight in males at 5000 ppm. The thymus and adrenal weight changes occurring at 5000 ppm in males were considered treatment-related.

Table 30. Findings of the five-week feeding study for DME sulfoxide acid in rats.

	Males					Females				
Dose (ppm)	**0**	**200**	**1000**	**5000**	**HCD**	**0**	**200**	**1000**	**5000**	**HCD**
Haematology										
RDW (%)	12.1	11.5*	11.9	11.6*	11.0–12.6	11.5	11.4	11.5	11.4	
MCH (fmol)	1.24	1.20*	1.19*	1.21	1.15–1.28	1.19	1.19	1.18	1.18	
MCHC (mmol)	21.77	20.62**	20.87**	20.78**	20.13–22.47	21.50	21.62	21.61	21.48	
Platelets (10^9/L)	971	1224	1120	1166	647–1502	1253	1029*	1143	1055*	647–1502
Clinical chemistry										
Sodium (mmol/L)	142.9	141.5*	141.5*	141.6*	134–146.1	143.3	141.3	140.9	141.2	
Potassium (mmol/L)	4.14	4.15	4.24	4.35*	3.55–4.87	3.84	3.91	4.06*	3.92	3.52–4.30
Calcium (mmol/L)	2.74	2.70	2.66*	2.75	2.45–2.79	2.81	2.85	2.90*	2.78	2.47–2.78
Inorganic P (mmol/L)	2.58	2.59	2.66	3.03**	2.03–3.24	2.16	2.32	2.43	2.23	
Organ weights										
Heart: relative (g)	0.329	0.348	0.349	0.348		0.349	0.354	0.378*	0.366	
Thymus: absolute (g) (± SD)	0.405 ± 0.073	0.444 ± 0.038	0.451 ± 0.029	0.502 ± 0.065*		0.344 ± 0.058	0.458 ± 0.065*	0.347 ± 0.056	0.345 ± 0.038	
Thymus: relative (g) (± SD)	0.109 ± 0.015	0.121 ± 0.016	0.124 ± 0.006	0.136 ± 0.02*		0.154 ± 0.019	0.215 ± 0.029**	0.159 ± 0.025	0.155 ± 0.013	
Adrenals: absolute (g) (± SD)	0.056 ± 0.005	0.065 ± 0.008	0.066 ± 0.012	0.067 ± 0.006		0.068 ± 0.001	0.064 ± 0.005	0.062 ± 0.011	0.063 ± 0.007	
Adrenals: relative (g) (± SD)	0.015 ± 0.001	0.018 ± 0.002	0.018 ± 0.003	0.018 ± 0.001*		0.031 ± 0.002	0.030 ± 0.003	0.028 ± 0.005	0.028 ± 0.001	

HCD Historical control data

RDW Red blood cell distribution width

* $p < 0.05$ ** $p < 0.01$ (Dunnett's test)

The NOAEL was 1000 ppm (80 mg/kg bw per day), based on thymus and adrenal weight changes in males at 5000 ppm (equal to 396 mg/kg bw per day) (Beerens-Heijnen, 2010b).

In a reverse gene mutation assay in bacteria (Ames test), DME sulfoxide acid (purity 99.51%) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation (S9 mix), in two independent experiments. Doses ranged from 100–5000 µg/plate, based on a dose range-finding test. No cytotoxicity and no precipitation were observed. There was no statistically dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed an appropriate increase in the number of revertant colonies, and both negative and positive control values were within the laboratory

historical control data ranges. DME sulfoxide acid was not mutagenic under these test conditions (Verspeek-Rip, 2009a).

The clastogenic potential of DME sulfoxide acid (purity 99.51%) was examined in an in vitro chromosomal aberration test using cultured peripheral human lymphocytes, with and without metabolic activation (S9 mix), in two independent experiments. The dose levels were 33–2503 µg/mL (10mM) for the toxicity test, and 100–2503 µg/mL in the presence and absence of S9 mix for the main assay. In the first experiment the test item was tested up to 2503 µg/mL during a 3 h exposure, in the absence and presence of S9 mix. In the second experiment DME sulfoxide acid was tested at concentrations up to 1000 µg/mL with 24 h and 48 h of exposure in the absence of S9 mix, and in the presence of S9 mix up to 2503 µg/mL with 3 h of exposure. Appropriate cytotoxicity and no precipitation were observed. DME sulfoxide acid did not increase the number of polyploidy cells and cells with endoreduplicated chromosomes either in the absence or presence of S9 mix, except in the first cytogenetic assay in the absence of S9 mix. In this first assay, two cells in one of the duplicates had an increase in endoreduplicated chromosomes at 2503 µg/mL, to just above the historical control data range (0–1) and was not observed in the quality control check (one of the duplicate slides was scored by a different scorer, not reported). As a result, this increase was not considered biologically relevant. Therefore, it can be concluded that DME sulfoxide acid does not disturb mitotic processes and cell cycle progression and does not induce numerical chromosome aberration under the experimental conditions. All negative and positive control cultures gave values of chromosomal aberrations within the expected range (Buskens, 2010).

DME sulfone acid (M18R)

DME sulfone acid (M18R) (3-[(2-Ethylsulfonyl) propyl]- pentanedioic acid) is a crop metabolite (spinach, carrot roots and foliage).

Table 31. Results of toxicity studies with the metabolite DME sulfone acid.

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Sprague Dawley)	99.58	LD_{50} > 5000 mg/kg bw	Beerens-Heijnen, 2010c
Ames test	*S. typhimurium, E. coli*	99.58	Negative (+/− S9)	Verspeek-Rip, 2009b

In an acute oral toxicity study in rats, DME sulfone acid (purity 99.58%) was administered by gavage, as a suspension in propylene glycol, to six female rats (three animals per step) in a single oral dose of 2000 mg/kg bw. All animals survived the observation period and no clinical signs were noted. All animals gained weight and no pathological findings were noted during necropsy. Therefore the oral LD_{50} of DME sulfone acid in rats was greater than 2000 mg/kg bw. According to the OECD 423 test guideline, the LD_{50} cut-off value was considered to exceed 5000 mg/kg bw (Beerens-Heijnen, 2010c).

In a reverse gene mutation assay in bacteria (Ames test), DME sulfone acid (purity 99.58%) was tested on four strains of *S. typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *E. coli* (WP2*uvrA)* in the presence and absence of metabolic activation (S9 mix), in two independent experiments. Doses ranged from 100–5000 µg/plate. No cytotoxicity and no precipitation were observed. There was no statistically dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed an appropriate increase in the number of revertant colonies. DME sulfone acid was not mutagenic under these test conditions (Verspeek-Rip, 2009b).

(e) Literature review

A review of the published literature for clethodim was performed. Articles of potential relevance were investigated in further detail by examining the abstract and/or the full article text. Where articles were considered to meet the criteria for relevance, an assessment of the reliability of the study was carried out based on the approach described by Klimisch, Andreae & Tillmann (1997). As a result one 90-day oral toxicity study in rats and a genotoxicity study (in vitro and in vivo) were identified.

In a non-GLP study, clethodim (purity 95%) was administered for 90 days to four groups of ten males and ten females Sprague Dawley rats at dose levels of 0, 14.9, 89.6 and 537.5 mg/kg bw per day. Rats were observed on a daily basis for clinical signs. Body weights were recorded once a week. At the

end of the study animals were subjected to overnight fasting before taking blood samples to investigate a limited number of haematological parameters: RBC, Hb, platelet count (PLT), white blood cell (WBC), lymphocyte, neutrophil and monocyte counts. The following biochemical serum parameters were investigated: ALT, aspartate aminotransferase (AST), ALP, TP, albumin, bilirubin, BU:N CREA (the ratio between blood urea nitrogen and serum creatinine), glucose, cholesterol, total glycerides (TG), cholinesterase, etc. At necropsy, no gross or histopathology investigation was conducted. Relative organ weights only were measured.

No treatment-related clinical signs or abnormal activities were reported. From week 2 of treatment body weight was significantly reduced in both sexes at 537.3 mg/kg bw per day. In males given 537.3 mg/kg bw per day, relative brain, heart, lung, liver, spleen, adrenal gland, kidney and testicle weights were significantly increased when compared to controls. In males given 89.6 mg/kg bw per day, relative kidney weights were significantly increased. In females given 537.3 mg/kg bw per day a significant increase in relative brain, heart, lung, kidney and liver weights was observed.

RBC and HGB for male rats from the high dosing group were significantly lower than controls, and WBC for male rats was significantly lower than controls in all dosed groups. The number of monocyte and neutrophil in both male and female rats from the medium and high dosing groups was significantly lower than controls, while the number of lymphocyte was significantly higher.

Study 1
Levels of albumin, TP, CHE and CHOL in male rats from the high-dose group and GLU from the medium- and high-dose groups were significantly higher than for controls. For female rats from the high-dose group CHE was significantly lower than for the reference group.

The study's author concluded that the maximum nontoxic dose was 14.9 mg/kg bw per day (He et al., 2013)

The study partially conformed to regulatory test guidelines (for example OECD TG408), with the following exceptions:

- Age of the animals was not reported.
- Food consumption was not reported.
- It is not clear if the compound intake was determined.
- Absolute organ weight was not reported/evaluated.
- The method of analysis used for cholinesterase was not reported.
- Urine analysis was not performed.
- Gross and histopathological investigation were not performed.

Given the above shortcomings, the results of the study cannot be appropriately evaluated, therefore, the reliability of the study is questionable. It is anyhow noted that the results of the study do not substantially change the compound's risk assessment.

Study 2
The potential ability of clethodim to induce DNA damage was investigated in a non-GLP study:

In an in vitro comet assay conducted on male mouse (ICR) bone marrow, femur bone marrow was exposed to 5, 10 and 20 mg/L clethodim (purity 95%) for 15 h. A positive control group was exposed to 100 mg/L methyl methanesulfonate. Double-distilled water (ddH_2O) was used as a blank reference, and DMSO as the vehicle control. Cytotoxicity was determined by trypan blue.

Changes in tail length and tail moment between the reference DMSO group and clethodim group were statistically significant at high doses (20 mg/L), however the difference in the fraction of DNA in the tail from the reference and from the clethodim groups was not statistically significant.

In an vivo comet assay using ten male (ICR) mice per group, clethodim (purity 95%) was administered in a single oral gavage dose of 250, 500 and 1000 mg/kg bw. An additional group of ten animals dosed at 50 mg/kg bw with cyclophosphamide served as a positive control. An extra ten animals were given DMSO as vehicle control group. The compound and the vehicle were administered at a

volume of 0.2 mL/10 g bw. Mice were dosed once per day and twice in total with a time gap of 24 h. Six hours after the second dosing, mice were executed and bone marrow cells extracted for comet assays.

No noticeable DNA damage in mice bone marrow cells was observed at any dose tested (Meng et al. 2012).

The study's experimental protocol is poorly detailed for both assays:

- The number of animals (in vitro) and age (in vitro and in vivo) is not indicated.
- The number of samples/animal, replicates and cells/sample analysed is not indicated.
- The description of the experimental methodology is incomplete and poorly described; slide preparation are not detailed, and the details of electrophoresis are missing. In addition, it is stated that the method used is modified from Hartmann et al., 2003, but no details are provided.
- The proficiency of the laboratory is not documented (positive and negative controls).
- Cytotoxicity is not evaluated in the in vivo assay.

Given the above experimental deficiencies these studies are considered of doubtful reliability. Nevertheless, the genotoxic potential of clethodim has been properly investigated with GLP and guideline compliant studies.

3. Observations in humans

No data are available.

Comments

Biochemical aspects

Male and female rats were given single oral doses of [propyl-1 ^{14}C]-clethodim at 4.4 or 468 mg/kg bw, or unlabelled test material at 4.5 mg/kg bw per day for 14 consecutive days before treatment with a single radiolabelled dose of 4.8 mg/kg bw. Oral absorption was 88–95% based on urine, tissue, expired CO_2, cage wash and residual carcass. Seven days after treatment, the total amount of radiolabel recovered from organs and tissues was less than 1% of the administered dose. Elimination was rapid, with 94–98% of the administered dose excreted by 48 h after administration. The principal route of excretion was the urine (87–93%), and a smaller percentage of the radioactivity (9–17%) was eliminated in the faeces. The amount of radioactivity excreted in expired air as carbon dioxide represented 0.5–1% of the administered dose. Although the elimination patterns were similar in all groups, the rate of elimination was somewhat faster in animals that were administered the single low dose of 4.4 mg/kg bw (84% eliminated within 24 h) than in those given the single high dose of 468 mg/kg bw (53% within 24 h), suggesting the tendency to saturation. No sex differences in elimination rate were seen in animals administered repeated low doses of clethodim. There were no significant dose-related or sex-specific differences in tissue distribution, when expressed as a proportion of the dose administered, and there was no evidence of bioaccumulation (Rose & Griffis, 1988).

Clethodim was extensively metabolized in all dosed groups. Unchanged parent was detected in urine of females (0.4% of the administered dose) of the high-dose group only, in faeces of females and males of the low-dose and high-dose groups (0.3–1% of the administered dose). The primary routes of metabolism were by oxidation and subsequent hydroxylation or demethylation with subsequent oxidation. The major metabolite in excreta was clethodim sulfoxide, which accounted for 46–61% and 2–5% of the administered dose in urine and faeces, respectively. *S*-methyl sulfoxide, accounted for 6–11% and 0.4–1% of the administered dose in urine and faeces, respectively. Imine sulfoxide was 6–9% of the administered dose in urine, and 1–2% in faeces, in females and males. Other identified compounds (≤ 5% of the administered dose), in urine and faeces of females and males were 5-OH sulfoxide (3–5%), oxazole sulfoxide (2–3%), trione sulfoxide (ca 1%), 5-OH sulfone (0.3–1%), clethodim sulfone (0.1–1%), aromatic sulfone (0.2–0.7%) and *S*-methyl sulfone (0.0–0.4%). No significant differences were noted in the pattern of metabolites between sexes and dose regimes (Rose & Griffis, 1988).

Toxicological data

In rats, the acute oral LD_{50} was 1133 mg/kg bw. In mice the acute oral LD_{50} was 1688 mg/kg bw, and the acute LC_{50} was greater than 3.25 mg/L. In rabbits, the acute dermal LD_{50} was greater than 4167 mg/kg bw. Clethodim was mildly irritating to eyes and skin, and was a skin sensitizer to the skin of guinea pigs (Magnusson & Kligman test) (Arcelin, 2006; Cox, 1986a; Cushman, 1986a, b, d,; Griffis, 1986; Pelcot, 2005).

The short-term toxicity of clethodim was tested in mice, rats and dogs, and the long-term toxicity and carcinogenicity was tested in mice and rats. In rodent short-term studies critical effects were observed on the liver (increased liver weight, hepatocyte hypertrophy and focal coagulative necrosis) and body weights. In dogs, toxicity effects on liver consisted of organ weight changes, associated with biochemical and histopathological changes. Sternum marrow hyperplasia and pigment in spleen were also observed in dogs.

In a 28-day study in mice in which clethodim was administered at dietary concentrations of 0, 100, 250, 625, 1500 and 4000 ppm (equivalent to 0, 11.9, 29.7, 74.4, 179 and 476 mg/kg bw per day), the NOAEL was 1500 ppm (equivalent to 179 mg/kg bw/day) based on increased liver weights, increased liver hypertrophy in both sexes and increased liver focal coagulative necrosis in males at the 4000 ppm (equivalent to 476 mg/kg bw per day) dose level (Cox, 1986b).

In a 35-day study in rats in which clethodim was administered at dietary concentrations of 0, 5, 200, 1000, 4000 and 8000 ppm (equal to 0, 0.26, 12.5, 65.6. 216 and 515 mg/kg bw per day for males, 0, 0.29, 13.9, 70.6, 291 and 554 mg/kg bw per day for females), the NOAEL was 1000 ppm (equal to 65.6 mg/kg bw per day) based on decreased body weights and food consumption in both sexes at 4000 ppm (equal to 216 mg/kg bw per day) (Eisenlord, 1986).

In a 90-day study in rats in which clethodim was administered at dietary concentrations of 0, 50, 500, 2500 and 5000 ppm (equal to 0, 2.3, 25, 134 and 279 mg/kg bw per day for males, 0, 2.8, 30, 159 and 341 mg/kg bw per day for females), the NOAEL was 500 ppm (equal to 25 mg/kg bw per day) based on changes in body weight in males at 2500 ppm (equal to 134 mg/kg bw per day) (Dougherty, 1986).

In a 90-day study in Beagle dogs in which clethodim was administered by gelatine capsules at dose levels of 0, 1, 25, 75 and 125 mg/kg bw per day (equal to 0, 0.83, 21, 62 and 104 mg/kg bw per day after correction for purity), the NOAEL was 62 mg/kg bw per day based on elevated liver weights and liver histopathology lesions (centrilobular vacuoles) in both sexes, and increased serum cholesterol and alkaline phosphatase at 104 mg/kg bw per day (Daly, 1987).

In a one-year study in Beagle dogs in which clethodim was administered by capsule at dose levels of 0, 1, 75 and 300 mg/kg bw per day (equal to 0, 0.83, 62 and 250 mg/kg bw per day after correction for purity), the NOAEL was 62 mg/kg bw per day, based on increased platelets, increased liver weights and associated increase of biochemical parameters (alkaline phosphatase and cholesterol) and histopathological effects (hypertrophy and pigment), hyperplasia in sternum marrow and pigments in the spleen in both sexes at 250 mg/kg bw per day (Cox, 1988a).

In an 18-month toxicity and carcinogenicity study in mice, clethodim was administered at dietary concentrations of 0, 20, 200, 1000 and 3000 ppm (equivalent to 0, 2.4, 24, 119 and 238 mg/kg bw per day until week 15, the highest dose increasing thereafter to 357 mg/kg bw per day, after correction for purity), the systemic NOAEL was 200 ppm equivalent 24 mg/kg bw per day based on hepatic changes, notably centrilobular hypertrophy, increased pigmentation and bile duct hyperplasia, and an increased incidence of alveolar macrophages in the lungs of mice given 1000 ppm (equivalent to 119 mg/kg bw per day). No evidence for a carcinogenic potential in mice was observed with clethodim (Cox, 1988b).

In a two-year toxicity and carcinogenicity study in rats, when clethodim was administered at dietary concentrations of 0, 5, 20, 500 and 2500 ppm (equal to 0, 0.15, 0.57, 16 and 86 mg/kg bw per day for males, 0, 0.20, 0.72, 21 and 113 mg/kg bw per day for females), the NOAEL for systemic toxicity was 500 ppm (equal to 16 mg/kg bw per day), based on decreased body weight gain, decreased food intake in both sexes, increased mortality in males and increased chronic pancreatitis in females at 2500 ppm (equal to 86 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 21 mg/kg bw per day), based on increased incidence of benign granulosa cell tumours in the ovary (2/63) at 2500 ppm (equal to 113 mg/kg bw per day) (Dougherty, 1988a).

The Meeting concluded that clethodim is carcinogenic in female rats, but not in mice or male rats.

Clethodim was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that clethodim is unlikely to be genotoxic.

In view of the lack of genotoxicity, and the fact that tumours were observed only at doses unlikely to occur in humans, by a mechanism that would exhibit a threshold, the Meeting concluded that clethodim is unlikely to pose a carcinogenic risk to humans due to exposure in the diet.

In a two-generation reproductive toxicity study in which rats were given clethodim at a dietary concentration of 0, 5, 20, 500 and 2500 ppm (equal to 0, 0.5, 1.2, 32.2 and 163 mg/kg bw per day for males, 0, 0.5, 1.5, 37.4 and 181 mg/kg bw per day for females, after correction for purity), the NOAEL for parental toxicity was 500 ppm (equal to 32.2 mg/kg bw per day), based on body weight changes and reduced food consumption in F0 and F1 generations at 2500 ppm (equal to 163 mg/kg bw per day). The NOAEL offspring toxicity was 2500 ppm (equal to 163 mg/kg bw per day). The NOAEL for reproductive toxicity was 2500 ppm (equal to 163 mg/kg bw per day) (Tellone, 1987).

In a developmental toxicity study in rats given clethodim by gavage at doses of 0, 10, 100, 300 and 700 mg/kg bw per day (equal to 0, 8.3, 83.3, 292 and 583 mg/kg bw per day, after correction for purity) the NOAEL for maternal toxicity was 83.3 mg/kg bw per day based on a reduction in body weight gain, reduced food consumption and clinical signs (excessive salivation, red/mucoid nasal discharged, alopecia, staining anogenital area) at 292 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 83.3 mg/kg bw per day, based on decreased fetal weight and delayed ossification at 292 mg/kg bw per day (Schroeder, 1987).

In a developmental toxicity study in rabbits given clethodim by gavage at 0, 25, 100 and 300 mg/kg bw per day (equal to 0, 20.8, 83.3 and 250 mg/kg bw per day after correction for purity), the NOAEL for maternal toxicity was 20.8 mg/kg bw per day, based on increased clinical signs (red substance in pan, dried faeces), decreased body weights and food consumption. The NOAEL for embryo and fetal toxicity was 250 mg/kg bw per day (Dearlove, 1987).

The Meeting concluded that clethodim is not teratogenic.

In an acute neurotoxicity study, clethodim was given to rats by gavage at a dose of 0, 10, 100 and 1000 mg/kg bw. The NOAEL for systemic toxicity was 100 mg/kg bw, based on transient decreased locomotor activity (total and/or ambulatory counts) at 1000 mg/kg bw. The NOAEL for neurotoxicity was 1000 mg/kg bw, the highest dose tested (Herberth, 2012a).

In a 90-day neurotoxicity study in rats given clethodim at a dietary concentration of 0, 500, 1500 and 5000 ppm (equal to 0, 31, 94 and 331 mg/kg bw per day for males, 0, 38, 115 and 380 mg/kg bw per day for females), the NOAEL for systemic toxicity was 1500 ppm (equal to 94 mg/kg bw per day) based on the treatment-related reductions in mean body weights, and food consumption in both sexes at 5000 ppm (equal to 331 mg/kg bw per day). The NOAEL for neurotoxicity was 5000 ppm (equal to 331 mg/kg bw per day), the highest dose tested (Herberth, 2012c).

The Meeting concluded that clethodim is not neurotoxic.

In a 28-day immunotoxicity study in female mice given clethodim at a dietary concentration of 0, 400, 2000 and 4000 ppm (equal to 0, 136, 603 and 1312 mg/kg bw per day), the NOAEL for immunotoxicity was 4000 ppm (equal to 1312 mg/kg bw per day), the highest dose tested (Burke, 2012b).

The Meeting concluded that clethodim is not immunotoxic.

In a mechanistic study to investigate liver microsomal cytochrome P450 induction, groups of eight male rats were given clethodim (purity 83.8%) by gavage at 0 or 250 mg/kg bw per day for 21 consecutive days. Results indicated that microsomal cytochrome P450 was not increased, indicating that the increased liver weight was not due to cytochrome P450 induction (Beatty, Brorby & Wong, 1989).

Toxicological data on metabolites and/or degradates

Metabolite clethodim imine sulfone (RE-47719)

Clethodim imine sulfone (5-(2-(ethylsulfinyl)propyl)-3-hydroxy-2-(1-iminopropyl) cyclohex-2-en-1-one) is a metabolite found in the leaves of soya bean, carrot and cotton, in rotational crops, and in goats.

The acute oral LD_{50} of clethodim imine sulfone in rats was greater than 1400 mg/kg bw (Dougherty, 1988b).

In a 35-day toxicity study in rats in which clethodim imine sulfone was administered at dietary concentrations of 0, 100, 1000 and 8000 ppm (equal to 0, 6.6, 70.9 and 604 mg/kg bw per day for males, 0, 7.8, 83.7 and 723 mg/kg bw per day for females), the NOAEL was 1000 ppm (equal to 70.9 mg/kg bw per day) based on increased reticulocytes, liver weights and higher serum cholesterol levels at 8000 ppm (equal to 604 mg/kg bw per day) (Bagos & Beatty, 1988a).

In a screening developmental toxicity study in which clethodim imine sulfone was administered to rats by gavage at dose levels of 0, 10, 100 and 700 mg/kg bw per day, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduction in body weight gain at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on reduced fetal weight and increased incidence of skeletal findings (increase in cervical ribs and decrease of sternebrae ossification sites) at 700 mg/kg bw per day (Hoberman, 1988a).

Clethodim imine sulfone was tested in a gene mutation assay in bacteria and an in vitro chromosomal aberration test. There was no evidence of genotoxicity (Machado, 1988; Putnam, 1988a).

The Meeting concluded that clethodim imine sulfone is of similar toxicity to clethodim, therefore is considered to be covered by the parent compound.

Metabolite clethodim imine sulfoxide

Clethodim imine sulfoxide (5-(2-(ethylsulfinyl)propyl)-3-hydroxy-2-(1-iminopropyl) cyclohex-2-en-1-one) is a metabolite in rats (up to 9% in urine), plants (soya bean, carrot, cotton leaves, rotational crops) and animals (goat).

No specific toxicological data are available, but the Meeting noted that clethodim imine sulfoxide has a similar structure to clethodim imine sulfone, and concluded that clethodim imine sulfoxide is of no greater toxicity than clethodim, therefore is considered to be covered by the parent compound.

Metabolite clethodim 5-OH sulfone (RE-51228)

Clethodim 5-OH sulfone, (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-5-(2-(ethylsulfonyl) propyl)-3,5-dihydroxycyclohex-2-en-1-one) is a metabolite in soya beans (seeds), carrots (roots), and also a minor rat metabolite (1% in urine).

The acute oral LD_{50} of clethodim 5-OH sulfone in rats was greater than 1400 mg/kg bw (Dougherty, 1988b).

In a 35-day toxicity study in rats in which clethodim 5-OH sulfone was administered at a dietary concentration of 0, 100, 1000 and 8000 ppm (equal to 0, 5.94, 67.7 and 588 mg/kg bw per day for males, 0, 6.43, 75.5 and 663 mg/kg bw per day for females), the NOAEL was 8000 ppm (equal to 588 mg/kg bw per day), the highest dose tested (Bagos & Beatty, 1988b).

In a screening developmental toxicity study in rats in which clethodim 5-OH sulfone by gavage at dose levels of 0, 10, 100 and 700 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on clinical signs at 700 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 700 mg/kg bw per day, the highest dose tested (Hoberman, 1988b).

Metabolite clethodim 5-OH sulfone was tested in a gene mutation assay in bacteria and an in vitro chromosomal aberration test. There was no evidence of genotoxicity (Machado, 1987).

The Meeting concluded that clethodim 5-OH sulfone is of lower toxicity than clethodim, and it would be covered by the parent compound.

Metabolite clethodim sulfone (RE-47253), free and conjugated

Clethodim sulfone (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-5-(2-(ethylsulfonyl) propyl)-3-hydroxycyclohex-2-en-1-one) is a metabolite in rats (≤ 1% in urine), spinach, carrot (outdoor, roots and leaves), soil, goats and hens. The conjugate form is a plant (soya bean) metabolite. The submitted studies were performed with the free form of the metabolite only.

Clethodim sulfone was tested for genotoxicity in an adequate range of in vitro and in vivo assays. Positive or equivocal results were observed in gene mutation assays in bacteria or mammalian cells and in an in vitro chromosomal aberration assay. However, negative results were observed with the same genotoxicity assays, using a purer batch of clethodim sulfone. Equivocal results were observed in an in vivo micronucleus test (Beevers, 2007a, b; Innes, 2003; Lloyd, 2009; Riach, 2003a; Stevenson, 2004a; Stone, 2009; Williams, 2008).

The Meeting concluded that a threshold of toxological concern (TTC) approach for genotoxicity could be applied to clethodim sulfone.

Metabolite clethodim oxazole sulfone (RE-47797)

Clethodim oxazole sulfone (2-ethyl-6-(2-(ethylsulfonyl)propyl)-6,7-dihydrobenzo[d]oxazol-4(5*H*)-one) is a soil and rotational crop metabolite.

Clethodim oxazole sulfone was tested for genotoxicity in an adequate range of in vitro and in vivo assays. Negative results were obtained in gene mutation assays in bacteria and mammalian cells. Positive results were obtained in an in vitro chromosomal aberration assay. Negative results were obtained in an in vivo micronucleus test (Beevers, 2007c; Hart & Stevenson, 2005; Riach, 2009b; Stevenson, 2004b).

For chronic toxicity of clethodim oxazole sulfone, the Meeting concluded that a TTC approach could be applied using Cramer class III.

Metabolite clethodim oxazole sulfoxide

Clethodim oxazole sulfoxide (2-ethyl-6-(2-(ethylsulfinyl)propyl)-6,7-dihydrobenzo[d]oxazol-4(5H)-one) is minor rat (≤ 3% in urine), soil and rotational crop metabolite.

No specific toxicological data are available, but the Meeting concluded that clethodim oxazole sulfoxide has a similar structure to clethodim oxazole sulfone. Therefore, for chronic toxicity, the Meeting concluded that a TTC approach could be applied using Cramer class III.

Metabolite DME sulfoxide acid (M17R)

Metabolite DME sulfoxide acid (3-[(2-ethylsulfinyl) propyl]-pentanedioic acid) is a crop metabolite found in spinach, carrot roots and foliage.

The acute oral LD_{50} of metabolite DME sulfoxide acid in rats was greater than 5000 mg/kg bw (Beerens-Heijnen, 2010a).

In a 28-day toxicity study in rats in which DME sulfoxide acid was administered at a dietary concentration of 0, 200, 1000 and 5000 ppm (equal to 0, 15, 80 and 396 mg/kg bw per day for males, 0, 16, 78, and 407 mg/kg bw per day for females), the NOAEL was 1000 ppm (equal to 80 mg/kg bw per day), based on thymus and adrenal weight changes in males at 5000 ppm (equal to 396 mg/kg bw per day) (Beerens-Heijnen, 2010b).

Metabolite DME sulfoxide acid was tested in a gene mutation assay in bacteria and an in vitro chromosomal aberration test (Buskens, 2010; Verspeek-Rip, 2009a). There was no evidence of genotoxicity.

The Meeting concluded that metabolite DME sulfoxide acid is of no greater toxicity than clethodim, and is therefore considered to be covered by the parent compound.

Metabolite DME sulfone acid (M18R)

DME sulfone acid (3-[(2-ethylsulfonyl) propyl]-pentanedioic acid) is a metabolite of crops (spinach, carrot roots and foliage).

The acute oral LD_{50} of metabolite DME sulfone acid in rats was greater than 5000 mg/kg bw (Beerens-Heijnen, 2010c).

DME sulfone acid was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Verspeek-Rip, 2009b).

The Meeting noted that metabolite DME sulfone acid (M18R) has a similar structure to metabolite DME sulfoxide acid (M17R), which is of no greater toxicity than clethodim, and concluded that metabolite DME sulfone acid (M18R) would be covered by the parent compound.

Metabolite M15R

M15R (hydroxy-3-[(2-ethylsulfinyl)propyl]-pentanedioic acid) is a plant metabolite (spinach, outdoor carrot roots).

No specific toxicological data are available, but the Meeting concluded that metabolite M15R has a similar structure to metabolite DME sulfoxide acid (M17R) and metabolite DME sulfone acid (M18R).

The Meeting concluded that metabolite M15R would be covered by the parent compound.

Metabolite clethodim sulfoxide (free and conjugated)

Clethodim sulfoxide (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-5-(2-(ethylsulfinyl) propyl)-3-hydroxycyclohex-2-en-1-one) is a major rat metabolite (up to 60% in urine), and a metabolite in plants (spinach, soya bean seeds, carrot roots and leaves, cotton seeds), soil and other animals (goat, hen).

No specific toxicological data are available, but the Meeting concluded that clethodim sulfoxide (free and conjugate) would be covered by toxicological studies on the parent.

Metabolite M19R

M19R (3-hydroxy-5-(2-hydroxypropyl)-2-(1-iminopropyl)cyclohex-2-en-1-one glucose conjugate) is a plant metabolite (carrots leaves).

No specific toxicological data are available. The Meeting concluded that a TTC approach for genotoxicity could be applied.

Metabolite M15A

M15A (3-chloroallyl alcohol glucoside) is a plant metabolite (spinach). No specific toxicological data are available. The Meeting noted that repeated-dose and in vitro and in vivo genotoxicity studies were available to another international authority, which had concluded that metabolite M15A appears to be more toxic than clethodim after repeated oral doses, and that no conclusion could be drawn as to its genotoxicity potential.

The Meeting concluded that a TTC approach for genotoxicity could be applied.

Metabolite *S*-methyl sulfoxide

S-methyl sulfoxide (2-((E)-1-((((E)-3-chloroallyl)oxy)imino)propyl)-3-hydroxy-5-(2-(methylsulfinyl) propyl)cyclohex-2-en-1-one) is a metabolite in rats (up to 11% in urine), rotational crops, and animals (goat).

No specific toxicological data are available, but the Meeting concluded that *S*-methyl sulfoxide would be covered by toxicological studies on the parent.

Human data

No information was provided on the health of workers involved in the manufacture or use of clethodim. No information on accidental or intentional poisoning in humans was available.

The Meeting concluded that the existing database on clethodim was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI for clethodim of 0–0.2 mg/kg bw, based on a NOAEL of 16 mg/kg bw per day on the basis of decreased body weight gain, decreased food intake in both sexes, marginally increased mortality in males and increased chronic pancreatitis in females, and using a safety factor of 100. The upper bound of this ADI provides a margin of about 565 times relative to the LOAEL for increased incidence of benign granulosa cell tumours in the ovary (2/63) of rats at the highest dose.

The Meeting concluded that it was not necessary to establish an ARfD for clethodim in view of its low acute oral toxicity and the absence of developmental toxicity or any other toxicological effects likely to be elicited by a single dose.

Levels relevant to risk assessment of clethodim

Species	Study	Effect	NOAEL	LOAEL
Mouse	Four-week toxicity[a] study	Toxicity	1500 ppm, equal to 179 mg/kg bw per day	4000 ppm, equal to 476 mg/kg bw per day
	18-months carcinogenicity[a] study	Toxicity	200 ppm, equivalent to 24 mg/kg bw per day	1000 ppm, equivalent to 119 mg/kg bw per day
		Carcinogenicity	3000 ppm, equivalent to 357 mg/kg bw per day[c]	-
Rat	Five-week toxicity[a] study	Toxicity	1000 ppm, equal to 65.6 mg/kg bw per day	4000 ppm, equal to 216 mg/kg bw per day
	13-week toxicity study[a]	Toxicity	500 ppm, equal to 25 mg/kg bw per day	2500 ppm, equal to 134 mg/kg bw per day
	Two-year toxicity and carcinogenicity[a] study	Toxicity	500 ppm, equal to 16 mg/kg bw per day	2500 ppm, equal to 86 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 21 mg/kg bw per day	2500 ppm, equal to 113 mg/kg bw per day
	Two-generation reproductive toxicity[a] study	Reproduction/ fertility	2500 ppm, equal to 163 mg/kg bw per day[c]	-
		Parental toxicity	500 ppm, equal to 32.2 mg/kg bw per day	2500 ppm, equal to 163 mg/kg bw per day
		Offspring toxicity	2500 ppm, equal to 163 mg/kg bw per day[c]	-
	Developmental toxicity[b] study	Maternal toxicity	83.3 mg/kg bw per day	292 mg/kg bw/day
		Fetal toxicity	83.3 mg/kg bw per day	292 mg/kg bw/day
Rabbit	Developmental toxicity[b] study	Maternal toxicity	20.8 mg/kg bw per day	250 mg/kg bw per day
		Fetal toxicity	250 mg/kg bw per day[c]	-
Dog	90-day toxicity[d] study	Toxicity	62 mg/kg bw per day (males)	104 mg/kg bw per day
	One-year toxicity[d] study	Toxicity	62 mg/kg bw per day (males)	250 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Clethodim imine sulfone (metabolite RE-47719)				
Rat	Five-week toxicity[a] study	Toxicity	1000 ppm, equal to 70.9 mg/kg bw per day	8000 ppm, equal to 604 mg/kg bw per day
	Developmental toxicity[b] study	Maternal toxicity	10 mg/kg bw per day	100 mg/kg bw per day
		Fetal toxicity	100 mg/kg bw per day	700 mg/kg bw per day
Clethodim 5-OH sulfone (metabolite RE-51228)				
Rat	Five-week toxicity[a] study	Toxicity	8000 ppm, equal to 588 mg/kg bw per day[c]	-
	Developmental toxicity[b] study	Maternal toxicity	100 mg/kg bw per day	700 mg/kg bw per day
		Fetal toxicity	700 mg/kg bw per day[c]	-
DME Sulfoxide acid (metabolite M17R)				
Rat	Five-week toxicity[a] study	Toxicity	1000 ppm, equal to 80 mg/kg bw per day	5000 ppm, equal to 396 mg/kg bw per day

[a] Dietary administration [b] Gavage administration [c] Highest dose tested [d] Capsule administration

Acceptable daily intake (ADI) for clethodim, clethodim sulfoxide (free and conjugated), 5-hydroxy sulfone, clethodim imine sulfoxide, clethodim imine sulfone, M15R, M17R, M18R and S-methyl sulfoxide, expressed as clethodim

0–0.2 mg/kg bw

Acute reference dose (ARfD) for clethodim, clethodim sulfoxide (free and conjugated), 5-hydroxy sulfone, clethodim imine sulfoxide, clethodim imine sulfone, M15R, M17R, M18R and S-methyl sulfoxide, expressed as clethodim

Not necessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to clethodim

Absorption, distribution, excretion, and metabolism in mammals	
Rate and extent of oral absorption	Approximately 90% based on urine, tissue, expired CO_2, cage wash and residual carcass within 168 h
Distribution	Widely (0.2–0.7% in tissues); highest residues in adrenals, liver and kidneys
Rate and extent of excretion	Urinary: 80–86% in 24 h; faecal 8.5–14% in 24 h
Potential for accumulation	No evidence for accumulation
Metabolism in mammals	Extensively metabolized; primary routes of metabolism are oxidation and subsequent hydroxylation or demethylation with subsequent oxidation
Toxicologically significant compounds (animals, plants, and the environment)	Clethodim, clethodim sulfoxide (free and conjugate), clethodim imine sulfone, clethodim imine sulfoxide, clethodim oxazole sulfoxide, clethodim oxazole sulfone, clethodim sulfone (free and conjugate), 5-hydroxy sulfone, M15R, DME sulfoxide acid (M17R), DME sulfone acid (M18R), M19R, M15A, *S*-methyl sulfoxide.

Acute toxicity	
Rat LD_{50} oral	1133 mg/kg bw
Rat LD_{50} dermal	> 4167 mg/kg bw
Rat LC_{50} inhalation	> 3.25 mg/L (4 h; whole body)
Rabbit, skin irritation	Mildly irritant
Rabbit, eye irritation	Mildly irritant
Guinea pig, skin sensitization	Sensitizing (Magnusson & Kligman test)
Short-term studies of toxicity	
Target/critical effect	Liver (increased weight and associated histopathological findings in all species, bone marrow and spleen in dog), red blood cells (rat, mouse, dog).
Lowest relevant oral NOAEL	25 mg/kg bw/day (90-day study in rat)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased body weight and food consumption, mortality in males and chronic pancreatitis in females (rat) Liver: increased weights and associated histopathological findings (rat, mouse) Lungs: increased incidence of multiple foci of amphophilic alveolar macrophages (mouse)
Lowest relevant oral NOAEL	16 mg/kg bw per day (two-year study in rat)
Carcinogenicity	Carcinogenic in female rats, but not in male rats or mice[a]
Genotoxicity	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Parental: decreased body weight and food consumption Offspring: no adverse effects Reproductive: no adverse effects
Lowest relevant parental NOAEL	32.2 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	163 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	163 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Maternal: clinical signs, decreased body weight and food consumption (rat, rabbit), increased mortality (rat) Developmental: reduced fetal weight and delayed ossification (rat)
Lowest relevant maternal NOAEL	20.8 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	83.3 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	1000 mg/kg bw, the highest dose tested (rat)
Subchronic neurotoxicity NOAEL	331 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	Not available
Immunotoxicity	1312 mg/kg bw per day, the highest dose tested (mice)

Mechanism studies	No in vivo evidence of liver cytochrome induction in male rats
Studies on metabolites or impurities	
Clethodim imine sulfone	Rat LD_{50} oral, > 1400 mg/kg bw No genotoxic potential (Ames, chromosome aberration in vitro) Subacute toxicity Oral NOAEL 70.9 mg/kg bw per day (rat) Developmental toxicity NOAEL maternal toxicity, 10 mg/kg bw per day (rat) NOAEL embryo/fetal toxicity, 100 mg/kg bw per day(rat) Not teratogenic.
Clethodim 5-OH sulfone	Rat LD_{50} oral, > 1400 mg/kg bw No genotoxic potential (Ames, chromosome aberration in vitro) Subacute toxicity Oral NOAEL 588 mg/kg bw per day (rat) highest dose tested Developmental toxicity, oral, rat: NOAEL maternal toxicity, 100 mg/kg bw per day NOAEL for embryo/fetal toxicity, 700 mg/kg bw per day Not teratogenic.
Clethodim oxazole sulfone	Genotoxicity: unlikely to be genotoxic (in vitro: negative Ames test, positive chromosome aberration, negative gene mutation; in vivo: negative (mouse micronucleus)
Clethodim sulfone	Genotoxicity: in vitro some positive results (some Ames tests, some chromosome aberration and some mammalian gene mutation), in vivo negative (mouse liver UDS), in vivo equivocal (mouse micronucleus)
DME sulfoxide acid M17R	LD_{50} oral > 5000 mg/kg bw (rat) Oral NOAEL, 80 mg/kg bw per day (rat) Ames test: negative
DME sulfone acid M18R	LD_{50} oral > 5000 mg/kg bw (rat) Ames test: negative
Human data	No data

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI[a]	0–0.2 mg/kg bw[a]	Two-year toxicity and carcinogenicity (rat)	100
ARfD	Not necessary		

[a] Applies to clethodim, clethodim sulfoxide (free and conjugated), 5-hydroxy sulfone, clethodim imine sulfoxide, clethodim imine sulfone, M15R, M17R, M18R and *S*-methyl sulfoxide)

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DIMETHOATE / OMETHOATE

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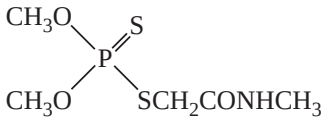
Explanation

Dimethoate (Fig. 1) is the ISO-approved common name for *O*,*O*-dimethyl-*S*-((methylcarbamoyl) methyl) phosphorodithioate-2-dimethoxyphosphinothioylsulfanyl-*N*-methylacetamide (IUPAC), with the Chemical Abstract Service number 60-51-5.

Figure 1 Chemical structure of dimethoate



Dimethoate is an organophosphate insecticide, having contact and systemic action, against a broad range of insects in agriculture and also used for the control of the housefly. It acts by inhibiting acetylcholinesterase (AChE).

Dimethoxon (also known as omethoate), the oxygen analogue metabolite of dimethoate, appears to play a dominant role in its toxicity for insects and mammals.

Dimethoate was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues in 1963, 1965, 1967, 1984, 1987 and 1996. An acceptable daily intake (ADI) of 0–0.002 mg/kg bw was established in 1996 on the basis of the apparent [sic] no-observed-adverse-effect level (NOAEL) of 1.2 mg/kg bw per day for reproductive performance in a study of reproductive toxicity in rats, and with a safety factor of 500. Although a safety factor of 100 would have normally been used in deriving an ADI from a study of this type, the Meeting was concerned about the possibility that reproductive performance might have been affected at 1.2 mg/kg bw per day in this study and therefore used a safety factor that was higher than normal. No data were available to assess whether the effects on reproductive performance were secondary to the inhibition of AChE activity. The 1996 JMPR concluded that it was not appropriate to base the ADI on the results of studies of volunteers, since the crucial end-point (reproductive performance) had not been assessed in humans.

The 2003 Meeting established an acute reference dose (ARfD) of 0.02 mg/kg bw on the basis of the overall NOAEL of 2 mg/kg bw for AChE inhibition in studies in rats, using a safety factor of 100. This ARfD was consistent with the NOAEL of about 0.2 mg/kg bw per day in studies on volunteers receiving single or repeated doses, which were evaluated by the 1996 JMPR.

Dimethoate was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues (CCPR).

A number of newly submitted studies, published and unpublished, covering a range of end-points, not available to the 1996/2003 Meetings, together with previous studies, were evaluated by the present Meeting. A literature search was performed which did not provide any further useful information for the evaluation of the compound.

Unless otherwise specified, critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines.

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Male and female Wistar rats, aged 7–10 weeks with body weights 195–224 g were used for the studies. Animals were provided with food and tap water ad libitum. ^{14}C-dimethoate (radiochemical purity > 98%), labelled at the methyl groups, was administered either:

- orally in water in a volume of about 2.5–5 mL/kg bw,
- intravenously, in isotonic saline (0.9% w/v aqueous sodium chloride solution) in a volume of approximately 5 mL/kg bw, or
- dermally, as a suspension in 1% w/v aqueous sodium carboxymethylcellulose in a volume of approximately 1 mL/kg bw (0.2 mL/rat).

Dose levels were selected on the basis of a series of dose range-finding preliminary studies, using non-radiolabelled dimethoate. A single oral dose of 10 mg/kg bw as a suitable no-observed-effect level for the 'low' dosage, and 100 mg/kg bw as a suitable lowest-observed-effect level (body tremors) for the 'high' dosage were deduced from those studies. For the purposes of comparison, 10 mg/kg bw was also selected for intravenous (i.v.) administration. For single dermal administration, the highest dose level investigated in the dose range-finding study due to technical reasons was 250 mg/kg bw, at which no toxic effects were noted. Since a high dose causing slightly toxic effects could not be achieved, dose levels of 100 and 10 mg/kg bw were also used for dermal administration to facilitate comparison with oral administration.

Dermal exposure was over 6 h, thereafter, the application site was decontaminated by washing with lukewarm soapy water and lukewarm water. In one of the excretion balance studies, rats received 10 mg/kg bw of unlabelled dimethoate daily by oral administration for 14 consecutive days prior to administration of a single oral dose of 10 mg/kg bw labelled with ^{14}C at dimethoate's *O*-methyl groups.

Studies to determine excretion balance (five rats/sex per group), plasma kinetics (nine rats/sex per group), biliary excretion (three rats/sex per group) and tissue distribution (nine rats/sex per group) were carried out. In the excretion studies, animals were individually housed in glass metabolism cages for the period from 24 h before dosing to sacrifice. Urine and faeces were collected from each animal separately and expired air was trapped in 2-ethoxyethanol/ethanolamine (3:1 mix). Dermally-dosed rats were housed in restraining cages for the 6 h exposure, then transferred to metabolism cages. Bile was collected by bile duct cannulation and these animals were housed in restraining cages. In the other experiments (plasmakinetic and tissue distribution) animals were housed in groups of the same sex in stainless steel battery cages. Rats were sacrificed at different intervals up to five days after dosing for tissue collection and for samples of muscle, fat, bone, bone marrow, skin and remaining carcass. After sacrifice, distribution of radioactivity in rats was investigated also by whole body autoradiography. Blood was taken from the tail vein at distinct intervals after dosing and collected in heparinized tubes and the plasma separated to determine plasma kinetics. Radioactivity in urine, bile, volatiles trapping solutions and plasma was assayed by liquid scintillation counting (LSC). Aliquots of faecal extracts, gastrointestinal tract (GIT), liver, spleen and blood were combusted prior to radioassay. Other organs/tissues were solubilized prior to radioassay. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used for metabolite separations and identification by comparison with reference compounds. Confirmatory identification was demonstrated by mass spectrometry.

Oral absorption was considered to be essentially complete (90% or greater). Excretion of administered radioactive dose was rapid with most of the applied dose eliminated within 6 h after i.v. dosing with 10 mg/kg bw and within 12 h and 24 h after oral dosing with 10 and 100 mg/kg bw, respectively. The main route of excretion was via urine. After oral or i.v. dosing 52–72% of the applied dose was excreted within 6 h in urine, 80–90% within 24 h and 85–91% after five days. Regarding minor routes of excretion, 1.2–1.6% and 1.6–2.5% of the applied dose were eliminated after five days in faeces and expired air, respectively (Table 1). Pretreatment with unlabelled dimethoate for 14 days did not affect the pattern and the rate of excretion. There were no differences between sexes. Experiments in rats with cannulated bile ducts following oral administration of 10 or 100 mg/kg bw ^{14}C-dimethoate indicated that bile was a minor route of excretion, with 3.5–4.5% of the dose excreted in bile after 48 h at both dose levels. Most of the radioactivity secreted in bile was subsequently reabsorbed in the gut (entero-hepatic recirculation), as shown by comparison with the proportion of the dose excreted in faeces in the previous experiment without bile duct cannulation, while the majority of the dose was excreted in urine.

More than 98% of an oral or i.v. dose of dimethoate was excreted within five days.

In dermally treated animals 9–10% and 1–2% of applied dose was absorbed after treatment with 10 and 100 mg/kg bw, respectively (Table 1). Absorption of radioactivity was about 1 mg/kg bw equivalents, that is about 0.02 mg/cm^2. The absorbed radioactivity was efficiently excreted mainly in urine. The unabsorbed radioactivity was mainly recovered in the 6 h skin wash. The radioactivity remaining in the treated skin had apparently not entered systemic circulation within five days after treatment.

Table 1. Absorption and excretion determined at five days after oral, dermal or intravenous administration of dimethoate. All values are % of applied dose.

Dose	10 mg/kg bw		10 mg/kg bw [a]		100 mg/kg bw	
Sex	**males**	**females**	**males**	**females**	**males**	**females**
Oral administration						
Urine	91.3	85.4	90.6	88.9	90.8	90.8
Expired air [b]	2.10	2.17	2.07	2.28	2.44	2.53
Faeces	1.15	1.56	1.30	1.22	1.45	1.45
Total excreted	94.6	89.1	94.0	92.4	94.7	94.8
Carcass	0.67	1.45	1.04	1.72	1.14	1.88
Recovery	95.3	90.6	95.0	94.1	95.9	96.6
Intravenous administration						
Urine	88.9	89.4	-	-	-	-
Expired air [b]	1.76	1.62	-	-	-	-
Faeces	1.15	1.35	-	-	-	-
Total excreted	91.8	92.4	-	-	-	-
Carcass	0.81	0.96	-	-	-	-
Recovery	92.6	93.3	-	-	-	-
Dermal application						
Urine	8.27	9.26	-	-	1.13	1.32
Faeces	0.21	0.58	-	-	0.05	0.13
Carcass	0.76	0.76	-	-	0.11	0.22
Skin wash (6 h after dosing)	62.5	62.1	-	-	84.1	83.7
Dressing	3.42	3.16	-	-	1.35	3.64
Treated skin	17.3	13.3	-	-	3.65	2.18
Recovery	92.5	89.1	-	-	90.2	91.1
Total absorbed	9.19	10.6	-	-	1.18	1.64

[a] Repeated oral administration of dimethoate at 10 mg kg bw per day for 14 consecutive days prior administration of radioactive dose

[b] Determined up to three days after dose administration

Source: Kirkpatrick, 1995

The highest concentrations of radioactivity were found in liver and kidney with highest concentrations in almost all tissues in animals sacrificed 0.5 h after dosing (Table 2). The maximum tissue levels in liver and kidney after a 10 mg/kg bw single oral dose were 8.6–11.7 and 20.0–24.6 mg dimethoate equivalent/kg bw, respectively. Concentrations in other tissues were much lower at this time (1–6 mg dimethoate equivalent/kg bw) with the lowest mean maximum concentrations occurring in brain and fat (about 1 mg dimethoate equivalent/kg bw). Concentrations declined with time and at 48 h were less than 1 mg dimethoate equivalent/kg bw in all tissues. After an oral dose of 100 mg/kg bw, the peak tissue concentrations were about 5–18 times higher, when compared with levels after administration of 10 mg/kg bw (see Tables 2–4 on the following pages).

Table 2. Mean tissue concentrations of radioactivity in rats sacrificed 0.5 h following oral single and repeated application. All values expressed as mg dimethoate equiv./kg tissue.

Application	Single		Repeated [a]		Single	
Dose	**10 mg/kg bw**		**10 mg/kg bw**		**100 mg/kg bw**	
Sex	**males**	**females**	**males**	**females**	**males**	**females**
Adrenal glands	2.22	4.23	3.70	6.34	0.74	0.59
Bone	1.12	1.39	1.84	2.27	0.21	0.21
Bone marrow	1.61	3.04	2.83	4.29	0.37	0.39
Brain	0.65	1.36	1.48	2.25	0.21	0.25
Fat	0.99	1.10	1.56	1.59	1.07	0.15
Heart	2.60	3.47	3.66	4.70	0.46	0.47
Intestines and contents	6.20	6.71	9.39	10.8	0.89	1.30
Kidneys	20.0	24.6	21.0	28.1	2.89	2.22
Liver	8.57	11.7	12.8	13.2	1.37	1.03
Lungs	3.28	5.47	4.94	7.00	0.59	0.62
Muscle	1.19	1.89	1.94	2.89	0.26	0.28
Ovaries	-	3.77	-	5.13	-	0.51
Pancreas	2.94	4.33	5.36	7.43	0.55	0.48
Skin	2.24	3.56	3.09	4.89	0.41	0.49
Spleen	1.76	2.93	3.04	4.41	0.35	0.38
Stomach and contents	146	178	250	240	22.8	38.1
Testes	1.72	-	2.62	-	0.40	-
Thyroid gland	1.99	3.31	3.48	4.63	0.49	0.40
Uterus	-	3.52	-	5.10	-	0.51
Whole blood	4.70	6.08	5.70	7.47	0.71	0.70
Plasma	6.09	7.81	6.54	8.96	1.00	1.00

[a] Rats received 14 oral daily doses of ^{14}C-dimethoate at a nominal level of 10 mg/kg bw per day

Source: Kirkpatrick, 1995

Repeated oral administration of dimethoate (10 mg/kg bw per day) showed similar results when compared to single administration of this dose, with tissue levels approximately 1.5–2 times higher than those after a single application of 10 mg/kg bw. No evidence of accumulation was observed following repeated oral administration (Tables 3 and 4).

Single i.v. administration resulted in similar tissue concentrations of radioactivity as with a single oral application of the same dose. Dermal application of 10 or 100 mg/kg bw showed lower levels in all tissues in general when compared to other routes of application (Table 4).

The results of qualitative tissue distribution by whole-body autoradiography were generally in agreement with those from quantitative experiments. There was some evidence for accumulation of radioactivity in the Harderian gland, the intraorbital and exorbital lachrymal glands and the preputial gland, whereas the central nervous system contained the lowest concentrations of radioactivity.

Table 3. Mean quantities of radioactivity following a single oral dose of dimethoate at 10 and 100 mg/kg bw. All values expressed as % of applied dose.

Sacrifice time	0.5 h		2 h		48 h	
Sex	males	females	males	females	males	females
10 mg/kg bw						
Gastrointestinal tract	54.2	46.8	28.1	14.6	0.18	0.67
Kidneys	1.70	1.87	0.58	0.61	0.03	0.03
Liver	4.25	5.46	2.96	3.41	0.31	0.29
Whole blood	3.29	4.29	1.14	1.46	0.08	0.09
Plasma	2.44	3.15	0.88	1.06	0.04	0.05
Other tissues	0.68	0.83	0.41	0.40	0.04	0.03
Residual carcass	11.9	17.9	8.18	10.7	0.90	3.32
100 mg/kg bw						
Gastrointestinal tract	58.2	53.1	43.0	27.1	0.20	0.28
Kidneys	1.29	0.91	1.59	0.35	0.02	0.02
Liver	3.65	2.64	1.39	1.60	0.24	0.20
Whole blood	2.77	2.77	0.50	0.88	0.09	0.10
Plasma	2.26	2.27	0.40	0.71	0.04	0.05
Other tissues	0.74	0.63	0.35	0.32	0.06	0.05
Residual carcass	12.2	13.0	5.22	8.83	1.16	1.87

Source: Kirkpatrick, 1995

Table 4. Mean concentrations of radioactivity in rats sacrificed 120 h following oral (single and repeated), dermal and intravenous application. All values expressed as mg dimethoate equiv./kg .

Application	Oral						i.v.		Dermal			
Dose	10 mg/kg bw		10 mg/kg bw [a]		100 mg/kg bw		10 mg/kg bw		10 mg/kg bw		100 mg/kg bw	
Sex	m	f	m	f	m	f	m	f	m	f	m	f
Adrenal glands	0.11	0.12	0.20	0.24	2.03	2.01	0.11	0.11	< 0.09	< 0.03	< 0.31	< 0.16
Bone	0.07	0.07	0.05	0.05	0.41	0.37	0.03	0.02	< 0.02	0.02	< 0.10	< 0.10
Bone-marrow	0.07	0.10	0.20	0.16	2.97	1.64	0.06	0.07	< 0.05	< 0.07	< 0.59	< 0.63
Brain	0.04	0.05	0.06	0.07	0.41	0.50	0.03	0.03	< 0.01	0.01	< 0.06	0.06
Fat	0.06	0.08	0.03	0.04	0.72	0.48	0.04	0.03	< 0.02	< 0.02	< 0.09	< 0.10
Heart	0.08	0.10	0.11	0.13	0.83	1.07	0.06	0.07	< 0.01	< 0.01	< 0.07	< 0.08
Intestines & contents	0.04	0.07	0.07	0.10	0.47	0.70	0.03	0.07	0.01	0.03	< 0.07	0.13
Kidneys	0.15	0.19	0.26	0.26	1.60	1.75	0.13	0.13	0.01	0.02	< 0.07	< 0.07
Liver	0.24	0.28	0.34	0.34	2.16	2.11	0.20	0.20	0.02	0.03	< 0.07	0.08
Lungs	0.10	0.14	0.15	0.17	0.91	1.19	0.08	0.09	0.01	0.02	< 0.07	< 0.07
Muscle	0.06	0.07	0.08	0.08	0.65	0.68	0.05	0.05	0.01	0.02	< 0.05	< 0.05
Ovaries	-	0.08	-	0.14	-	1.03	-	0.07	-	< 0.02	-	< 0.10
Pancreas	0.19	0.26	0.19	0.37	5.15	6.63	0.17	0.29	0.02	0.03	< 0.07	0.08
Skin	0.09	0.11	0.11	0.44	0.77	0.82	0.07	0.06	0.02	0.12	0.20	0.30
Spleen	0.08	0.10	0.14	0.16	0.87	1.17	0.06	0.07	< 0.02	< 0.02	< 0.08	< 0.09
Stomach & contents	0.03	0.09	0.14	0.14	0.30	0.49	0.03	0.07	< 0.02	0.04	< 0.08	< 0.08
Testes	0.06	-	0.10	-	0.63	-	0.05	-	< 0.01	-	< 0.05	-
Thyroid gland	< 0.14	0.13	0.18	0.23	5.18	1.99	< 0.12	< 0.11	< 0.15	< 0.14	< 0.88	< 0.74
Uterus	-	0.11	-	0.12	-	0.76	-	0.06	-	< 0.02	-	< 0.12
Whole blood	0.06	0.09	0.11	0.14	0.79	1.19	0.06	0.07	0.01	0.01	< 0.03	< 0.03
Plasma	0.03	0.04	0.06	0.07	0.36	0.52	0.03	0.04	0.01	0.01	< 0.02	< 0.02

[a] Rats received 10 mg/kg bw/day by oral administration once daily for 14 consecutive days prior to administration of a single oral 10 mg/kg bw dose of radiolabelled dimethoate

Source: Kirkpatrick, 1995

Peak plasma concentrations for both dose levels were reached quickly within 0.25–0.5 h, and thereafter, concentrations declined biphasically with an initial half-life of < 3 h and a terminal half-life of 36–59 h (Table 5).

At 100 mg/kg bw, mean peak plasma radioactivity concentrations and areas under plasma radioactivity against time curves (AUCs) were higher in female rats, such that AUCs were about 8 times greater in males and 14 times greater in females, respectively, when compared to those after a 10 mg/kg bw administration. Only after a 100 mg/kg bw application dimethoate was detectable in plasma for up to 6 h after administration, whereas after 24 h dimethoate was not detectable (< 0.05 mg/L).

Table 5. Toxicokinetic parameters of plasma radioactivity

	10 mg/kg bw		100 mg/kg bw	
	male	**female**	**male**	**female**
Maximum concentration, C_{max} (mg equiv./L)	8.62	7.68	50.7	93.2
Time to reach C_{max} T_{max} (h)	0.5	0.5	0.25	0.5
AUC (mg equiv. h/L)	49.4	48.9	417.0	686.6
Half-life, $t_{½}$ (h)	42.0	59.3	36.1	46.4

Source: Kirkpatrick, 1995

In summary, oral absorption of dimethoate was extensive (≥ 90%). Excretion of radioactivity was rapid, with 52–72% of the dose excreted in urine within the first 6 h, and 80–90% by 24 h. In general, concentrations in tissues were highest at 0.5 h after dosing, though maximum concentrations were occasionally reached at 2 h in males. In all cases, concentrations in tissues declined rapidly after reaching the maximum concentration, with only low levels present at 48 h after dosing. Highest concentrations were found in kidneys and liver, with low levels in brain and fat. There was clear evidence of radioactivity in the bone marrow following oral dosing. The results of this study do not indicate any potential for accumulation. The results of whole-body autoradiography experiments (one rat/sex at five time points) were generally in agreement with the quantitative experiments. Highest concentrations of radioactivity were present in the contents of the gastrointestinal GI tract, liver and kidneys; the central nervous system contained the lowest concentrations. (Kirkpatrick, 1995).

1.2. Biotransformation

Oral, intravenous and dermal administration resulted in comparable metabolite profiles. At least eight metabolites and unchanged dimethoate were isolated in urine with two metabolites representing most of the urinary excreted radioactivity over 48 h (Table.6). These metabolites were identified as dimethyl dithiophosphate and dimethoate carboxylic acid. Dimethyl dithiophosphate represented between 20% and 30% of the oral or intravenous applied dose, while dimethoate carboxylic acid represented 29–46% of the dose. Further metabolites representing 4–11% and 1–5.5% of oral and intravenous dose were identified as dimethyl thiophosphoric acid and omethoate, respectively. Incubation of urine samples with β-glucuronidase and sulfatase showed that conjugation with glucuronic acid and sulfate had not occurred. Parent dimethoate in urine accounted for only 1% and 2% of oral and i.v. dose. About 1% of the absorbed dose in dermally treated rats was excreted as the parent compound.

Dimethyl dithiophosphate was also a major metabolite in liver (9–22% radioactivity, 0.4–1% of applied dose) and kidney (40–41% radioactivity, 0.7–0.8% of applied dose).

Biliary, plasma, tissue and urinary metabolites that were identified, accounted for 70–80% of the orally or i.v. administered dose. Biotransformation of dimethoate proceeded mainly by cleavage of the C–N bond to yield initially dimethoate carboxylic acid, and subsequently, thiophosphate and phosphate esters, and by oxidative metabolism dimethoate's oxon analogue, omethoate. A minor pathway resulted in loss of the methoxy groups yielding carbon dioxide.

Table 6. Identified metabolites in urine following oral, intravenous and dermal application. Values are expressed as % of applied dose. Additionally for dermal route, results are expressed as % of dose absorbed (values given in brackets).

Application	**Oral**						**i.v.**		**Dermal**			
Dose (mg/kg bw)	**10**		**10***		**100**		**10**		**10**		**100**	
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Omethoate	1.5	2.5	3.2	5.6	3.7	3.7	1.3	1.8	0.2 (1.9)	0.2 (1.9)	< 0.1 (< 6.4)	< 0.1 (1.8)
Dimethyl thiophosphoric acid	8.3	5.7	10.8	7.3	8.7	4.7	6.5	4.0	0.8 (8.3)	06 (5.6)	0.1 (6.0)	0.1 (5.6)
Dimethyl dithiophosphate	26.6	25.2	29.7	27.4	20.3	22.1	22.5	24.1	2.9 (31.5	3.2 (30.3)	0.3 (23.7)	0.4 (24.0)
dimethoate carboxylic acid	37.8	35.1	29.1	31.0	43.2	44.4	42.7	45.7	2.5 (27.2)	2.8 (26.7)	0.3 (25.1)	0.3 (19.2)
dimethoate	1.4	0.7	0.9	0.6	0.7	2.0	0.4	1.7	0.1 (1.3)	0.1 (0.8)	< 0.1 (0.6)	< 0.1 (0.9)

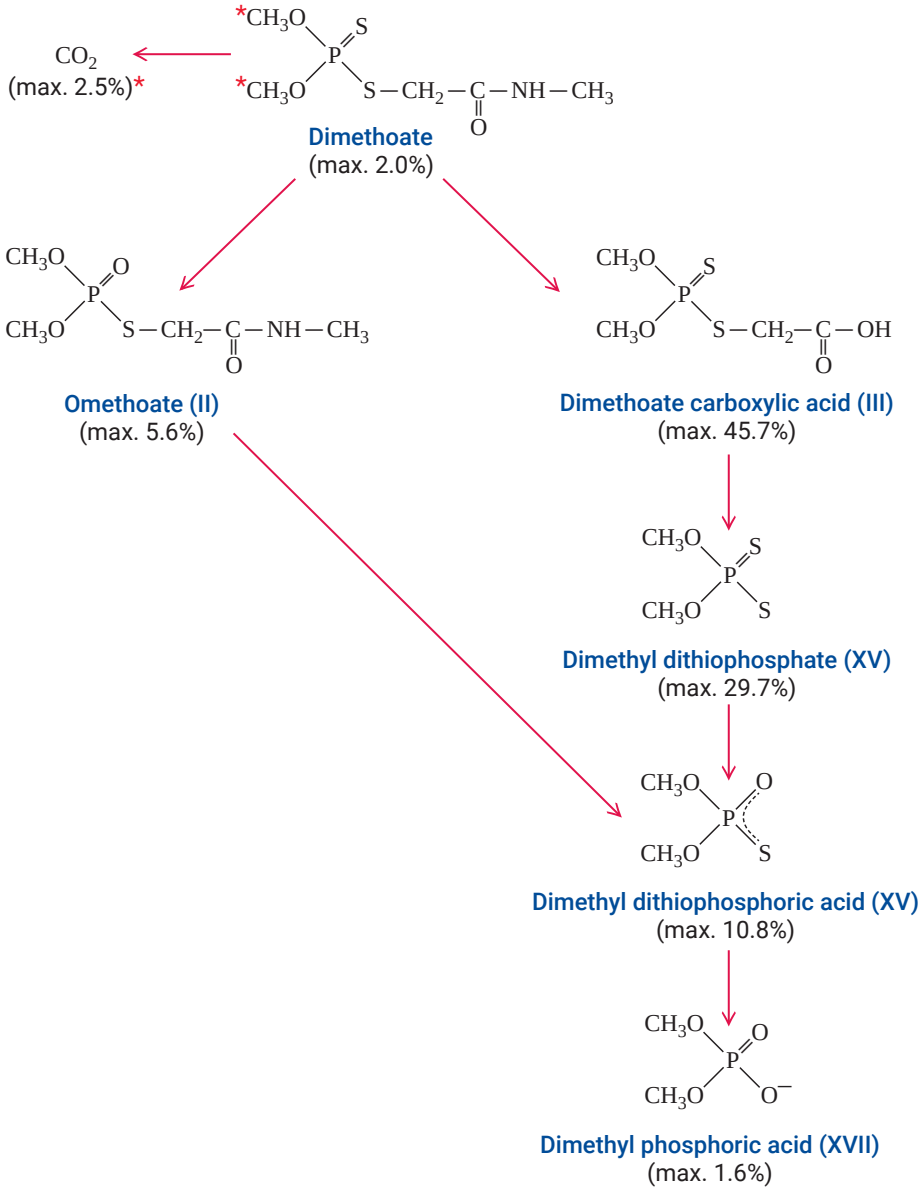
* Rats received 10 mg/kg bw per day by oral administration once daily for 14 consecutive days prior to administration of a single oral 10 mg/kg bw dose of radiolabelled dimethoate

FromSource: Kirkpatrick, 1995

In summary, dimethoate is rapidly and quantitatively absorbed, extensively metabolised and quickly eliminated independent of the route of application. The predominant route of excretion is within the urine. The compound does not accumulate in tissues. There were no qualitative differences in the metabolite profiles after dosing by different routes (oral, i.v. or dermal) or at different dose levels, and there were no significant differences in the proportions of the various metabolites between the sexes.

Dimethoate is mainly metabolised via initial cleavage of the C–N bond yielding dimethoate carboxylic acid, dithiophosphate, thiophosphate and phosphate esters, while a subordinate biotransformation pathway is oxidation to the oxygen analogue, omethoate (Kirkpatrick, 1995).

Figure 2. Scheme of proposed biotransformation pathway for dimethoate in rats



(*Redrawn from Kirkpatrick, 1995.*)

2. Toxicological studies on dimethoate

2.1 Acute toxicity

A summary of all essential data concerning acute toxicity of dimethoate is presented in Table 7.

Table 7. Summary of acute toxicity studies with dimethoate

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L air)	Reference
Mouse	Albino	F	Oral	NR	60	Sanderson & Edson, 1964
Mouse	KMF-NMRI	M + F	Oral	97.3	160	Ullman et al., 1985
Rat	Sprague Dawley	M + F	Oral	96–98	387	Kynoch,1986a
		M	Oral	96–98	358	Kynoch, 1986a
		F	Oral	96–98	414	Kynoch, 1986a
Rat	Albino	M	Oral	> 95	245	Shaffer, 1957
Rat	Sprague Dawley	M + F	Dermal	96–98	> 2000	Kynoch, 1986b
Rat	Albino Wistar	M + F	Inhalation	NR	"Chemathoate 60%" at 2.8 Dimethoate: at 1.68[§]	Jackson et al., 1986

LC_{50} Median lethal concentration; LD_{50} Median lethal dose

[§] Mass median aerodynamic diameter (MMAD) of < 5.5 μm

NR Not reported

Notes on studies in Table 7

Kynoch, 1986a

Dimethoate was orally administered via gastric intubation to five groups of 5 male and 5 female Sprague Dawley rats (weight range 96–150 g prior to dosing; 4–6 weeks old) at dosage levels of 250, 320, 400, 500 and 640 mg/kg bw. Mortality occurred at 400 mg/kg bw and above between 4 h and 46 h. Clinical signs of toxicity were seen at all doses and included piloerection, hunched posture, abnormal gait, lethargy and reduced respiratory rate. Increased salivation and body tremors were seen in rats at 320 mg/kg bw. Recovery as judged by external appearance and behaviour was apparently complete by day 3–7. No changes due to treatment were apparent upon postmortem examination.

Shaffer, 1957

A poorly reported (non-GLP, non-guideline) study. Dimethoate (as a 4% solution in corn oil) was administered to groups of five male albino rats by gavage at dose levels of 100, 200, 400 or 800 mg/kg bw. One death occurred at 200 mg/kg bw; all animals died at 400 and 800 mg/kg bw, largely on the day of administration.

Ullmann, 1985

Dimethoate was administered by oral gavage to six groups of 5 male and 5 female KFM-NMRI mice (6–9 weeks old at start of treatment) at dosage levels of 10, 20, 40, 80, 160 and 320 mg/kg bw. Mortality was observed at dose levels of 160 and 320 mg/kg bw. For mice treated with 10 mg/kg bw no clinical symptoms were observed. For treatment groups 20, 40 and 80 mg/kg bw, clinical signs included sedation, dyspnea, hunched posture, ruffled fur and reddish discharge, with spasms also in the 80 mg/kg bw test group. At 160 and 320 mg/kg bw additional clinical signs including ventral body position, coma, and tremors were seen. Mice treated with 20, 40 and 80 mg/kg bw had recovered within 2–6 observation days. The surviving mice from test groups on 160 and 320 mg/kg bw had also recovered within 2–6 observation days. No changes in body weight gain were recorded in any test group. In surviving mice no pathologic changes were observed at necropsy. In mice found dead during the observation period, changes mainly in lungs, liver, stomach/intestines were observed in all animals except one at 160 mg/kg bw. Mortalities occurred between 3 h and 48 h of dosing.

Kynoch, 1986b

Dimethoate was applied dermally to the shaved intact skin of five male and five female CD rats of a Sprague Dawley-derived strain (weight range 205–251 g prior to dosing; 6–8 weeks old) at a dose level of 2000 mg/kg bw. After application of the test substance the test sites were covered with semi-occlusive gauze bandaging held in place with a dressing. After 24 h, the dressings were removed and the skin was washed with water. None of the rats died. No signs due to treatment were seen in male rats. Hunched posture, body tremor and abnormal gait was noted in females on days 8 and 9. Recovery was complete by day 13. No signs of dermal irritation were noted. A body weight loss was recorded for two female rats on days 8 and 2 respectively; additional female rats showed very low body weight gains at this time. However, normal body weight gains were recorded for all rats on day 15. There were no significant external or internal changes at the terminal postmortem macroscopical examination.

Jackson, 1986

Wistar rats (5/sex per group) were exposed (whole body) for 4 h to atmospheres containing liquid aerosols of the test substance 'Chemathoate 60%', a manufacturing concentrate containing dimethoate (616 g/L) and cyclohexanone (499 g/L), at the concentrations 0, 0.9, 1.2, 1.3, 1.9 and 2.8 mg/L, and observed for 14 days. Deaths occurred in all treated groups, The majority of deaths at the highest concentration occurred during the exposure period. Signs of toxicity (dyspnoea, abnormal posture, ptosis, salivation and lacrimation) were observed in the majority of treated rats during the exposure period. These signs are stated by the report author to be consistent with exposure to an irritant aerosol, however some of the signs are equally characteristic of anticholinesterase activity. Signs of toxicity immediately following exposure included abnormal breathing, tremors, lacrimation and salivation number of animals were stated to be immobile and/or unresponsive to stimuli. Signs of toxicity persisted for several days. The single surviving rat at the highest concentration lost weight over the study period. Reduced weight gain or weight loss were seen in other treated groups over the week following exposure; normal weight gains were seen during the second week of the study. Food and water consumption were reduced in all treated groups following exposure. Relative lung weights were generally higher in all treated groups, most notably in decedents. Gross necropsy revealed pulmonary congestion in a number of treated rats and corneal opacity in males exposed to the highest concentration. Histopathology showed pulmonary congestion/oedema in decedents at 1.9 and 2.8 mg/L.The LC_{50} was calculated as 1.68 mg/L of air.

A summary of all essential data concerning dermal and eye irritation as well as skin sensitization is presented in Table 8.

Table 8. Summary of dermal and eye irritation and skin sensitization studies with dimethoate

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
Rabbit	New Zealand White	M + F	Skin irritation	96–98	Very slightly irritating	Liggett & Parcell, 1985a
Rabbit	New Zealand White	M + F	Eye irritation	96–98	Mild irritating	Liggett & Parcell, 1985b
Guinea pig	Hsd Poc.	F	Skin sensitization	99.4	Not sensitizing (Magnusson & Kligman test)	Albrecht, 2008
Guinea pig	Hartley	M	Skin sensitization	NS	Not sensitizing (Buehler test)	Madison, 1984

NS Not specified

2.2 Short-term studies of toxicity

(a) Oral administration

Short-term studies of oral toxicity in rats and dogs were conducted.

Rat

Study 1

Dimethoate (batch number 82/326; purity 97.3%) was administered to eight male and eight female Wistar rats per group (35 days old; mean weights: male 130.3 g, female 119.1 g) over a period of four weeks via the feed. Dose levels were 0 (control), 5, 25 and 75 ppm (equivalent to 0, 0.5, 2.5 and 7.5 mg/kg bw per day).

The animals were observed for mortality and clinical symptoms daily; body weight and food intake was measured weekly. A physical examination was performed weekly. Clinical chemistry and haematological examinations, but no urinalyses, were performed on all rats at termination. Acetylcholinesterase (AChE) activity (plasma and erythrocyte at days 8, 15, 22 and 28; brain at termination) was determined by the methods of Ellman et al. (1961) for plasma and brain or Augustinsson, Eriksson & Faijersson (1978) for erythrocytes. Blood was taken from nonfasted rats between 07.00 and noon. *S*-acetylthiocholine iodide was the substrate for cholinesterase (ChE) measurement. After study termination a complete gross necropsy was conducted, liver, spleen and thymus weights being determined. Histopathological examinations of 11 organs and of all gross lesions were conducted.

There were no mortalities and no clinical signs of toxicity due to dimethoate. A slight decrease in body weight gain was seen in male rats at 7.5 mg/kg bw per day in week 2. There were no effects due to dimethoate on haematology, gross pathology or on organ weights. Plasma ChE activity was decreased significantly in male rats at 2.5 and 7.5 mg/kg bw per day and in females at 7.5 mg/kg bw per day. Erythrocyte ChE activity was decreased significantly in male and female rats at 2.5 and 7.5 mg/kg bw per day. Brain ChE activity was decreased significantly in male and female rats at 2.5 and 7.5 mg/kg bw per day and slightly, but significantly, in female rats at 0.5 mg/kg bw per day (Table 9**)**. However, the marginal effects at 0.5 mg/kg bw per day are not regarded as biologically relevant.

Table 9. Influence of dimethoate on acetylcholinesterase activity (µKat/L or µKat/g) of rats after four weeks of treatment (percentages related to control shown in brackets)

Test group	PChE		EChE		BChE	
	Male	Female	Male	Female	Male	Female
Dose						
0 mg/kg bw per day	11.1 (100)	32.5 (100)	20.3 (100)	24.6 (100)	0.85 (100)	0.96 (100)
0.5 mg/kg bw per day	9.3 (84)	36.7 (113)	19.8 (98)	20.9 (85)	0.79 (93)	0.81** (84)
2.5 mg/kg bw per day	6.8** (61)	33.5 (103)	12.8** (63)	14.1** (57)	0.41** (48)	0.55** (57)
7.5 mg/kg bw per day	5.7** (51)	23.1** (71)	7.2** (35)	8.6** (35)	0.27** (32)	0.27** (28)

PChE Plasma cholinesterase; EChE Erythrocyte cholinesterase; BChE Brain cholinesterase

** Probability $p < 0.01$

Source: Hellwig, 1983

The main effect of dimethoate in the study was upon AchE activity in plasma, erythrocytes and brain at dose levels of 2.5 and 7.5 mg/kg bw per day. Male plasma and female brain ChE activities were only slightly affected at 0.5 mg/kg bw per day. These marginal effects (15% reduction) are not regarded as being toxicologically relevant. Therefore, the NOAEL in this short-term 28-day study is identified as 5 ppm (equivalent to 0.5 mg/kg bw per day) based on decreased ChE activity in plasma, erythrocytes and brain at dose levels 25 and 75 ppm (equivalent to of 2.5 and 7.5 mg/kg bw per day respectively) (Hellwig, 1983).

Study 2

In a four-week dose range-finding study dimethoate (lot/batch no. 2052200; purity 99.1%) was administered via the diet to groups of five male and five female Wistar rats (CrlGlxBrlHan:WI; ca 49 days old; average body weight: males,193 g, females,144 g at first administration of test substance) at dose levels of 0 (control), 0.83, 2.48 and 10.38 mg/kg bw per day for males and 0 (control), 0.85, 2.60 and 11.00 mg/kg bw per day for females.

There were no mortalities and no clinical signs of toxicity due to dimethoate. Food consumption and body weights were not affected. Neither clinical chemistry examinations nor haematology showed any signs of treatment-related effects.

At study termination AChE activities were significantly decreased in the serum, red blood cells and brain of the high-dose animals of either sex. In females, statistically significantly reduced AChE activities in erythrocytes were also observed on day 8 in the high-dose group and at study termination in the mid-dose group. A trend towards reduced AChE activity was also observed in the brain samples of the mid-dose animals, however, without attaining statistical significance.

Table 10. Serum cholinesterase activity (µkat/L) in rats after administration of dimethoate and its inhibition compared to control (%).

Males					**Females**				
Time point	**Day –7**	**Day 2**	**Day 8**	**Day 29**	**Time point**	**Day –7**	**Day 2**	**Day 8**	**Day 29**
Dose level (mg/kg bw/day)					***Dose level*** (mg/kg bw/day)				
0 (control)	13.9	13.0	12.4	11.0	0 (control)	25.2	29.5	33.7	46.5
0.83	13.5 2%	13.0 0%	12.6 –1%	11.5 –5%	0.85	24.5 2%	24.8 16%	28.7 15%	40.1 14%
2.48	12.8 8%	11.7 10%	11.3 9%	10.1 8%	2.60	28.1 –12%	33.3 –13%	39.0 –16%	47.7 –2%
10.38	15.1 –8%	13.5 –4%	11.7 6%	8.5* 23%	11.0	26.5 –5%	29.6 –1%	27.5 18%	28.6** 38%

* Statistically significant different from control; $p < 0.05$

** Statistically significant different from control; $p < 0.01$

Source: Kaspers, 2005

Table 11. Erythrocyte cholinesterase activity (µkat/L erythrocytes) in rats after administration of dimethoate and its inhibition compared to control (%).

Males					**Females**				
Time point	**Day –7**	**Day 2**	**Day 8**	**Day 29**	**Time point**	**Day –7**	**Day 2**	**Day 8**	**Day 29**
Dose level (mg/kg bw/day)					***Dose level*** (mg/kg bw/day)				
0 (control)	32.3	37.2	34.2	38.7	0 (control)	35.9	37.2	32.3	35.9
0.83	34.2 –6%	36.6 2%	33.1 3%	35.1 9%	0.85	39.0 –9%	40.4* –9%	36.2 –12%	36.8 –3%
2.48	32.8 –2%	37.0 1%	34.4 –1%	33.9 12%	2.60	35.2 2%	36.4 2%	31.4 3%	26.7** 26%
10.38	33.9 –5%	34.7 7%	24.9 27%	11.8** 70%	11.0	39.7 –11%	35.2 5%	21.2** 34%	10.9** 70%

* Statistically significant different from control; $p < 0.05$

** Statistically significant different from control; $p < 0.01$

Source: Kaspers, 2005

Table 12. Brain cholinesterase activity (μkat/g protein) in rats after administration of dimethoate and its inhibition compared to control (%).

Males		Females	
Time point	**Day 29**	**Time point**	**Day 29**
Dose level (mg/kg bw/day)		***Dose level*** (mg/kg bw/day)	
0 (control)	2.24	0 (control)	2.18
0.83	2.27 –1%	0.85	3.11** –43%
2.48	1.78 20%	2.60	1.53 30%
10.38	0.78** 65%	11.0	0.65** 70%

* Statistically significant different from control; $p < 0.05$

** Statistically significant different from control; $p < 0.01$

Source: Kaspers, 2005

At the doses administered in the course of this four-week study there were no adverse effects due to dimethoate other than reduction of the acetylcholinesterase activity. Acetylcholinesterases in serum and brain showed a significant decrease in activity at 10.38 mg/kg bw per day for males and 11 mg/kg bw per day for females, erythrocyte ChE at 10.38 mg/kg bw per day in males and 2.60 mg/kg bw per day in females. Thus, the NOAEL under the conditions of this study was identified as 0.83 mg/kg bw per day for males and 0.85 mg/kg bw per day for females, based on decrease erythrocyte ChE activity at 2.68 mg/kg bw per day in female on day 29, and possibly decrease in brain ChE in both sexes (males at, 2.48 mg/kg bw per day; females at 2.60 mg/kg bw per day) (Kaspers, 2005).

Dog

Study 1

Dimethoate (batch no.611A; purity 96.44%) was administered in the diet for 28 days (7 days/week) to two male and two female Beagle dogs per group; each dog was offered 400 g diet each day. The dogs' mean age was 28 weeks for males, 26 weeks for females; their mean weights 8.9 kg and 8.8 kg respectively. The dose levels were 0 (control), 2, 10, 50, 250 and 1250 ppm in the diet (equal to 0, 0.09, 0.43, 2.20, 11.12 and 49.81 mg/kg bw per day respectively).

The animals on the highest dose (1250 ppm) were killed in week 3 for humane reasons. A low incidence of vomiting was observed in one animal in each of the 10 and 250 ppm groups and in three animals at 1250 ppm. Marked body weight loss and reduced food consumption was shown in the top dose animals and in one dog at 250 ppm dimethoate. Plasma and erythrocyte ChE activity was markedly inhibited in dogs at 250 and 1250 ppm. There was a gradual decrease of erythrocyte ChE activity during the course of the study at the 50 ppm dose level. The effects on erythrocyte ChE at 24 h were broadly similar to values observed at 4 h. Thus, the effects of dimethoate on erythrocyte ChE in dogs can be reliably detected within a time frame of about 24 h after last dosing. Brain ChE activity was inhibited in dogs at 50 and 250 ppm. In surviving animals there were no findings of note from gross postmortem examination or organ weight measurements. In animals killed for humane reasons in week 3, several macroscopic findings at postmortem examination included unusual colour of some organs, liquid intestinal contents, congestion and notably small organs (for example, liver, spleen, and thymus). In general these changes are to be regarded as nonspecific.

Table 13. Plasma cholinesterase (µmol/mL per min) group mean values (combined, 24 h after feeding)

Dose level (ppm)	Day 6	Day 13	Day 20	Day 27
0 (Control)	1.38	1.52	1.31	1.28
2	1.47	1.40	1.52	1.65
10	1.61	1.49	1.37	1.64
50	1.42	1.49	1.48	1.40
250	0.99	1.03	1.06	0.96
1250	0.95	0.90	NA	NA

NA Not applicable as animals of this high dose group have been sacrificed in week 3 for humane reasons

Source: Buford et al., 1989

Table.14. Erythrocyte cholinesterase (µmol/mL per min) group mean values(combined, 24 h after feeding)

Dose level (ppm)	Day 6	Day 13	Day 20	Day 27
0 (Control)	1.77	1.76	1.86	1.66
2	2.22	2.45	2.26	2.11
10	2.11	2.07	2.10	1.85
50	1.82	1.64	1.51	1.30
250	1.24	0.87	0.62	0.41
1250	0.54	0.51	NA	NA

NA Not applicable as animals of this high dose group have been sacrificed in week 3 for humane reasons

From Buford, et.al, 1989

Table 15. Brain cholinesterase (BChE) and percentage difference in comparison with the control; Group mean values (combined)

Dose level (ppm)	BChE µmol/g per min	% inhibition (compared with control)
0 (Control)	3.86	-
2	4.30	–11.4
10	4.21	–9.0
50	3.14	18.7
250	1.81	53.2

From Buford, et.al, 1989

Dimethoate affected erythrocyte and brain cholinesterase activity at a concentration of 50 ppm and above in the diet. Concentrations of 2 and 10 ppm dimethoate in diet did not affect the parameters examined in this four-week dog study. Therefore, the NOAEL was identified in this 28-day study in this species as 10 ppm (equal to 0.43 mg/kg bw per day), based on erythrocyte and brain ChE inhibition at the lowest-observed-adverse-effect level (LOAEL) of 50 ppm (equal to 2.20 mg/kg bw per day)

The study is GLP compliant and a quality assurance (QA) statement was attached (Buford et al., 1989).

Study 2

Dimethoate (batch no. 1341–90; purity > 99.8%) was administered via their feed to groups of four male and four female Beagle dogs (males and females ca six and eight weeks old, respectively) over a period of 98 days. The dose levels were 0 (control), 4, 6 and 9 ppm (equivalent to 0, 0.1, 0.15 and 0.225 mg/kg bw per day respectively) in the diet.

All dogs survived their respective feeding periods. No treatment-related clinical signs were observed in any test group. Neither food intake nor body weight gain was affected by treatment with

dimethoate. There were no effects due to dimethoate administration for the haematological and clinical chemistry parameters tested.

Except for one male dog from the 9 ppm group, which showed a significant reduction in erythrocyte ChE activity solely at week 7 (56% of the predose value), there was no evidence of ChE inhibition. Since this low value was preceded and followed by erythrocyte ChE activities which were approximately the same as the pre-exposure value and the value at week 12, this finding is considered unrelated to the treatment. Therefore, it is concluded that a dietary level as high as 9 ppm of dimethoate does not inhibit erythrocyte or plasma ChE activity in male or female dogs.

Results of this 98-day feeding study identified 9 ppm (equivalent to ca 0.225 mg/kg bw per day) as the NOAEL, the highest dose tested.

This study is neither GLP-compliant nor has any QA statement attached as it was undertaken before the implementation of GLP (Hutchison & Shaffer, 1968).

Study 3

Groups of six male and six female pure bred Beagle dogs received dietary concentrations of dimethoate (batch no. 611A; purity 96.44%) for 52 weeks at 0 (control), 5, 20 or 125 ppm (equal to 0, 0.18, 0.70, 4.81 mg/kg bw for males, 0, 0.19, 0.76, 4.31 mg/kg bw per day for females). At commencement of dosage, dogs' body weights ranged from 7.3–13.5 kg and their ages from 25–32 weeks.

There were no deaths or clinical signs of reaction to treatment. There was no effect of treatment on body weight or food consumption. Dogs' eyes were unaffected by treatment.

Plasma ChE activity measurements indicated reductions relative to controls of greater than 20% in animals treated at 125 ppm in weeks 13 and 26 (both sexes), and in week 52 (only males). With the exception of females at week 13 (36% lower than controls) these reductions did not exceed 22%. Erythrocyte ChE reductions were seen in weeks 13 and 26 in animals treated at 20 or 125 ppm. The percentage reduction seen in animals treated at 20 ppm was between 27% and 20%; for higher dosage animals this figure was between 76% and 63%. In week 52 males treated at 5 or 20 ppm had marginally lower erythrocyte ChE activities than the controls, indicating a 20% reduction; the difference from controls was not statistically significant. Females of these groups were unaffected at this time. Clear reductions in erythrocyte ChE were still evident in animals treated at 125 ppm (approximately 65% reduction; Table 16). Statistically significant reductions in brain ChE were evident at all treatment levels after 52 weeks; these were slight at 5 or 20 ppm (approximately 90% and 83% of controls at 5 and 20 ppm respectively) but pronounced at 125 ppm (45% of controls). There were no other biochemical differences in the blood attributable to treatment. Urine was unaffected by treatment.

Table 16. Acetylcholinesterase activity in plasma (PChE), erythrocytes (EChE), brain (BChE). Group mean values (µmol/g/mm).

		Cholinesterase activity; group mean values (µmol/g per mm)						
		PChE			EChE			BChE
Sex	Dose (ppm)	Week 13	Week 26	Week 52	Week 13	Week 26	Week 52	Week 52
Males	(Control) 0	1.64	1.33	1.45	2.08	2.23	2.23	2.98
	5	1.50	1.20	1.49	1.90	1.99	1.78	2.70*
	20	1.53	1.22	1.51	1.60	1.62*	1.76	2.55**
	125	1.28*	1.07*	1.15**	0.68**	0.70**	0.78**	1.34**
Females	(Control) 0	1.84	1.48	1.70	2.20	2.25	2.16	3.31
	5	1.83	1.38	1.72	2.10	1.97	2.12	2.98*
	20	1.65	1.42	1.68	1.72*	1.80	2.02	2.70**
	125	1.18**	1.15*	1.47	0.53**	0.59**	0.79**	1.46**

* Statistically significant at $p < 0.05$ ** Statistically significant at $p < 0.01$

Source: Burford et al.,1991

There were no findings at necropsy that were attributed to treatment. The liver weights of animals treated at 125 ppm were lower than those of the controls. There was a marginally greater incidence of pigment, presumed to be haemosiderin, in the livers of treated animals of all groups, but there was no clear relationship to dosage. In the absence of other effects of treatment, notably on haematology, this effect was considered to be of no toxicological significance.

It was considered, that in this 52-week feeding study in dogs the only significant evidence of toxicity attributable to dimethoate was the reduction in AChE activity. This was clearly significant and > 20% at dose levels of 20 and 125 ppm in respect of erythrocyte and brain activities. Effects at 5 ppm were confined to minimal reductions on brain ChE (10% lower than controls) and a minimal reduction in male plasma ChE after 52 weeks and were not considered of toxicological relevance. Thus, a NOAEL of 5 ppm (equal to ca 0.18 mg/kg bw per day for males and 0.19 mg/kg bw per day for females) was identified, based on reduction in erythrocyte ChE activity in males at 20 ppm (equal to 0.70 mg/kg bw per day for males and 0.76 mg/kg bw per day for females).

The study is GLP compliant and a QA statement was attached (Burford et al.,1991).

(b) Dermal application

A short-term study of dermal toxicity in rabbits was conducted.

Rabbit

Groups of six male and six female New Zealand White rabbits (weight 2–3 kg) were dosed with dimethoate at levels of 0 (untreated and vehicle controls), 100, 300 or 1000 mg/kg bw per day. The vehicle used was paraffin oil. Dimethoate (batch no. 611A; purity 97.3%) was applied (total volume 2 mL) evenly to an area of 10 cm² of the clipped back of each animal, covered with gauze and taped into position. For half the animals in each group, the test areas were abraded before dose application. After 6 h, the dressings were removed and the test sites washed with soap and water. The applications were made daily for 5 days/week for three weeks (a total of 15 applications per animal). Rabbits were examined daily for dermal reactions and clinical signs and weighed at the start and end of the study.

No animal died during the study. There were no adverse clinical signs due to dimethoate. Dermal reactions occurred to a similar extent in the vehicle control and dimethoate groups. There was no discernible difference between the treatments on abraded or intact skin. Pustules were seen at the treatment sites of the majority of animals including vehicle controls during the study. There were no statistically significant or dose-related changes due to dimethoate in haematology, clinical chemistry or organ weight parameters. There was no effect on plasma or erythrocyte ChE activities. No gross or microscopic changes due to dimethoate were observed for skin or other examined tissues. The NOAEL for dimethoate was 1000 mg/kg bw per day, the highest dose tested.

The study was GLP compliant. (Madison, 1986)

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a 78-week study, groups of 50 male and 50 female albino B6C3F1 mice (plus satellite groups of 10 males and 10 females per group for interim sacrifice after 52 weeks of treatment) were given diets containing dimethoate (batch 611A; purity 96.71%) at concentrations of 0, 25, 100 or 200 ppm (equal to dosages of 0, and ca 3.6, 13.7 and 31.1 mg/kg bw per day in males, 0, 5.2, 18.2 and 35.6 mg/kg bw per day in females).The animals were 38 days old at study start, body weights were 16–23 g for males, 14.5–21 g for females. Treatment groups were randomly assigned and were housed individually. Food and tap water was available to animals ad libitum.

Animals were examined daily for clinical signs. Food consumption and body weights were measured weekly for the first three months. Thereafter, food consumption was measured every two weeks up to six months, and then at four-weekly intervals. Body weights were measured every two weeks after the first three months of treatment.

Blood for haematology was sampled from 10 mice/sex per group from the satellite and main groups at 51 and 78 weeks, respectively. Plasma and erythrocyte ChE activities were determined in samples obtained at 51 weeks. Blood samples were taken from nonfasted animals in the morning (at about 07.00).

At termination, gross necropsy was carried out. Heart, liver, kidneys, spleen, testes/ovaries and brain were weighed and a wide range of tissues from all animals in the main groups were examined histopathologically.

The mortality rates were not affected by dimethoate administration and no indications of intergroup differences in survival were seen during this 78-week feeding study with mice.

Male body weight gain was lower in all treated groups in the early stages of the study when compared to controls, but only 200 ppm group weights remained depressed throughout the study. Body weights in all treated female groups were greater than the control group throughout most of the study. However, these increases in mean body weight are often inconsistent at different time points and not always clearly related to dose. No notable differences between treated and control groups were observed.

Plasma and erythrocyte ChE activities were inhibited in males and females in a dose-dependent manner. Marked inhibition was observed at 100 and 200 ppm dimethoate, whereas only small, but statistically significant decreases in these activities were observed at 25 ppm. Activity in erythrocytes was inhibited to a greater extent than enzyme activity in plasma and no differences between males and females were observed. No characteristic clinical signs of organophosphate poisoning were seen, suggesting a somewhat lower inhibition of brain ChE.

Table 17. Acetycholinesterase activity in plasma and erythrocytes after 51 weeks oral administration of dimethoate to male and female mice

	Plasma cholinesterase (µkat/L) [% when compared to control]		**Erythrocyte cholinesterase (µkat/L erythrocytes) [% when compared to control]**	
Dose (ppm)	**Males**	**Females**	**Males**	**Females**
0 (control)	74.43 [100]	101.56 [100]	36.76 [100]	31.84 [100]
25	66.73** [90]	90.67** [89]	25.93** [71]	23.12** [73]
100	38.31** [51]	49.09** [48]	8.64** [24]	10.08** [32]
200	16.40** [22]	22.90** [23]	3.87** [11]	4.48** [14]

** Significance $p < 0.01$

Source: Hellwig, Deckardt. & Mirea, 1986a

Small increases in absolute and relative liver weights in males at 100 and 200 ppm dosage were observed. In females, absolute, but not relative, liver weight was slightly increased at these dose levels. Absolute and relative ovary weights were decreased in all treated female groups, but without any histological correlation.

Spontaneous non-neoplastic lesions were detected in males and females of all test groups including the controls. Only hepatic vacuolization and extramedullary haematopoiesis were more pronounced in animals of both sexes at 100 and 200 ppm and in males only at 200 ppm, respectively, when compared to the control group. No such effects were found in the 25 ppm treatment group. Incidences of tumours in the lung, liver and hemolymphoreticular system revealed no statistical significance Therefore, in conclusion, no treatment-related increased incidences of neoplasia were observed.

Dimethoate in the diet at 200 ppm caused a reduction in body weight gain, plasma and erythrocyte chloinesterase inhibition, increased liver weight and, in male animals, hepatic vacuolization and extramedullary haematopoiesis. At 100 ppm, plasma and erythrocyte chloinesterase inhibition, increased liver weight and hepatic vacuolization were noted in both sexes. Decreased ovary weight with no histopathological correlation at 25 ppm was considered to be of no toxicological significance. Significant reduction in erythrocyte chloinesterase activity was also observed at this dose (29% and 27% for males and females, respectively). Therefore, the LOAEL in this study was 25 ppm (equal to 3.6 mg/kg bw per day in males, 5.2 mg/kg bw per day in females), the lowest dose tested.

The NOAEL for carcinogenicity mice is more than 200 ppm (equal to 31.1 mg/kg bw per day in males and 35.6 mg/kg bw per day in females), the highest dose tested.

The study was not accompanied by GLP certification, though a QA statement was attached (Hellwig, Deckardt. & Mirea, 1986a).

Rat

Study 1

In a two-year combined chronic toxicity feeding and carcinogenicity study, groups of 50 male and 50 female Wistar rats (plus satellite groups for intermediate bleeding and urinalysis of 15 rats/sex per group each, except for the 1 ppm group, which consisted of 20 rats/sex per group) were given diets containing dimethoate (batch 611A; purity 96.71%) at concentrations of 0, 1, 5, 25 and 100 ppm, (equal to mean 0, 0.04, 0.23, 1.2 and 4.8 mg/kg bw per day in males, 0.06, 0.3, 1.5 and 6.3 mg/kg bw per day in females). The animals were 35 days old at study start, with body weights 112–180 g for males, 111–159 g for females.

Animals were dosed continuously for 104 weeks and were examined daily for clinical signs.

There were no notable intergroup differences in clinical signs. Mortality in females was slightly increased towards the end of the study in the 100 ppm group, but this effect revealed no statistical significance. Male and female body weight gain was slightly reduced in the 100 ppm group for the first year of the study. There were no significant differences in food consumption between treated animals and controls, although there seemed to be a trend towards increased food consumption, particularly in the high-dose group. Reduced haemoglobin, erythrocyte and haematocrit indicated a slight anaemic process in males given 100 ppm dimethoate. A slight increase in leucocyte values was seen in both sexes at 100 ppm.

Significant reductions in erythrocyte and brain ChE activity were seen at ≥ 5 ppm. No toxicologically relevant effects on AChE activity were seen at 1 ppm.

Table 18. Acetylcholinesterase activity in plasma (µkat/L plasma); percentage compared with control group shown in brackets

	Males				
	Control	**1 ppm**	**5 ppm**	**25 ppm**	**100 ppm**
Pre-treatment	13.0 (100)	13.1 (101)	12.8 (98)	12.8 (98)	12.3 (95)
1 month	9.6 (100)	9.8 (102)	9.3 (97)	8.4 (88)	4.4** (46)
3 months	12.6 (100)	13.0 (103)	11.8 (94)	10.3 (82)	5.7** (45)
6 months	12.5 (100)	14.8 (118)	12.9 (103)	12.6 (101)	6.6** (53)
12 months	16.0 (100)	16.6 (104)	15.5 (97)	13.8 (86)	7.5** (47)
18 months	14.7 (100)	17.1 (116)	15.7 (107)	13.3 (90)	6.8** (46)
24 months	14.5 (100)	17.5 (116)	17.3* (119)	15.2 (105)	7.6** (52)
	Females				
	control	**1 ppm**	**5 ppm**	**25 ppm**	**100 ppm**
Pre-treatment	15.5 (100)	15.6 (101)	15.8 (102)	14.8 (95)	16.1 (104)
1 month	32.2 (100)	33.8 (105)	34.1 (106)	27.8* (86)	15.5** (48)

(*Table 18 continued on the following page*)

3 months	43.6 (100)	45.4 (104)	46.4 (106)	39.2 (90)	20.3** (47)
6 months	48.6 (100)	54.0 (111)	54.9 (113)	50.6 (104)	30.3** (62)
12 months	53.5 (100)	50.9 (95)	52.8 (99)	50.2 (94)	24.0** (45)
18 months	30.7 (100)	35.7 (116)	40.0 (130)	30.7 (100)	14.2** (46)
24 months	29.1 (100)	26.6 (91)	31.3 (108)	25.6 (88)	12.9** (44)

* Significance $p < 0.05$ ** Significance $p < 0.01$

Table 19. Acetylcholinesterase activity in erythrocytes (µkat/L erythrocytes); percentage compared with control group shown in brackets

	Males				
	control	**1 ppm**	**5 ppm**	**25 ppm**	**100 ppm**
Pre-treatment	8.1 (100)	7.6 (94)	9.5 (117)	9.3 (115)	9.1 (112)
1 month	23.0 (100)	24.0 (104)	20.5 (89)	14.0** (61)	4.7** (20)
3 months	21.0 (100)	25.8 (123)	17.9 (85)	13.6** (65)	4.5** (21)
6 months	19.7 (100)	22.8 (116)	19.4 (98)	13.4** (68)	5.6** (28)
12 months	20.4 (100)	23.5 (115)	20.7 (101)	15.3 (75)	5.1** (25)
18 months	16.0 (100)	13.9 (87)	17.1 (107)	12.2 (76)	6.0** (38)
24 months	27.3 (100)	28.0 (103)	22.5** (82)	16.3** (60)	5.3** (19)
	Females				
	control	**1 ppm**	**5 ppm**	**25 ppm**	**100 ppm**
Pre-treatment	17.4 (100)	19.0 (109)	17.2 (99)	15.8 (91)	19.3 (111)
1 month	25.4 (100)	24.5 (96)	19.3* (76)	15.0** (59)	5.6** (22)
3 months	27.4 (100)	25.6 (93)	20.7** (76)	16.1** (59)	6.4** (23)
6 months	24.6 (100)	25.0 (102)	19.8** (80)	14.1** (57)	5.8** (24)
12 months	27.3 (100)	28.8 (105)	18.0** (66)	13.8** (51)	12.0** (44)
18 months	16.4 (100)	15.7 (96)	15.7 (96)	12.9 (79)	5.8** (35)
24 months	24.8 (100)	23.6 (95)	22.4 (90)	17.9** (72)	6.0** (24)

* Significance $p < 0.05$ ** Significance $p < 0.01$

Table 20. Cholinesterase activity in brain (µkat/g protein); percentage compared with the control group shown in brackets

Dose levels	Males	Females
0 ppm	0.48 (100)	0.50 (100)
1 ppm	0.44 (92)	0.50 (100)
5 ppm	0.37* (77)	0.45 (90)
25 ppm	0.33** (69)	0.30** (60)
100 ppm	0.18** (38)	0.19** (38)

* Significance $p < 0.05$ ** Significance $p < 0.01$

An increased mean spleen weight and a decreased mean ovary weight were seen in animals at the 100 ppm dose level. In treated males a higher incidence of tumours when compared to controls was found, but no dose relationship was obtained and for females, with even the hint of a negative correlation apparent. Most of these tumours were located in the mesenteric lymph nodes.

In none of the organs in which angiogenic tumours (hemangioma and/or hemangiosarcoma) were detected (mesenteric lymph node, spleen, kidney and skin) did the incidences of any one of these neoplasms show statistically significant deviations. However, when the incidences of animals with angiogenic tumours at any sites were compared, the Fisher's exact test showed a significant increase for males in all dose groups, with an obvious dose–response relationship. In the female, an opposite trend was demonstrated (Table 21).

The vascular and proliferative lesions from the above study were evaluated by a second group of pathologists (Squire, 1988a, b), who were unaware of the previous interpretation of each slide.

The tabular presentations are shown in Tables 21 and 22.

Table 21. Incidence of vascular proliferative lesions in spleen (male Wistar rats[§])

Dose (ppm)	0	5	25	100
Number examined	55	51	56	56
Endothelial proliferation (% affected)	3(5.4)	3(5.8)	1(1.8)	1(1.8)
Mean grade	1.3	1.3	4.0	5.0
Haemangioma (% affected)	0(0)	1(2.0)	0(0)	1(1.8)
Mean grade	0	2.0	0	2.0
Haemangiosarcoma (% affected)	0(0)	2(3.9)	1(1.8)	4(7.1)
Mean grade	0	3.0	3.0	2.7
Combined haemangioma/haemangiosarcoma	0(0)	3(5.9)	1(1.8)	5(8.9)**

[§] There was also a satellite group of eight animals intended for ancillary studies.They are not included here because numbers were inadequate for meaningful comparison and the animals were premature decendents

* Significant by Fisber's exact test at $p = 0.05$; for combined tumours only

Source: Squire, 1988a, b

Table 22. Incidence of vascular proliferative lesions in mesenteric lymph nodes (male Wistar rats[§])

Dose (ppm)	0	5	25	100
Number examined	55	51	55	56
Endothelial proliferation (% affected)	4(7.2)	6(11.8)	11(20.0)	9(16.0)
Mean grade	1.5	2.8	4.0	1.9
Haemangioma (% affected)	0(0)	0(0)	1(1.8)	0(0)
Mean grade	0	0	3.0	0
Haemangiosarcoma (% affected)	0(0)	4(7.8)	2(3.6)	2(3.6)
Mean grade	0	1.7	2.0	2.5
Combined haemangioma/haemangiosarcoma	0(0)	4(7.8)	3(5.4)	2(3.6)

[§] There was also a satellite group of eight animals intended for ancillary studies.They are not included here because numbers were inadequate for meaningful comparison and the animals were premature decendents

Statistical analysis performed based upon Fisher's exact test and the Cochran–Armitage trend test, showed there was no significant increases in any lesions in mesenteric lymph nodes.

Source: Squire, 1988a, b

Table 23 shows the historical control incidence of vascular tumours in the spleen and lymph nodes from two control groups in the BASF Laboratory which varies from 16% to 22.0%. In the light of this evidence the control group incidence in this study (0%) appear to have been unusually low. According to the pathologist, the incidence of combined splenic-lymph node vascular tumours in each of the treated group (7.2–13.7%) did not exceed the reported historical control limits although diagnostic criteria were not necessarily the same.

Table 23. Historical control incidence of male Wistar rats with one or more vascular tumours[§]

Study	Tumour	Spleen	Node	Total All Sites
A	Hemangioma	3/50 (6%)	8/50 (16%)	16/50 (32%)[#]
	Hemangiosacrcoma	0/50 (0%)	0/50 (0%)	-
B	Hemangioma	3/100 (3%)	8/100 (8%)	17/100 (17%)[#]
	Hemangiosacrcoma	1/100 (1%)	4/100 (4%)	-

[§] Data derived from two year studies in Wistar rats completed during 1987 at BASF, Ludwigshafen, FRG

[#] Combined hemangioma/hemangiosarcoma in spleen, mesenteric lymph node and other tissues

The total vascular tumours at any sites for the two historical control groups were 17% and 32%. If one combines all the endothelial proliferation/endothelial tumours diagnosed from this study, plus the two hemangiosarcomas at other sites, the total incidences in groups 1 to 4 are: 14.5%, 31.3%, 30.9% and 30.3% respectively. Consequently the total endothelial proliferation/endothelial tumour incidences in this study did not exceed the reported endothelial tumour incidences for the two historical control groups, and again did not show a dose relationship.

Table 24. Incidence of all combined endothelial proliferation/endothelial tumours diagnosed from this study, plus the two haemangiosarcomas at other sites,

Dose (ppm)	0	5	25	100
Number examined	55	51	55	56
Endothelial proliferation combined	7	9	12	10
Haemangiosarcoma from other sites	1	0	1	0
Combined angioma and angiosarcoma from spleen and mesenteric lymph nodes	0	7	4	7
Total (%)	8 (14.5%)	16 (31.3%)	17 (30.9%)	17 (30.3%)

However, it can be concluded that in this study as haemangioma/haemangiosarcoma are the most common vascular tumours in rodents, and they exist in various tissues/organ of the body, combining only the tumours of two organs where the vascular tumour showed increased tendency might mislead or bias the interpretation of their carcinogenicity.

The review by Squire (1988a, b) supported the conclusion of the original pathologist.

Although rarely occurring in humans, haemangiosarcomas have become important in evaluating the potential human risk from several chemicals which are known to induce the vascular tumour in rodents. However, their mode of action (MOA) is considered to be via hypoxia, and not relevant to the human haemangioma/haemangiosarcoma MOA. (Cohen et al., 2009) A number of surveys of two-year bioassay data for control animals revealed that hemangiosarcomas occur more commonly than hemangiomas, that these tumours are more common in mice than rats and that the liver and spleen are the most common anatomic sites of origin (Berridge et al., 2016).

In addition, there was no evidence for dimethoate reducing the latency period of those vascular tumours, which supports the view that they were spontaneous tumours of ageing animals. Consequently, these tumours were considered unrelated to treatment.

Increased incidences of some other tumours, including mammary gland fibroadenoma, mammary gland adenocarcinoma and granular cell tumours of the brain in females, were noted but are not considered to be substance-related because of the lack of statistical significance and/or lack of dose–response, as well as that the effects are in line with published historical control incidences.

A NOAEL of 1 ppm (equal to 0.04 and 0.06 mg/kg bw per day in males and females, respectively) was identified in this study, based on toxicologically relevant inhibition of brain cholinesterase (males) and erythrocyte ChE (females) at 5 ppm (equal to 0.23 mg/kg bw per day for males, 0. 3 mg/kg bw per day for females). The NOAEL for carcinogenicity was 100 ppm (equal to 4.8 mg/kg bw per day in males and 6.3 mg/kg bw per day in females) the highest dose tested.

The study is GLP compliant (Hellwig & Gembart, 1986; Hellwig, Deckardt & Mirea, 1986b).

Study 2

In a non-GLP study, Wistar rats (30 rats/sex per group) were administered dimethoate (94–97% purity) in the diet at concentrations of 0, 0.1, 1, 10 (equal to 0, 0.02, 0.2 and 2 mg/kg bw per day) or 75 ppm (equal to 20 mg/kg bw for males and 21 mg/kg bw per day for females) for up to 104 weeks. An interim sacrifice of 6 rats/sex per group was made at week 52. Body weights and food consumption were recorded weekly for four weeks and then monthly. Haematology was performed four times between weeks 32 and 100. Plasma and erythrocyte ChE activities (6 rats/sex per group) were measured during weeks 1, 3, 12, 50, 75 and 100; brain ChE activity was measured at weeks 52 and 104. Other (limited) clinical chemistry was performed on blood and urine during the study. All animals were examined macroscopically and organs weighed. Histological examination was performed on 6 rats/sex per group at each sacrifice.

Signs of toxicity (slight piloerection, exophthalmos and fine tremor) were noted at 75 ppm during the early part of the study; signs had resolved by week 4. There was no treatment-related mortality. High mortality resulting from a respiratory infection was seen at 78 weeks; deaths were evenly distributed among the groups. Body weight gain was reduced in animals treated at 75 ppm, up to week 20 in females and throughout the study in males. Food consumption was unaffected; food conversion efficiency was reduced in animals treated at 75 ppm in line with body weight effects. Haematology indicated no effects due to treatment. Cholinesterase activity was clearly reduced in the plasma, erythrocytes and brain in animals receiving 10 or 75 ppm. Brain ChE activity at 1 ppm was 19% lower than controls at one year. Standard clinical chemistry investigations did not reveal any treatment-related findings. Organ weight analysis showed lower liver, spleen, adrenal and testis weights in 75 ppm males after one year and lower adrenal and liver weights at two years. Necropsy did not reveal any gross or microscopic treatment-related findings.

No evidence of carcinogenicity was seen, however the incomplete data presentation, and evidence of midstudy infection significantly compromises the validity of this study. Hence, no NOAEL was identified.

The study is not GLP-compliant (Lewerenz, Lewerenz & Engler, 1970).

Study 3
In a US National Cancer Institute (NCI) bioassay, groups of 50 male Osborne–Mendel rats were administered dimethoate (94–96% purity) in the diet at concentrations of 250 or 500 ppm for 19 weeks, after which the dose levels were reduced to 125 and 250 ppm and treatment continued for a further 61 weeks. A period of 33 to 35 weeks without treatment followed before termination. Groups of females were administered the same dietary concentrations; these were reduced after 43 weeks to 125 and 250 ppm and treatment continued for a further 37 weeks. The total treatment period for both males and females was 80 weeks. A control group of 10 males and 10 females was supplemented by similar groups of animals from concurrent studies, giving a pooled control group of 50–60 rats/sex. All animals were observed daily and body weights recorded at regular intervals. All animals were necropsied and tissues retained for histopathological examination.

Treatment at 500 ppm was associated with lower body weight gains in males and females during the first 20 weeks of treatment. Following the reduction to 250 ppm, body weight gain in males increased but remained lower than in controls until treatment ceased at week 80. Body weight gains to week 80 were also lower in low-dose males and high-dose females. Clinical signs of ChE inhibition were seen in animals of the high-dose group, particularly during the first week of treatment. Conjunctivitis of viral origin was diagnosed in the animals at week 38; animals surviving to termination were reported to be in poor physical condition. Higher mortality was seen at the top dose level. There were no treatment-related differences in the distribution of non-neoplastic or neoplastic histopathological findings.

This study does not show any evidence of carcinogenicity, however, the evaluation of carcinogenic potential is severely limited by the duration of dosing. Cholinesterase activity was not investigated, therefore a reliable NOAEL cannot be determined for this study.The study was GLP compliant (US NIC, 1977).

2.4 Genotoxicity

As a general strategy, the genotoxicity studies were evaluated as per the criteria devised by the JMPR Meeting of May 2016.

Genotoxicity has been tested in a battery of tests both in vivo and in vitro, Unpublished studies used for regulatory purposes and in addition several publications have been provided. The overall data package is suggests that higher concentrations of dimethoate potentially produce equivocal, or weakly positive results in certain in vitro tests, although the relevance of some of these findings is questionable due to the purity of the material tested, methodological considerations and the dose levels employed. The weight of evidence from the package of in vivo studies confirms a lack of any appreciable genotoxicity due to dimethoate.

Of the in vivo studies in the more comprehensive rodent bone marrow cytogenetics and micronucleus studies (Sorg, 1985; San Sebastian, 1985), negative results were obtained for numerical and structural chromosome changes. Although only a single dose level was tested by Sorg (1985), in a mouse micronucleus assay there was evidence of systemic (and bone marrow) toxicity and therefore the study was considered to be valid in the context of hazard identification. The mouse dominant lethal assay (Becker, 1985) and the rat liver unscheduled DNA synthesis assay (Jäckh & Hoffman, 1991) were also found to be negative.

The submitted studies provide evidence for dimethoate to have the potential to elicit equivocal, or weakly positive results in vitro (at higher dose concentrations) with in vivo results supporting a lack of genotoxic hazard in vivo. The weight of evidence from the package of in vivo studies confirms a lack of any appreciable genotoxicity due to dimethoate.

A summary of the studies provided is shown on the following page in Table 25.

Table 25. Summary of genotoxicity studies

End-point	Test object	Concentration or dose	Purity (%)	Results	Reference
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* strain WP2*uvr*A; plate incorporation and preincubation assay; with/without S9 mix	20 to 5000 µg/plate for strains TA 98, TA 1535, TA 1537 and from 20 to 8000 µg/plate for strains TA 100 and WP2	98.45	Weakly positive in TA 100 from 2000–2500 µg/plate (factor 1.5–1.6) *E. coli* WP2 uvrA-A slight increase in number of trp^+ revertants was found without S9 mix from about 2500 µg/plate(factor 2.0–2.2).Without S9 enhanced colony number detected from 2500 µg/plate	Engelhardt, 1993
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* strain WP2*uvr*A; plate incorporation and preincubation assay; with/without S9 mix	0, 10, 100, 1000 and 10 000 µg/plate	NA	Negative in *S. typhimurium* but very weakly positive in *E. coli* strain WP2*uvr*A	Allen, 1977
Comet assay	Human lymphocytes;	0, 10, 50, 100 and 200 µg/mL	98.0	Increase in comet tail length and tail intensity at 100 and 200 µg/mL Weak correlation with dose level for tail length	Undeger & Başaran, 2005
Micronucleus test	Human hepatocellular carcinoma cells (HepG2)	25, 50, 100 and 200 µg/mL	98.0	Increase in micronucleus frequency at 100 and 200 µg/mL	Jiong-Lin et al., 2010
Forward mutation assay in mammalian cells (HPRT test)	Chinese hamster ovary (CHO) cells with/without S9 mix	1000, 1500, 200, 2700 and 3500 µg/mL	97.3	Negative	Johnson & Allen, 1985
Unscheduled DNA synthesis in mammalian cells	Rat hepatocytes	23, 76, 229, 763, and 2290 µg/mL	96.38	Positive, but reservations about method	Fautz, 1990a
Unscheduled DNA synthesis in mammalian cells	Rat hepatocytes; (autoradiography)	7.63, 22.90, 76.33, 229, 763.33, 1526.67 and 2290 µg/mL	96.38	Positive, some increases in UDS in absence of toxicity; reservations about method	Fautz, 1990b

End-point	Test object	Concentration or dose	Purity (%)	Results	Reference
In vivo					
Micronucleus test	NMRI mouse, male and female (single i.p. administration; vehicle 0.9% saline)	2 × 55 mg/kg bw	97.3	Negative	Sorg, 1985
Chromosomal aberration assay	Sprague Dawley rats, i.p. administration; analysis of femoral bone marrow cell	0, 15; 75; and 150 mg/kg bw	97.3	Negative	San Sebastian, 1985
Chromosomal aberration assay	SPF Wistar rats, by gavage: analysis of the bone marrow cells	0. 7.04, 14.1 or 28.2 mg/kg bw per day for 28 days	98	Inconclusive (poorly reported)	Ündeger, 2000
Unscheduled DNA synthesis	Rat hepatocytes	50, 100 and 200 mg/kg bw (single gavage dose)	96.41	Negative	Jäckh & Hoffmann, 1991
Dominant lethal test	NMRI mice (oral administration)	0 (control), 5, 10, and 20 mg/kg bw per day	96.89	Negative	Becker, 1985

i.p. Intraperitoneal

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Mouse

Study 1

A multigeneration study was undertaken in male and female albino CF1 mice, four weeks old at the start of the study. Prior to first mating of each generation, animals were housed in pairs of like sex. At mating they were housed in groups of three (one male, two females) and between the first and second matings animals were housed individually. Mating groups were housed together until females were obviously pregnant or after a maximum of 18 days. After separation from the males, females were housed individually in nesting cages (standard cage with sheet metal tray containing wood shavings as floor). F0 animals were randomly assigned in treatment groups and mated at random. Animals from F1b and F2b litters were randomly selected for subsequent mating. Dimethoate (batch AC 888–73; purity 98.3%) was admixed in the diet at concentrations of 0, 5, 15 or 50 ppm (equal to 0, 1.3, 4.1 and 13.6 for males, 0, 1.5, 4.6 and 15.3 for females).

The study commenced with groups of eight male and 16 female mice which were mated one male with two females to produce the first litter. When the offspring had been weaned, the parents were remated on the same basis to produce a second litter. After weaning, the pups from this second litter were taken to produce a second generation. This process was repeated so that three generations with two litters per generation were produced for the study overall. Each successive generation was about three months of age at the time of first mating.

The number of pups born alive and at weaning, the mean weaning weight and fertility, gestation, viability and lactation indices were recorded for each generation. Animals of the F2b and

F3b generations were examined by gross necropsy and one male and one female from the F3b litters was histologically examined.

The appearance and behaviour of the test and control animals was similar throughout the study period except for occasional tremors. Three dams from the F2b generation of 50 ppm groups lost their litters. At this time occasional tremors in these dams and one more dam of this group were noted. As reproductive parameters were not affected by these findings, they are considered to be related to parental toxicity only.

The number of litters, pups born alive, pups weaned and mean weaning weights were similar in the treated and control groups. Nor were fertility, gestation, viability and lactation indices affected by treatment with dimethoate. No effects were seen on the parameters examined at postmortem gross necropsy or microscopical assessments.

Since dimethoate, at a dietary concentration of up to 50 ppm in the diet (the highest test dose) did not affect the reproductive capacity of mice over three generations, the NOAEL for reproductive as well as for offspring toxicity was 50 ppm (equal to 13.6 mg/kg bw per day for males and 15.3 mg/kg bw per day for females). The NOAEL for parental toxicity was 15 ppm (equal to 4.1 mg/kg bw per day for males and 4.6 mg/kg bw per day for females) based on the tremors in three dams of F2b generation at 50 ppm (equal to 13.6 mg/kg bw per day for males and 15.3 mg/kg bw per day for females). However it is to be noted that ChE activity was not measured.

The study was non-GLP as it was generated before implementation of GLP (Ribelin & Levinskas, 1965).

Study 2

In a published study, the effects of dimethoate (batch no.106–52A; purity 98%) on male reproduction were studied in ICR (CD-1) mice (10 weeks old). Males were treated with dimethoate at concentrations of 0, 7, 15 and 28 mg/kg bw per day via oral gavage for 20 days. Twenty-four hours after the end of the treatment course, groups of dimethoate-treated male mice were mated 1:1 with untreated females for seven days. The day that a vaginal plug was found was considered day 0 of gestation. Pregnant females were sacrificed on day 14 of gestation. No mortality was observed during the study. No differences in feed consumption were observed up to and including the highest dose group. However, body weights and body weight gain were significantly reduced at 28 mg/kg bw per day. Signs of cholinergic effects were observed at 15 and 28 mg/kg bw per day. While the mating index was decreased only at 28 mg/kg bw per day, the fertility index was significantly decreased at 15 and 28 mg/kg bw per day. Furthermore, the spermatozoa counts, spermatid counts and percent of motile sperm were significantly reduced at 15 and 28 mg/kg bw per day. No morphological sperm abnormalities were recorded up to and including the highest dose group. No statistically significant differences in the absolute and relative weight of brain and epididymides were noted in any of treatment groups. However, the absolute and relative testes weights were significantly reduced at 15 and 28 mg/kg bw per day. In addition, brain and muscle AChE activity was significantly decreased at 15 and 28 mg/kg bw per day. In conclusion, in this study dimethoate caused cholinergic toxicity and adverse effects on fertility, reproductive performance and sperm parameters in male mice at 15 and 28 mg/kg bw per day. The NOAEL in the present study for reproductive performance was 7 mg/kg bw per day based on adverse effects on fertility, reproductive performance and sperm parameters in male mice at the LOAEL of 15 mg/kg bw per day. Cholinesterase activity was not measured in this study.

The study is not GLP-compliant (Farag, El-Aswad & Shaaban, 2007).

Study 3

In another published paper, the effects of dimethoate, deltamethrin or a mixture of both substances were studied in male Swiss Albino mice. Animals were examined for their general health condition and randomly divided into three groups consisting of 10 animals to give each group an approximately equal mean body weight. Animals were treated with dimethoate at concentrations of 0, 5, 15 and 28 mg/kg bw per day via oral gavage for 21 days. After the treatment period, animals were weighed and sacrificed, and sperm parameters (e.g. motility, count, viability and morphology) determined. Dimethoate administered at concentrations of 0, 5, 15 and 28 mg/kg bw per day produced adverse effects on several sperm parameters at the highest dose. An increase in the percentage of morphologically abnormal spermatozoa was observed in animals administered 15 and 28 mg/kg bw per day compared with the control group.

Furthermore, the evaluation of sperm parameters revealed a significant decrease in sperm counts, motility and viability in animals administered 28 mg/kg bw per day, compared with the control group. No effects on body weight and absolute testis or epididymides weights were observed in any of the treatment groups. Cholinesterase activity was not measured in this study.

Data on the nonrelevant substances included in this study are not summarized here. Only data on dimethoate are included.

The study is not GLP compliant (Abdallah et al, 2009).

Rat

Study 1

A dose range-finding study for two-generation reproduction in rats (Crl: CD (SD) BR VAF/Plus; 11 weeks old at study start; body weight for males 420–430 g, for females 228–232 g) and their offspring was performed in four groups. Dimethoate (batch no.611A; purity 96.44%) was incorporated into the diet at concentrations of 0, 50, 75 or 100 ppm (equal to approximately 0, 2.9, 4.4 and 6.1 mg/kg bw per day for males, 0, 3.9, 5.8 and 7.5 mg/kg bw per day for females, respectively).

Statistical analyses were not performed in view of the small group size. In F0 generation, a proportion of females showed tremors at doses of 75 and 100 ppm group. Decreased body weight gains were also recorded in females at these dose levels and, to a lesser extent, at 50 ppm. Marked ChE inhibition was found in plasma, erythrocytes and brain at all treatment levels. A dose-dependent reduction in the implantation rate, a higher number of prebirth losses and pup losses were observed in treated animals compared to controls. Litter and pup weights in treated groups were lower than control values in all treated groups. There was no effect on mating performance (pregnancy rate, precoital time or gestation time).

In the offspring (F1 generation), no clinical signs of toxicity were noted. Body weight gain was slightly reduced in treated animals compared to controls. Cholinesterase activity in plasma, erythrocytes and brain was markedly inhibited in animals of the treated groups.

The treatment-related effects on the most sensitive parameter, ChE inhibition, and on reproductive parameters produced in this study provide sufficient information for the setting of appropriate dose levels in a subsequent two-generation rat reproduction study on dimethoate.

The study is not GLP compliant (Brooker & Stubbs, 1990).

In a two-generation reproductive study, male and female Crl: CD (SD) BR VAF/Plus rats, seven weeks old at study start (weight range for males 109–155 g and for females 100–139 g) in four groups, were administered dimethoate (batch 611A; purity 96.44%) through their diet at levels of 0, 1, 15 or 65 ppm (during premating equal to ca 0, 0.08, 1.2 and 5.46 mg/kg bw per day for males, 0, 0.09, 1.30 and 6.04 mg/kg bw per day for females).

The F0 parental animals (28 rats/sex per group) were treated for 10 weeks prior to the first of two matings. F1 parents were selected from the F1a litters (24 rats/sex per group) and mated at week 16. A second mating of the F1 parents was conducted, followed by a partial third mating for animals which had not been successful at previous pairings.

Organ weight analysis was conducted on all adults (F0 and F1 generations). Histopathological examinations on a range of reproductive organs and the pituitary was conducted on all control and high-dose (65 ppm) animals as well as on apparently infertile adults in the 1 ppm and 15 ppm groups.

In parents on 65 ppm dimethoate, several effects were recorded including marked inhibition of ChE activity (in plasma, erythrocyte and brain) on all occasions. Mean reductions in erythrocyte ChE in males and females ranged from 55–57% and 60–71%, respectively. A slightly reduced body weight gain in 65 ppm-treated parental females during the first two weeks of pregnancy was observed. Thereafter, to day 20, body weight gain was comparable or superior to controls at first mate of both generations, whereas they remained lower during second mate of both generations. In the offspring of the 65 ppm dimethoate treatment group a lower litter size at birth was observed in both F0 mates and second mate of F1; also a slight increase in pup mortality at lactation as well as a slower pup growth in all four mates was seen. Additionally, a slight delay in the attainment of startle reflex in first matings of both generations was observed.

A reduction in the number of successful mating was observed in the study overall, and an increased number of apparently infertile males and females in the F1 generation noted. However, these findings were re-analysed independently by the US-EPA in 1993 and opined to be not related to treatment.

At 15 ppm in F0 and F1 generations there was a less marked inhibition of erythrocyte ChE activity of 17–27% in males and 18–48% in females. Brain ChE activity inhibition in the range of 18–32% was observed in both sexes. Also a slightly reduced body weight gain was seen in females during the first two weeks of pregnancy only. There was a reduction in the number of successful matings overall and a slight increase in the number of apparently infertile males and females in the F1 generation. These findings were re-evaluated by US-EPA in 1993 and considered not to be treatment-related. The possibly adverse effects on pregnancy rate at 15 ppm were also thoroughly discussed and finally discounted by the JMPR Meeting in 1996. Taking historical control data into account, the observed reduction in pregnancy rate was not considered to be a substance-related effect, indicative of reproductive toxicity. Among the offspring, treatment at 15 ppm was without significant adverse effect, apart from a reduction in litter size at birth in three out of the four complete matings and reduced litter weight in the second mating of each generation.

There were no effects on reproduction in parents or offspring at the dietary level of 1 ppm dimethoate In addition, no effects regarding ChE activity were observed in animals treated with 1 ppm dimethoate in the diet.

There were no significant effects of treatment on organ weights. Histological examination of the reproductive organs tissues revealed no treatment-related changes. A marginal increase in testicular atrophy or reduction in spermatogenesis were seen in the 65 ppm treatment group in 4/23 animals compared to 1/24 in control animals. These changes were considered not treatment-related since, in three of four of the males affected at 65 ppm, the changes were unilateral and these animals had no reduction in fertility. Additionally, the upper historical range for this observation found in control animals is 4/24 animals. No such effects were apparent in F1 males at 1 and 15 ppm.

Compound-related reproductive toxicity was observed at 65 ppm. It was manifested as significantly decreased pup body weight during lactation in both sexes and generations. In addition the litter size at birth among F1a and F2b litters was significantly decreased. The EPA reviewers recalculated the mating index and fertility index as separate parameters. There was an apparent effect on the fertility rate following F1b, F2a and F2b mating (this end-point has not been statistically analysed). The slightly decreased mating index at 15 and 65 ppm in the F1a mating was neither dose-related nor confirmed in later matings, and was therefore not considered to be compound-related.

The pregnancy index (defined as % of surviving paired females that became pregnant) was reported by the study author and is shown in Table 26.

Table 26. Pregnancy rate (%) by study author

Dose level (ppm)	0	1	15	65
F0 Generation				
First mating	93	96	86	89
Second mating	89	93	89	71
F1 Generation				
First mating	96	71*	71*	63*[#]
Second mating	73	67	58	50

* Significantly different from control (but *p* value not given)

[#] Outside the historical range (but range not given)

The above table indicates that pregnancy may have been affected by the test compound at 65 ppm in the first generation, second mating, at 1, 15 and 65 ppm in the second generation, first mating, and at 15 and 65 ppm in the second generation, second mating.

However, the above pregnancy rates did not differentiate between dams that were pregnant but delivered no pups and females that were never pregnant (as confirmed by Salewski's method). These females were all considered by the authors to be nonpregnant. The EPA reviewer recalculated the pregnancy rates based on individual data and confirmed pregnancy status at the time of sacrifice (Table 27) and concluded that there was no compound-related effect on pregnancy rates. Even though the rates were decreased at 1 and 65 ppm in second generation mating, there was no dose–response. Therefore, these decreases were considered to be incidental in nature.

Table 27. Pregnancy rate (%) as assessed by EPA reviewer

Dose level (ppm)	0	1	15	65
F0 Generation				
First mating	100	100	100	96
Second mating	100	100	100	96
F1 Generation				
First mating	100	79	92	75
Second mating	100	79	92	75

A third mating in the last generation was conducted between the animals that had previously been unsuccessful in mating and proven breeders. When these results were included in the overall mating performances, the total number of males that failed to induced pregnancy was 2, 1, 2, and 2 in the F0 generation and 0, 2, 3, and 3 in the F1 generation at 0, 1, 15 and 65 ppm respectively. For females it was 0, 0, 0, and 1 in the F0 generation and 0, 5, 3, and 6 in the F1 generation in these same dose group.

It is also believed that the fertility in these animals may have been compromised by the fact that after two successive matings, the animals might have been the beyond the period of peak reproductive performance, when the second mating was attempted. Additionally, it is possible that the cumulative systemic effects of the compound might be manifested clinically as dullness in behaviour, leading to a real effect on reproduction (fertility).

The amount of time required for half of the paired females to mate in the first mating of F0 generation was 2.5, 3.0, 3.0, and 3.0 days for the control, low-, mid-, and high-dose groups respectively. By the second mating in this generation, the time required for mating was 3, 3, 2, and 4 days for the control, low-, mid-, and high-dose groups respectively. During the second mating, three animals of the high-dose group were cohabited for a period that was in excess of two weeks.

Similar findings were also apparent in F1 second mating, with precoital times being 3, 4 and 5 days for control, low- and mid-dose groups. Precoital times were not calculated for high-dose group F1 animals at the second matings because only half of the females in this dose group mated during the twenty day cohabition period. The precoital times tend to support the suggestion that the animals may have been disinterested in mating and the effects on fertility are secondary to the systemic effects of dimethoate.

In view of the above it can be concluded that dimethoate induces effects on adult rats in the 15 and 65 ppm groups. Reproductive parameters were only adversely affected at the treatment level of 65 ppm,. The NOAEL for parental toxicity was 1 ppm (equal to 0.08 mg/kg bw per day) on the basis of cholinesterase inhibition at 15 ppm (equal to 1.2 mg/kg bw per day). The NOAEL for reproductive toxicity was 15 ppm (equal to 1.2 mg/kg bw per day) based on decreased fertility in the F1b, F2a and F2b matings, decreased body weight during lactation in both sexes and generations and decreased litter size at birth among F1a and F2b litters, at a LOAEL of 65 ppm (equal to 5.46 mg/kg bw per day). These are secondary effects on reproduction at the high dose (65 ppm) level as a result of marked inhibition of cholinesterase. The NOAEL for offspring toxicity was 1 ppm: (equal to 0.08 mg/kg bw per day) based on reduced brain and erythrocyte cholinesterase activity at 15 ppm (equal to 1.2 mg/kg bw per day) in the F1a generation

The study was GLP compliant (Brooker, et.al, 1992).

Study 2
In another two generation dietary reproductive toxicity study, dimethoate (batch 20522-00; purity 99.1%) was administered at dose levels adjusted to provide target concentration of 0, 0.2, 1.0 and 6.5 mg/kg bw per day to groups of 25 male and 25 female Wistar rats (strain CrlGlxBrlHan:WI; 35 days old at the beginning of treatment; weight of F0 parents in the range 80.6–108.0 g for males, 78.0–101.0 g for females) during two generations producing two litters per generation, designated F1A/F1B and F2A/F2B

F1 parental animals were selected from the F1A litters and dietary concentrations were regularly adjusted according to a certain schedule throughout the treatment period. After treatment for a period of 75 days during the premating phase, parental animals in both generations were mated in a 1:1 ratio to produce F1A and F2A litters. F0 and F1 parental females were allowed to litter and rear their pups until day 4 (culling) or day 21 after parturition. At least 10 days after weaning of F1A or F2A generation pups F0 and F1 generation parental animals were mated again with different partners and females were allowed to litter and rear their F1B and F2B offspring as describe above.

Parental animals were checked twice daily for mortality and moribundity, clinical signs were observed daily. Littering and lactation behaviour of dams was evaluated in the morning and in the afternoon (littering behaviour only). Drinking water consumption was determined weekly for F0 and F1 generation parental animals during the premating period and in all F0/F1 females on gestation days (GDs) 0–1, 6–7, 13–14 and 19–20 and on lactation days (LDs) 1–2, 4–5, 7–8 and 14–15, respectively. Food consumption was determined weekly for F0 and F1 generation parental animals during the premating period and in all F0/F1 females on GDs 0–7, 7–14 and 14–20 and on LDs 1–4, 4–7 and 7–14. Food and water consumption was not determined in females without evidence of positive sperm or without litter. Body weights including body weight changes were recorded on the day of first administration and weekly thereafter for F0 and F1 parental animals. F0 and F1 parental females were weighed on the day of positive sperm and on days 7, 14 and 20 postcoitum as well as on days 1, 4, 7, 14 and 21 postpartum. F0 and F1 generation parents were weighed once weekly after weaning of F1A/F1B and F2A/F2B pups until sacrifice. Estrous cycle length was evaluated daily in all F0 and F1 parental females for at least three weeks prior to mating and thereafter until evidence of mating was observed. In male parental animals sperm parameters (sperm motility, morphology and head count) were determined. Mating, fertility, gestation and live birth index were calculated and the number of implantations recorded. At necropsy, organ weights (liver, kidney, epididymides, testes, uterus, ovaries with oviducts, seminal vesicles, prostate gland, brain, pituitary gland, adrenal gland, spleen and thyroid/parathyroid gland of F0 and F1 parental animals were determined. In addition to organs being weighed, cervix uteri and gross lesions were examined histopathologically.

After delivery of F1 and F2 litters, pups were sexed and the number of live and stillborn pups determined. Pups were regularly checked for mortality, moribundity or clinical signs. Survival rates were determined on the day of birth and on LDs 4 (viability index), 7, 14 and 21 (lactation index). Pup body weights were recorded on the days 1, 4 (before standardization), 7, 14 and 21 after birth. Brain weight was taken on day 4 postpartum from one male and one female culled or surplus pup per litter and brain ChE activity was determined. Brain ChE activity was determined for all high-dose F1B and F2B pups. All F1A pups not selected as F1 parental animals were sacrificed after standardization or weaning and all F1B as well as F2A/F2B pups were sacrificed after weaning. Sacrificed pups including stillborns and those that died during rearing were subject to macroscopic (external and visceral) examination. Serum and erythrocyte ChE activity was determined in fasted animals, that is all F0 parents during acclimatization, all F0 and F1 parents prior to mating and in all survivors at the end of treatment. On necropsy, brain samples were taken from fasted F0 and F1 parents and brain ChE activity determined.

There were no substance-related or spontaneous mortalities in any group of treated male orfemale F0 parental animals. All F0 rats were terminated on scheduled sacrifice.

No clinical signs or changes of general behaviour which may be attributed to the test substance were detected in male or female F0 generation parental animals. In addition, there were no substance- or dose-related clinical findings in F0 females during the gestation and lactation period for F1A and F1B litters nor findings such as spontaneous alopecia or kinked tail seen in individual animals only. There were no effects on mating and fertility.

At the dose of 6.5 mg/kg bw per day group, F0 animals showed statistically decreased erythrocyte and brain ChE activity in both sexes, but more pronounced in females (up to 60%–70% below control values). There was focal vacuolization of the epithelium of the corpus epididymidis in 7 of 25 animals. In F1A and F1B no test substance-related adverse effect were observed. In F1 parental animals there was statistically significant body weight gain of the dams during lactation of the F2B litters (about 60% below control).There was statistically significantly decreased erythrocyte and brain ChE activity in both sexes, but more pronounced in females (up to 60%–70% below control values). There was also significantly decreased mean absolute and relative weights of the prostate gland. Increased incidence of males with diffuse atrophy of the epithelium of the dorso-lateral part of the prostate gland was seen in 15 of 25 rats. There was an increased number of animals with reduced secretion in the dorsolateral part of the prostate gland in 19 out of 25 males. Focal vacuolization of the epithelium of the corpus epididymidis was seen in 13 of 25 animals (Table 28). There were higher grades of focal vacuolization of the epithelium of the cauda epididymidis in 6 of 25 animals.

However there was no test substance-related adverse effects seen in F2A and F2B pups.

Table 28. Findings in the epididymides and prostate of F1 parental males on necropsy

	Dose level (mg/kg bw per day)			
	0	**0.2**	**1.0**	**6.5**
Organ	**Incidences**			
Epididymides				
Slight to severe focal vacuolization of the epididymal epithelium	0	0	0	13
Dorso-lateral part of prostate				
Moderate and severe diffuse atrophy of the glandular epithelium	7	4	2	15
Minimally to severely reduced secretion	12	14	15	19

Source: Mellert *et al.,* 2003

No test substance-related adverse effect on any of the parameters was seen in either the 1 mg/kg bw per day group or the 0.2 mg/kg bw per day group.

In view of the above, it can be concluded that no developmental toxicity in the offspring was evident and effects seen, such as reduced pup body weight and pup body weight gain in F1B pups or reduced viability index (F1B pups) and reduced lactation index (F2B pups), were either secondary to maternal toxicity or covered by the range of historical controls.

The NOAEL for parental toxicity and offspring toxicity was 1.0 mg/kg bw per day based on statistically significant reductions on body weight gain of high dose F1 females during lactation of F2B pups, histopathological findings in prostate and epididymides of high dose F0 and F1 males as well as statistically significant and toxicologically relevant reductions of erythrocyte and brain ChE activities in high-dose F0 and F1 parental generations of both sexes at 6.5 mg/kg bw per day. The NOAEL for reproductive toxicity was 6.5 mg/kg bw per day, the highest dose tested.

The study was GLP compliant (Mellert, et al., 2003).

Study 3

A dietary, one-generation study was carried out aimed at clarifying the effect of maternal exposure during lactation on offspring viability and growth. Dimethoate (batch 611/A; purity 98%) was administered to Crl:CD,(SD) rats. F0 generation male rats (ca six weeks old) were exposed to the test substance (6.5 mg/kg bw per day) and/or the carrier at least 90 days before cohabitation, through cohabitation (maximum of 17 days), and continuing until euthanasia. Male rats were euthanized after completion of the cohabitation period. F0 generation female rats (ca six weeks old) were exposed to the test substance (6.5 mg/kg bw per day) and/or the carrier at least 90 days before cohabitation, through cohabitation (maximum of 17 days), and continuing through GD 25 (rats that did not deliver) or day 21 postpartum (rats that delivered a litter). The F1 generation pups may have been exposed to the test substance or carrier in utero during gestation or via maternal milk and maternal food during the postpartum period. The experimental design is shown on the following page in Table 29.

Table 29. Experimental design

Group No.	No. of F0 generation rats		Dose Material	Dose (mg/kg bw per day)
	Males	Females		
1	14	14	Certified Rodent Diet® #5002 (PMI® Nutrition International)	0 (Carrier control)
2	14	14	dimethoate[a]	6.5[b]
3	14	14	dimethoate[a]	6.5[c]

[a] The test substance was considered 98.0% pure for the purpose of dosage calculations

[b] Rats were exposed to a dietary concentration calculated to give a constant dose of 6.5 mg/kg bw per day of dimethoate throughout the study (pre-mating, mating, gestation and lactation periods). The concentration of the test substance in the diet was determined based on the mean body weights and food consumption for the female rats and the male rats received the same diet concentration as the female rats.

[c] The rats were exposed to a dietary concentration calculated to give a constant dose of 6.5 mg/kg bw per day of dimethoate throughout the pre-mating, mating and gestation periods. The dietary concentration was not adjusted during the lactation period but remained at the level administered during gestation. The concentration of the test substance in the diet was determined based on the mean body weights and food consumption for the female rats and the male rats received the same diet concentration as the female rats.

Mortality was observed in both dimethoate exposure groups in the female rats during the lactation phase of the study. In both dimethoate exposure groups there was also a reduction in body weight gains observed in the male rats, a treatment-related decrease in the number of pregnancies that occurred, an increase in the absolute and relative food consumption values that occurred in the female rats during the gestation period and a dose-dependent decrease in the brain and erythrocyte ChE values in both male and female rats. There were also changes in organ weights observed in male rats in both dimethoate exposure groups. In the F1 generation pups there was an increase in pup mortality, adverse clinical signs and necropsy observations, along with a decrease in pup body weights; these effects were observed in both dimethoate exposure groups. Apart from common findings in females of both Group 2 and Group 3 during the lactation period, there were additional findings in Group 3 female rats, including adverse clinical signs, body weight loss or reductions in body weight gain and reductions in food consumption during the lactation period. Two female rats had slight or moderate continuous whole body tremors during the postpartum period, and one of these females was also observed with a hunched posture. A statistically significant body weight loss occurred on LDs 4 to 7 and during the entire lactation period in Group 3. There was also a statistically significant decrease observed in the mean body weight on LD 17. There was an increase in the number of pups observed with coldness to the touch (whole body), pups observed not to be nursing, and pups with no milk band present in their stomachs.

There were statistically significant dose-dependent decreases in the both the brain and the erythrocyte ChE values of female rats in both dimethoate exposure groups.

Dose levels of Group 2 and Group 3 were similar during precohabitation and gestation. During lactation dose levels for Group 3 were distinctly higher compared to Group 2, resulting in more pronounced toxicity affecting the Group 3 F0 generation females and F1 generation pup development. In view of the above it can be seen that the dose of dimethoate to which parental females in Group 3 were exposed during lactation was higher than that during the precohabitation and gestation periods, resulting in more pronounced toxicity and effects on F1 generation pup development.

The study was GLP compliant (Barnett, 2015a).

Study 4

In another published study, the effects of dimethoate (purity 98%) on the reproductive system of Wistar rats (male's weights at dosing 180–210 g, female's 160–180 g) were investigated. Accordingly, male and female Wistar rats were treated with dimethoate at concentrations of 0.01 and 0.1 mg/kg bw per day via oral gavage for 10 weeks. Subsequently, treated males and females were mated with untreated animals. The study was terminated on day 20 of pregnancy and reproductive parameters were recorded. In conclusion, dimethoate administered at concentrations of 0.01 and 0.1 mg/kg bw per day via oral gavage for 10 weeks produced no signs of systemic toxicity in parental animals. No significant effects on sperm parameters were observed in males in any of the treatment groups. Variations in the frequency

and duration of the estrous cycle were observed in treated females during the last two weeks of the treatment period. The conception and fertility indices were significantly lower in females treated with 0.1 mg dimethoate/kg bw per day, compared with the control. Significantly reduced fetal weight was observed at 0.01 mg/kg bw per day. However, the effect was only observed at the lower dose level. Macroscopic examination did not reveal any treatment-related abnormalities.

The study was not GLP compliant (Prodanchuk & Shepelska, 2007).

(b) Developmental Toxicity

Rat

Dimethoate (batch: 611A; purity 97.3%) was assessed in a preliminary study with Sprague Dawley rats (CrL: COBS CD (SD) BR strain) to help set dose levels for a subsequent embryotoxicity/teratogenicity study.

Mated female rats (body weight range 176–207 g), six per group, were dosed with dimethoate by gavage at levels of 0, 3, 10 or 30 mg/kg bw per day from day 6 to day 15 of pregnancy. The test substance was suspended in 1% aqueous methylcellulose and was freshly prepared each day.

Parent animals treated with 30 mg/kg bw per day showed clinical signs due to treatment (for example body tremor, increased respiration, abnormal faeces) and a marked decrease in body weight gain. Litters showed slightly reduced mean fetal weight at this dose, but there were no obvious external fetal abnormalities. The clinical reaction to treatment was slight in the parental animals at 10 mg/kg bw per day. The proportion of dams with immediate postdosing salivation increased with increasing dose: 0/6 (control), 1/6 (3 mg/kg bw per day), 3/6 (10 mg/kg bw per day), 5/6 (30 mg/kg bw per day).

Taking into account the marked effects at 30 mg/kg bw per day and the slight effects at 10 mg/kg bw per day observed in parental animals the following dose levels were selected for the main study: 0, 3, 6, 18 mg/kg bw per day. The study was GLP compliant (Edward, Leeming and Clark, 1984a).

Time-mated female Crl:COBS CD (SD) BR rats, supplied by Charles River UK, body weight 170–201 g, were used. Animals were housed five to a cage (galvanized metal cages). Dimethoate (batch 611A; purity 97.3%) in 1% aqueous methylcellulose was administered daily by gavage on GDs 6–15 to randomly assigned groups of 25 mated female rats at dose levels of 0, 3, 6 and 18 mg/kg bw per day. Dosing suspensions were prepared freshly each day. The animals were sacrificed on GD 20. Animals had free access to diet and tap water. Parent animals were observed for clinical signs. Body weight and food consumption were recorded. The uterus and ovaries were examined histologically at termination. Litter parameters recorded were: number of corpora lutea, number and sex-distribution of live young, embryonic and fetal deaths, fetal weight and foetal abnormalities.

In dams, signs of toxicity due to dimethoate were seen at 18 mg/kg bw per day and included body tremor, hypersensitivity and abnormal gait. Body weight gain and food consumption were also reduced at this dose level. Signs at 3 mg/kg bw per day were confined to postdose salivation and at 6 mg/kg bw per day this was accompanied by some faeces being small and rounded. It is noted that the proportion of dams with immediate postdosing salivation increased with increasing dose: 0/25 (control), 5/25 (3 mg/kg bw per day), 6/25 (6 mg/kg bw per day), 12/25 (18 mg/kg bw per day). However, a more detailed evaluation revealed that salivation was slight and apparently transient at all dose levels. Also, whilst at 18 mg/kg bw per day salivation was seen in 3–7 rats per day from days 8–15, at 3 mg/kg bw per day and 6 mg/kg bw per day it was seen only once in any individual over the whole study period (with the effect seen only from days 8–12). In addition, salivation is a quite common effect in gavage studies and is generally not considered to be of toxicological relevance. For organophosphate-related salivation a rather prolonged effect would be expected, but the observed effect was described as “immediately post dosing” further supporting the supposition that effects noted occurred incidentally and were most likely related to gavage application.

There were no adverse effects on any litter parameter due to treatment and no effect on the incidences of malformations, visceral or skeletal anomalies or variants.

Table 30. Group mean litter parameters

(Dose)	Group 1 0 (control)	Group 2 3 mg/kg bw/day	Group 3 6 mg/kg bw/day	Group 4 18 mg/kg bw/day
Nunber of animals	25	22	23	23
Pregnancy rate (%)	100.0	88.0	92.0	92.0
Live young	11.0	11.9	11.2	11.0
Embryonic deaths				
Early	0.5	0.3	0.7	0.4
Late	0.2	0.1	0.1	0.1
Total	0.7	0.4	0.8	0.5
Implants	11.8	12.2	12.0	11.6
Corpora lutea	12.6	12.7	12.7	12.7
Preimplant loss (%)	6.0	3.7	4.7	7.9
Postimplant loss (%)	6.1	3.1	7.0	4.7
Litter weight. (g)	39.16	41.44	39.86	39.02
Mean fetal weight. (g)	3.55	3.51	3.57	3.53

In view of the above, the NOAEL for offspring toxicity was 18 mg/kg bw per day, the highest dose tested, as dimethoate did not cause any embryotoxic, fetotoxic or teratogenic effects in rats in this study. The observed salivation postdosing in the 3 and 6 mg/kg bw per day groups were not considered an adverse effect. In dams, changes of toxicological significance (body tremor, hypersensitivity and abnormal gait, redued body weight gain and food consumption) occurred at the top dose level only, giving a NOAEL for maternal toxicity of 6 mg/kg bw per day. Acetylcholinesterase activity was not measured in this study.

The study was GLP compliant (Edwards, Leeming, & Clark, 1984b).

Rabbit

Dimethoate (batch 611A; purity 97.3%) was assessed in a preliminary study with New Zealand White rabbits (body weight range 2.9–4.1 kg) to determine dose levels for a subsequent embryotoxicity/ teratogenicity study.

The rabbits (six females/group) were mated and dosed by gavage at 0, 3, 10 or 30 mg /kg bw per day from GDs 7–19. Additionally, in a separate assessment unmated females (two rabbits/group) were orally treated with 50 or 75 mg/kg bw per day for 13 days. Dimethoate was applied within 1% aqueous methylcellulose and dosing suspensions were prepared freshly each day. Apart from a slightly lower body weight gain at 30 mg/kg bw per day, there were no treatment-related effects seen in parental animals or their offspring. In a separate assessment with unmated female rabbits (two rabbits/group) dosed at 50 or 75 mg /kg bw per day orally for 13 days, the animals showed muscle tremor, lethargy, prostration and weight loss.

In face of the findings observed up to 30 mg/kg bw per day in pregnant rabbits and at 50 or 75 mg/kg bw per day in unmated female rabbits, dose levels for the following main study were set at 0, 10, 20 and 40 mg/kg bw/day.

The study was GLP compliant (Edwards, Leeming, & Clark, 1984c).

In the main study, non-pregnant female New Zealand White rabbits (weight 2.9–4.1 kg) were used. After coitus, each female received an injection of luteinizing hormone. Dimethoate (batch 611A; purity 97.3%) in 1% aqueous methylcellulose was administered at levels of 0, 10, 20 or 40 mg/kg bw per day to groups of 16 mated female rabbits by daily gavage on GDs 7–19. The animals were sacrificed on GD 29. During this time, adult animals were observed for clinical signs and body weights and food consumption were recorded. The uterus and ovaries were examined at termination. Pups were examined in vivo (in utero) and subsequently sacrificed for further examinations. Litter parameters recorded

included: number of corpora lutea, number and distribution of live young, embryonic and fetal deaths, fetal weight and fetal abnormalities.

No clinical signs in mother animals at 10 and 20 mg/kg bw per day were observed. At 40 mg/kg bw per day muscle tremor, unsteady gait, cold ears and abnormal faeces were observed at a late stage of treatment. Mean body weight gain and food consumption were reduced at this dose level. At 20 mg/kg bw per day there was a slight and statistically not significant reduction in the overall body weight gain (14% less compared to controls). There was no effect on the incidences of malformations, visceral or skeletal anomalies or variants (see Table 34). There were no adverse effects on litter parameters due to treatment, except for a slight (9%), but statistically significant reduction in mean fetal weight at 40 mg/kg bw per day (see Table 35). This slight decrease might be linked to the fact, that a parallel increase in mean number of live young (not statistically significant) had also been noted.

Dimethoate did not cause embryotoxic or teratogenic effects in this study. The effects on fetal weight at 40 mg/kg bw per day were not considered toxicologically relevant due to the parallel increase in mean number of live young. Thus, a NOAEL of 10 mg/kg bw per day for maternal toxicity was identified based upon effects on weight gain at a 20 mg/kg bw per day dose level in dams. The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, the highest dose tested.

The study was GLP-compliant (Edwards, Leeming & Clark, 1984d).

2.6 Special studies

(a) Acute neurotoxicity

Gavage administration

In a dose range-finding acute study, male and female Sprague Dawley Crl: CD BR rats, 25–31 days old were taken to investigate the neurotoxicity potential of dimethoate (batch 20522-00; purity 99.1%). On the basis of this study, the dose levels of 2, 20 and 200 mg/kg bw were selected for a rat acute neurotoxicity study with dimethoate. The study was GLP compliant (Lamb, 1993a).

In the main study that complied with the United States Environmental Protection Agency GLP regulations (EPA, 1999) groups of 12 male and 12 female Sprague Dawley Crl:CD BR rats (aged 43 days) were given dimethoate (purity 99.1%) at 0, 2 or 20 mg/kg bw by gavage as a single dose in water. Additional groups of 15 male and 15 female rats received dimethoate at a dose of 200 mg/kg bw.

Observations and examinations were carried out until day 14 after dosing, when surviving animals were sacrificed. Viability, clinical signs and body weight were recorded. A functional observational battery (FOB) and motor activity evaluations were performed before treatment, at the time of peak effect 2 h after dosing (as determined in a dose range-finding study), and on days 7 and 14. Brain weight and dimensions were measured at termination. All animals were perfused in situ and neuropathological examinations were carried out on five randomly selected animals of each sex in the control group and in the group receiving a dose of 200 mg/kg bw.

No mortalities were observed during the study. A lower body weight gain was measured in males over days 0–7 (−38%) at 200 mg/kg bw. The most remarkable clinical signs at this dose were observed on days 1 and/or 2 and included gait alterations (rocking, lurching or swaying), tremors (whole body or forelimbs/hindlimbs) and constricted pupils. Other clinical signs at 200 mg/kg bw consisted mainly of coloured material on the body and decreased defaecation, and were observed on days 1, 2 and/or 3, but persisted until day 5 for one male. The most notable effects on FOB parameters in males and females were alterations in posture, convulsions, tremors and changes in faeces consistency during the home cage observations; lacrimation, salivation and changes in fur appearance during the handling observations; impaired mobility, gait alterations and decreased rearing activity during the open-field observations; alterations in approach, touch, startle, tail pinch and pupil responses, forelimb extension and air-righting reflex during the sensory observations; reduced hindlimb extensor strength, reduced forelimb grip strength and impairments in rotarod performance during the neuromuscular observations; increased catalepsy times and decreased body temperature during the physiological observations; these were noted at 200 mg/kg bw. Additionally, treatment-related reductions in mean ambulatory and total motor activity in males and females were apparent at this dose. The effects were noted approximately 2 h after treatment

on day 0, and were transient in nature (gait alterations, tremors and constricted pupils persisted until days 1 and/or 2 for some animals), but on the basis of cageside observations some symptoms persisted until day 5. None of the above signs were apparent on days 7 or 14. The only treatment-related effect on FOB parameters at 20 mg/kg bw was the absence of pupil response (5/12 males and 6/12 females affected versus 0/12 and 2/12 among the controls). No treatment-related changes in brain weights and dimensions or in central or peripheral nervous system tissues examined microscopically were observed at any dose. The presence of one female (of the five examined) at 200 mg/kg bw with minimally swollen sciatic nerve axons (none in concurrent control group) was not considered to be significant compared with the maximum incidence of 17% among historical controls. Acetylcholinesterase activity was not measured in this study.

The NOAEL for acute neurotoxicity in rats treated by gavage was 2 mg/kg bw on the basis of an absence of pupil response at 20 mg/kg bw (Lamb, 1993b; JMPR, 2003).

Administration in the diet

Groups of 24 male and 24 female Sprague Dawley Crl:CD BR VAF Plus rats (aged 8–9 weeks) received diets containing dimethoate (purity 99.1%) at 0, 25.8, 51.7, 77.5 and 387 ppm. for males, 0, 23.8, 54.4, 81.5 and 407.5 ppm for females. This provided a single nominal dose of 0, 1, 2, 3 or 15 mg/kg bw, in the study. In a two-week feeding adaptation phase before administration of the test material, the animals were conditioned to eat their daily ration of food over a short period of time. During this phase, animals received two-thirds of their daily food ration for 1 h between 06:00 and 07:00 and one-third of their daily ration of food for 1 h between 17:00 and 18:00. The test material was administered once on day 1 during the first daily feeding. Calculated intakes of dimethoate, which were close to the intended intakes, were 0, 0.96, 1, 95, 2.94 and 15.01 mg/kg bw per day for males and 0, 0.98, 2.11, 3.23 and 15.01 mg/kg bw per day for females. For the remainder of the study, rats had access to the control diet ad libitum until termination on day 15. Doses and time of peak effect (2.5–3 h after dosing) for FOB and/or detailed clinical observation, and for effects on ChE activity were determined in two preliminary studies. Eight animals of each sex per dose were sacrificed for determination of ChE acivity (in plasma, erythrocytes and brain) on day 1, at time of peak effect. The remaining 16 animals of each sex per group were subjected to FOB and determination of locomotion activity on day 1 (time of peak effect) and on day 15. After these observations on day 15 all animals were sacrificed and determinations of ChE activity were conducted on eight animals of each sex per group, and gross necropsy conducted on the remaining eight animals of each sex per group. Clinical observations and determination of body weights and food consumption were made during the study. No histopathological examination was carried out. Determinations of ChE activity (also measured before dosing in all animals) involved reaction with acetylthiocholine and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The separated plasma and erythrocyte samples and brain sections were stored frozen at −70°C for up to two weeks if determination of ChE activity was not possible on the day of sample collection.

There were no deaths or treatment-related clinical signs of toxicity and no effects on body weight or food consumption. FOB parameters and examination of motor activity showed no effects that could be attributed to the test material. Plasma ChE activity was statistically significantly reduced on day 1 at 15 mg/kg bw in males (50% of control value) and in females (60% of control value); no significant reduction was observed on day 15. Erythrocyte ChE activity was statistically significantly reduced on day 1 at 15 mg/kg bw in males (42% of control value) and in females (35% of control value) and at 3 mg/kg bw in males (71% of control value), while the reduction in males at 2 mg/kg bw (75% of control value) was not statistically significant. No significant differences were observed on day 15. Brain ChE activity was significantly reduced on day 1 in both sexes at 15 mg/kg bw (hippocampus: 67% and 62% of control value in males and females, respectively; cortex: 64% and 57% of control value in males and females, respectively; striatum: 59% and 53% of control value in males and females, respectively) and in females at 3 mg/kg bw (cortex, 89% of control value). On day 15, inhibition of ChE activity in the hippocampus was still statistically significant in both sexes at 15 mg/kg bw (84% and 85% of control value in males and females, respectively). There were no treatment-related effects on weight of the brain or on ChE activity in the three regions of the brain investigated, and no macroscopic findings on days 1 or 15.

The NOAEL was 2 mg/kg bw on the basis of a statistically significant inhibition of ChE activity in erythrocytes in males (29%) and in the brain cortex in females (11%) at 3 mg/kg bw. Recovery at 14 days after dosing appeared to be incomplete at the highest dose of 15 mg/kg bw, since there was still a slight (biologically not relevant as the reduction is below the trigger value of 20%). but statistically significant reduction of ChE activity (15–16%) in the hippocampus.

The study was GLP compliant (Schaefer, 1999; JMPR, 2003).

(b) Short-term study of neurotoxicity

In a study, groups of 10 male and 10 female (12 male and 12 female at the highest dose) Sprague Dawley strain Crl:CD BR rats (aged 50 days) were given diets containing dimethoate (purity 99.1%) at concentrations of 0, 1, 50 or 125 ppm for 91, 92, 93 or 94 days. These dietary concentrations provided intakes of 0, 0.06, 3.22 or 8.13 mg/kg bw per day for males and 0, 0.08, 3.78 or 9.88 mg/kg bw per day for females, Rats were randomly assigned to one of four study replicates on which to conduct FOB and tests for locomotor activity. Five rats of each sex from the control group and from the groups receiving the lowest and intermediate doses, and seven males and five females from the group receiving the highest dose were allocated for determination of plasma, erythrocyte and brain ChE activities; the remaining five rats of each sex per group were allocated for neuropathological investigations. Clinical observations were recorded daily, body weights and food consumption were measured weekly, FOB and locomotor activity were assessed at weeks 3, 7 and 12. Cholinesterase activity was measured in plasma and erythrocytes before dosing and at weeks 3, 7 and 13, and in brain at week 13. Animals were not fasted before blood collection. Cholinesterase measurements in brain, and initial measurements in blood were performed according to the method of Dietz, Rubinstein & Lubrano (1973); this method uses the Ellman reaction, but the substrate is propionylthiocholine, not acetylthiocholine as proposed by Ellman et al. (1961). Later in the study, measurements of ChE activity in erythrocytes and plasma were conducted at another laboratory by a method that used the Ellman reaction with acetylthiocholine as the substrate. All samples were stored at −20°C until analysis. Erythrocytes, plasma and brain from week 13 were stored for 3–7 days until analysis. Brains were weighed and dissected for measurement of brain ChE activity at termination. Tissue from the central and peripheral nervous system was dissected and processed for histopathological examination after perfusion.

No mortalities occurred during the study. Small faeces in females at 50 mg/kg and in males and females at 125 mg/kg were noted primarily during weeks 2–6 of treatment. At 125 mg/kg the mean body weights of males were slightly reduced during weeks 3–13, and cumulative mean body weight gains were lower in weeks 0–1 compared to weeks 0–13. No treatment-related effects on food consumption, FOB (home cage, handling, open field, sensorimotor, neuromuscular and physiological observations) and locomotor activity (total and ambulatory motor activity) were apparent at any dose. Plasma ChE activity was statistically significantly inhibited in males at 50 mg/kg (weeks 7 and 13) and at 125 mg/kg (weeks 3, 7 and 13). Erythrocyte ChE activity was statistically significantly inhibited at weeks 7 and 13 in both sexes at 50 mg/kg (53% and 51% of control value in males, 66% and 58% in females) and at 125 mg/kg (40% and 47% of control value in males, 45% and 43% in females). At 1 mg/kg, erythrocyte ChE activity in males was slightly, but statistically significantly reduced at week 7 (89% of control value), but no significant inhibition was seen at week 13. Statistically significant inhibition of ChE activity was observed at 125 mg/kg in both sexes in the following brain regions: olfactory region (82% of control value in males), midbrain (85% of control value in males, 82% in females), brainstem (83% of control value in males, 80% in females) and cortex (88% of control value in males). No differences were seen in absolute or relative brain and brain-region weights and no treatment-related effects were observed on neuropathological examination.

The NOAEL for systemic toxicity and neurotoxicity was 1 mg/kg (equal to 0.06 mg/ kg bw per day) on the basis of inhibition of erythrocyte ChE activity (34–49%) and small faeces at 50 mg/kg (equal to 3.22 mg/kg bw per day).

The study was GLP compliant (Lamb, 1994; JMPR, 2003).

(c) Developmental neurotoxicity

In a dose range-finding study, groups of 15 pregnant female rats (strain Crl: CD®BR; 10–11 weeks old; weight range 215–277 g), were administered dimethoate (batch 20522-00; purity 99.1%) in water by gavage (5 mL/kg bw) at dose levels of 0, 0.2, 3 and 6.0 mg/kg bw per day.

The study was conducted in two groups.

- **Study group A** Five of the 15 females were treated from GD 6 to 20 and were killed within three hours after last dosing. Litter data were assessed. Cholinesterase activity was determined in maternal and fetal plasma, erythrocytes and brain.
- **Study group B** The remaining 10 of the 15 animals were treated from GD 6 to postnatal day (PND) 10. Offspring (two females and two males) from each of these females were also dosed by gavage from PND 11 to 21.

Litter observation took place throughout the study and detailed examination of the offspring was performed daily from PND 1 to 21. Number of offspring (live or dead) body weight, sex ratio and dam/litter interaction were noted. The treated offspring were killed two hours after the last dosing on PND 21 and ChE activity was determined in plasma, erythrocytes and brain The remaining offspring in each litter were retained undosed to act as a "within-litter" control group.

Clinical signs, body weight and food consumption of the dams were recorded several times throughout the study. Date of parturition and duration of gestation were noted.

Necropsy of dams, fetuses and offspring was performed at termination. In addition, necropsies were performed on sections of the perfused brains of four undosed pups (PND 21) to gain experience in prefusion procedure and histological sectioning of PND 21 brains.

Acetylcholinesterase activity

Blood samples were collected under light isoflurane anaesthesia from the retro-orbital sinus (adults and pups) and umbilical cord (fetuses). The blood samples on GD 20 were taken 3 h after dosing and the samples on PNDs 11–21 taken 2 hours after dosing. The fetal blood samples on GD 20 were pooled per litter. The blood samples were cooled on ice-water until processing and centrifugation. The resulting plasma samples and erythrocyte haemolysates were stored at −80°C until analysis.

Brains were removed immediately after sacrifice, weighed and frozen in liquid nitrogen and stored until analysis. The GD 20 fetal brains were pooled for male and female fetuses per litter. Cholinesterase activity was determined according to a modified Ellman method (EPA, 1996). Erythrocyte ChE activity was measured using DNTA, plasma and brain activity was measured using DNTB as a colour component.

At GD 20 dams were sacrificed by carbon dioxide directly after blood sampling and their brains collected immediately. The reproductive tract was examined for the following parameters: number of corpora lutea, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each uterine horn. Fetuses were weighed and sexed, and sacrificed on a cool plate at 0 °C. The brains of the fetuses were then sampled.

All dams were examined thoroughly macroscopically and specimens of any abnormal tissue retained.

Pups treated up until PND 21 were killed immediately after blood sampling by inhaled carbon dioxide. Their brains were removed, weighed and snap frozen in liquid nitrogen.

All pups were examined carefully macroscopically and specimens of any abnormal tissue retained.

The culled PND 4 pups were euthanized with an injection of barbiturate and not examined further.

Effects in dams

There were no clinical signs that were considered treatment-related. No mortalities occurred throughout the study. No biologically relevant effect on any of the investigated parameters was noted at 0.2 mg/kg bw per day.

Body weight was affected at doses of 6 mg/kg bw per day and 3 mg/kg bw per day. A statistically significantly reduced maternal body weight gain from GD 6 until 20 was observed at 6 mg/kg bw per day and 3 mg/kg bw per day. A lower mean body weight (but not statistically significantly so) persisting to PND 17 was noted for both doses. Food consumption was not affected by the treatment. No treatment-related findings were noted at necropsy at GD 20 and PND 21. Brain weight was also unaffected.

At GD 20 (study Group A) there was a clear inhibition of plasma, erythrocyte and brain ChE in the dams of the mid- and high-dose groups. For plasma, erythrocytes and brain ChE the inhibition figures at 3 mg/kg bw per day and 6 mg/kg bw per day were 25%, 78%, 75% and 57%, 85%, 88% , respectively. No effects were noted at 0.2 mg/kg bw per day.

Effects in fetuses/offspring (study Groups A + B)

Study A

Litter parameters at GD 20 and the number of implantation sites were unaffected by the treatment. However, a clear inhibition of plasma, erythrocyte and brain ChE in the fetuses of the mid- and high-dose groups was noted. For plasma, erythrocytes and brain ChE the inhibition figures in male fetuses were 75%, 70%, 22% at 3 mg/kg bw per day and and 79% 87%, 35% at 6 mg/kg bw per day. The corresponding figures for females treated with 3 mg/kg bw per day and 6 mg/kg bw per day were 66%, 82%, 24% and 73% 96%, 42%, respectively. No significant sex difference in the inhibitory effects on ChE can be deduced from these findings.

Study B

The number of total litter size at PND 1 was not affected by the treatment, confirming that there was no effect on postimplantation survival up to the time of birth. An increased postnatal mortality was observed at 6 mg/kg bw per day. Two normal-sized litters died at PNDs 2 and 5. One dam lost four out of 14 offspring at PND 4, resulting in lower litter size and viability indices at PND 4. At this dose, group mean body weight and body weight gain of the offspring from PNDs 1–11 was reduced. No effects were noted for these parameters at 3 mg/kg bw per day. Direct dosing of offspring from PND 11 to 21 did not affect offspring survival, body weight or body weight gain at any dose level.

A clear inhibition of plasma, erythrocyte and brain ChE in the offspring in the mid- and high dose-groups was noted. For plasma, erythrocytes and brain ChE the inhibition figures in male offspring were 39%, 60%, 45% at 3 mg/kg bw per day and 60% 70%, 55% at 6 mg/kg bw per day, respectively. The corresponding figures for females treated with 3 mg/kg bw per day and 6 mg/kg bw per day were 40%, 65%, 42% and 60% 80%, 66%, respectively. No significant sex difference in the inhibitory effects ChE was found in the offspring for the doses tested.

Based on the findings in this preliminary study, it can be concluded that 6 mg/kg bw per day is not suitable for use in a developmental neurotoxicity study, as a lower birth weight at this dose and evidence of an increase in early postnatal pup death was observed. Direct dosing of offspring between PNDs 11 and 21 was without obvious toxicity at all dose levels, but marked ChE inhibition (up to 80% for erythrocyte inhibition in females) was noted at termination. A dose of 0.2 mg/kg bw per day was considered to represent the NOAEL for dams and their offspring under the conditions of this study.

The study generally followed GLP, but no specific study-related QA procedures or analysis of dose form were performed (Myers, 2001a).

In the main study, groups of 24 pregnant female Crl:CD BR rats (aged 10–11 weeks) were given dimethoate (purity 99.1%) at doses of 0, 0.1, 0.5 or 3 mg/kg bw per day by oral gavage in water from GD 6 until PND 10. Offspring received the same doses by oral gavage on PNDs 11–21. This study complied with United Kingdom GLP regulations 1999, European Commission Directive 1999/11/EC, OECD GLP principles 1997, and EPA guideline OPPTS 870.6300. Clinical observation was performed on all dams at least twice daily throughout the study. Body weight and food consumption were recorded several times throughout the study; parturition and duration of gestation were noted. Ten dams per group were subjected to a detailed clinical examination and open arena observations on GDs 12 and 18, and on PNDs 4 and 10. On PND 4, litters were culled to eight pups each (four males and four females when possible). Five pups from each litter were allocated to undergo further functional investigations (motor activity on PNDs 13, 17, 22 and 59; auditory startle response habituation and prepulse inhibition of startle on PNDs 23/24 and 60/61; learning and memory on PNDs 23/24 and 61/62), while a sixth pup was

sacrificed on PND 11 for examination of the brain. In all litters except two in the group receiving a dose of 3mg/ kg bw per day, a different pup was allocated to each behavioural test. Physical development of the pups was assessed by measurement of body weight. Sexual maturation of female pups was assessed by age at vaginal opening, and maturation of males was assessed by age at balano-preputial separation. Dams were sacrificed on PND 21 and subjected to gross necropsy, abnormal tissues being retained for possible histopathological examination. Groups of 10 male and 10 female offspring were selected for detailed neuropathological examination and sacrificed on PND 21 (males) or PND 65 ± 2 (females).

Treatment of dams with a dose of 0.1, 0.5 or 3 mg/kg bw per day had no adverse effect on clinical condition, survival, body weight gain or food intake during gestation and lactation, gestation length, macroscopic necropsy findings or brain weights. There was no evidence for neurotoxicity in the dams, according to FOB assessments. There was no effect of treatment on mean implantation rate, litter size or on mean pup weights on PND 1. At 3 mg/kg bw per day, all offspring in six litters showed signs of poor general condition or retarded development during early lactation. Three affected litters at 3 mg/kg bw per day and one litter at 0.5 mg/kg bw per day were killed on PNDs 2–4 for reasons of animal welfare. In addition to these deaths of entire litters during early lactation, there was an increase in pup mortality among litters that survived to weaning in the group receiving a dose of 3 mg/kg bw per day. The number of pups found dead or that were killed up to PND 21 was 15, 11, 24 and 44 respectively for the control group and the groups receiving the low, intermediate and high doses. Background data on controls from five studies in which littering took place between October 2000 and September 2002 showed that up to one litter was found dead or killed for reasons of animal welfare and a range of 10 to 33 pups were missing, found dead or killed among litters surviving to weaning at PND 21. At 3 mg/kg bw per day, body weight gains of male and female offspring during PNDs 1–4 were about 30% lower than those of controls, but the differences did not attain statistical significance. Thereafter, weight gains were comparable or only marginally inferior to those of controls, such that overall gains during PNDs 1–21 were about 10% lower than those of controls. Direct dosing of offspring during PNDs 11–21 did not adversely affect body weight gains and no clinical signs were observed that were considered to be related to treatment. Among offspring maintained until PND 65 ± 2, there was no effect of treatment on general clinical condition, survival, body weight gains during PNDs 21–63 or age at attainment of sexual maturation. Treatment-related differences in the functional performance of offspring were limited to the groups receiving a dose of 3 mg/kg bw per day, before weaning. On PND 4, male and female offspring tended to be less active than the controls, as shown by lower values for maximum pivoting angle, maximum distance travelled and number of sections entered in the arena. Although these differences did not achieve statistical significance, the consistency between all three measures, and between males and females did indicate an effect of treatment. Reduced arena activity was also observed in males and females on PND 21. There was no evidence of any treatment-related effect in offspring performance during postweaning observations and functional testing, including monitoring of learning and memory, auditory startle response and sexual maturation. There was no effect of treatment on findings made on macroscopic necropsy or on brain weights of selected offspring killed on PNDs 11, 21 or 65 ± 2, or on brain length and width on PNDs 21 and 65 ± 2. Also, there was no effect of treatment on histopathological findings or brain morphometry for selected offspring killed on PNDs 21 or 65 ± 2.

Treatment with dimethoate at a dose of up to 3 mg/kg bw per day (the highest dose tested) was not associated with any selective developmental neurotoxicity. The NOAEL for functional development of the nervous system and systemic toxicity in the offspring was 0.5 mg/kg bw per day, on the basis of developmental delay in some functional parameters and increased pup mortality at a dose of 3 mg/kg bw per day, after maternal treatment by oral gavage from GD 6 until PND 10, and direct treatment of the offspring on PNDs 11–21.

The study was GLP compliant and a QA statement was attached (Myers, 2001b; JMPR, 2003).

A study was supplemental to, and conducted to further interpret the data on offspring survival obtained from a dimethoate developmental neurotoxicity study (Myers, 2001c). The objective of this study was to further assess the influence on offspring survival of maternal exposure to dimethoate (98%) during gestation and the postnatal period. In the DNT study, pup survival was particularly affected up to PND 4 with a smaller effect observed up to PND 11. In the present study, litters from dimethoate-treated dams were cross fostered with dams that were untreated to better interpret whether

pup deaths were related to maternal exposure to dimethoate in the DNT study and/or exposure of pups to dimethoate in utero and/or through milk. Mated females (Crl:CD BR) were allocated to treatment groups in following way:

- 100 females to the control group;
- 25 females to the 3 mg/kg bw/day group;
- 50 females to the 6 mg/kg bw/day group.

Dose levels were chosen based on those used in the DNT range-finding and definitive studies; 3 mg/kg bw per day (DNT) and 6 mg/kg bw per day (range-finding study) clearly had an effect on pup survival. As in the DNT study, dams were treated with dimethoate or vehicle (water for formulation) from GD 6 to PND 10. Offspring received no direct exposure to dimethoate. As in the DNT study, dams were subjected to a neurobehavioural screen on GDs 12 and 18, PNDs 4 and 10.

On PND 1 (defined as beginning six hours after the completion of parturition), litter size, sex ratio, offspring body weights, visual assessment of the presence/absence of milk in stomach, and offspring clinical signs were recorded. Dams and offspring were then allocated to treatment groups after birth as shown in Table 31.

Table 31. Study design

Group	Maternal dose (mg/kg bw per day)	Litter of origin (prenatal maternal dosage) (mg/kg bw per day)	Number of litter units
1A	0	3§	23
1B	0	6§	24¶
1C	0	0#	25
2	3	0§	23
3A	6	0§	24¶
3B	6	6#	22

§ Indicates litters that were cross-fostered

# Indicates litters that were raised by their own mothers

¶ One litter allocated to cross fostering in error – dam and litter culled on day 2

Source: Myers, 2004

Table 32. Comparision design of the study

Route of potential exposure to offspring	Study group	Exposure design
No Exposure to offspring	Group 1C	Control dams given daily oral gavage doses of vehicle (water); raised their own offspring
Potential exposure to offspring in utero (3mg/kg bw level)	Group 1A	Offspring from dams given daily oral gavage doses of 3mg/kg bw per day; raised by control dams
(6mg/kg bw level)	Group 1B	Offspring from dams given daily oral gavage doses of 6mg/kg bw per day; raised by control dams
Potential exposure to offspring via milk only (3mg/kg bw level)	Group 2	Offspring of control dams are raised by dams given daily oral gavage doses of 3mg/kg bw per day
(6mg/kg bw level)	Group 3A	Offspring of control dams are raised by dams given daily oral gavage doses of 6mg/kg bw per day
Potential exposure to offspring in utero and via the milk	Group 3B	Offspring from dams given daily oral gavage doses of 6mg/kg bw per day are raised by their own mothers

Table 33. Principal effect on offspring

Treatment group	**1C**	**1A**	**1B**	**2**	**3A**	**3B**
Dosage of dam (mg/kg bw per day)	**0**	**0**	**0**	**3**	**6**	**6**
Dosage of litter (mg/kg bw per day)	**0**	**3**	**6**	**0**	**0**	**6**
Number of dams/litters	25	23	23 [§]	23	23 [§]	22
Mean no. of offspring per litter [litters involved] killed or found dead during PNDs 1–11***	0.2 [5]	0.4 [8]	0.6 [6] 0.2 [5][#]	0.4 [8]	**1.2 [14]** ***	**1.4 [13]** **1.1 [12]**[¶]
Mean percentage of offspring per litter [litters involved] killed or found dead during PNDs 1–11	1.4(5)	2.5(8)	3.4(6) 1.6(5)[#]	2.5(8)	**7.9(14)**	**8.8(13)** **7.7(12)**[¶]
Male offspring bw gain PNDs 1–11(g)***	13.6	13.9	13.7	12.5	**10.9*****	**11.5*****
Female offspring bw gain PNDs 1–11 (g)***	13.2	13.6	13.2	12.3	**10.6*****	**11.1*****
Male offspring taking more than 2 s to complete surface righting on PND 10***	3	4	2	5	**9*****	**9*****
Blood urea (mmol/L) – males***	6.67	7.10	7.35	**8.59*****	**8.90*****	**10.25*****
Blood urea (mmol/L) – females***	7.51	8.16	8.20	**9.35*****	**9.58*****	**11.32*****
Blood creatinine phosphokinase –females (U/L)*	12 184	10 592	11 594	9507	11 776	**6798***
Packed cell volume (L/L) – males*	0.289	0.292	0.294	0.301	**0.308***	**0.310***
Packed cell volume (L/L) – females*	0.293	0.289	0.298	0.297	**0.313***	**0.304***
Mean cell haemoglobin conc. (g/dL) – males*	30.5	30.7	30.9	30.9	30.1	**29.6***
Mean cell haemoglobin conc. (g/dL) – females*	30.7	30.4	30.6	30.8	30.3	**29.7***
Mean cell volume (fL) – males**	85.4	84.6	84.4	86.3	**88.4****	**89.8****
Mean cell volume (fL) – females**	85.8	85.9	85.4	86.0	**88.9****	**89.0****
Neutrophils ($\times 10^9$/L) – males**	0.70	0.66	0.55	0.84	**0.99****	**1.24****
Monocytes ($\times 10^9$/L) – fales**	0.21	0.30	0.25	0.39	**0.43****	**0.53****

[§] Excludes one liner allocated to cross fostering in error – dam and litter culled on day 2

[#] Excludes litter 126 which showed an atypically high level of pup mortality

[¶] Excludes litter 139 which had an atypically large litter size and showed a high level ot pup mortality

* $p < 0\ 05$ ** $p < 0.01$ *** $p < 0.00$l by the general or generalised linear model

Values in bold show treatment-related effects inferred following review of results of statistical analysis.
All treatment-related effects inferred were still apparent when data for litters 126 and 139 were excluded from all analyses.

Dams and offspring were observed for maternal behaviour and clinical signs five times per day commencing at PND 1. Up to one male and one female offspring per litter were subjected to a neurobehavioural screen on PND 4 and PND 10.

On PND 11 blood samples were obtained from male and female offspring in 10 litters per subgroup or group for haematology and blood chemistry assessments. Offspring and dams were subjected to a necropsy examination. Blood samples were taken to evaluate the health status of the offspring.

Treatment of pregnant females with dimethoate at 6 or 3 mg/kg bw per day was not considered to be associated with any effect of treatment on body weight gain during gestation, length of gestation or gestation index.

During the postnatal period, there was an increased incidence of maternal restlessness and scattering of offspring on two or more days in the litters raised by dimethoate-treated dams. Other signs consisted of a higher proportion of dams with hair loss on their forelimbs and a lower proportion of dams defaecating or urinating in the arena in the 6 mg/kg bw per day treatment group. Additionally, a lower proportion of treated dams showing some resistance or avoidance on removal from the cage on PNDs 4/10 was observed. Dams in both treated groups also had lower body weight gain (PNDs 1–7) compared to untreated animals.

Treatment of the dams was associated with a number of effects among the offspring reared by these dams during the lactation period up to PND 11. The principal effects on the offspring were as follows.

Dams treated at 6 mg/kg bw per day and rearing control group or their own offspring, the offspring showed:

- a slight but clear increase in offspring mortality,
- a clear increase in scattering of offspring in the cage,
- a slight increase in the number of occasions on which offspring were noted to have no milk apparent in the stomach,
- absence of milk in the stomach was the principal necropsy finding among decedents,
- lower body weight gains of males and females,
- a higher number of male offspring taking more than two seconds to complete surface righting on PND 10,
- higher blood area levels, packed cell volumes and mean erythrocyte cell volumes among males and females,
- higher neutrophil and monocyte counts among males.

Dams treated at 3 mg/kg bw per day and rearing control group offspring, the offspring showed:

- higher blood urea levels,
- a clear increase in scattering of offspring in the cage,
- a slight increase in the number of occasions on which offspring were noted to have no milk apparent in the stomach.

It is concluded that excess mortality in pups reared by treated dams, seen in this study as well as in the prior DNT study (Myers, 2001c) that this study supplements, and the behavioural and clinical effects observed, are very strongly associated with postnatal rearing by dimethoate-treated dams. The excess mortality is not associated with in utero exposure of the offspring.

The study was GLP compliant (Myers, 2004).

(d) Effects on cholinesterase activity in rats

Study of cholinesterase activity after single and repeated doses of dimethoate

The purpose of this study (which complied with UK GLP regulations 1999, European Commission Directive 1999/11/EC and OECD GLP principles 1997) was to assess the effect of single and/or repeated doses of dimethoate (purity 99.1%) on plasma, erythrocyte and brain ChE activities in pregnant female Sprague Dawley Crl:CD(SD)IGS BR rats and their preterm fetuses, in pre-weaning offspring and in young adult rats. Recovery of ChE activity was also assessed in young adults 39 days after cessation of treatment with repeated doses.

In trial A, groups of 19 mated female rats (aged 10–11 weeks) received dimethoate at doses of 0, 0.1, 0.5 or 3 mg/kg bw per day by gavage in water. Ten females per group were treated from GD 6 until PND 10, while the remaining nine females were treated on GDs 6–20. For the females treated until GD 20, eight dams per group were killed 3 h after dosing on that day; data on litters were assessed and ChE activity was determined for maternal and fetal plasma, erythrocytes and brain. For the females that were allowed to litter and were treated until PND 10, offspring in eight litters per group were treated on PNDs 11–21 inclusive in order to assess effects on survival, body weight gain and ChE activity. Selected offspring from these litters were killed on PNDs 4, 21 or 60, and ChE activity was determined for plasma, erythrocytes and brain. Time of parturition and duration of gestation were noted. Body weight was noted several days during gestation and after parturition, until PND 21.

In trial B, a group of eight pregnant female rats was not treated throughout the study. Of the offspring of these females, one male and one female per litter were given dimethoate as a single dose of 0, 0.1, 0.5 or 3 mg/kg bw by gavage in water on PND 11. Eight male and eight female offspring per group (one male and one female from each litter) were killed 2 h after dosing and ChE activity was

determined for plasma, erythrocytes and brain.

In trial C, groups of 16 male and 16 female naive adult rats (aged 7–8 weeks) received dimethoate at doses of 0, 0.1, 0.5 or 3 mg/kg bw per day by gavage in water. Eight males and eight females per group were treated for one day and were killed 2 h after dosing, while the remaining eight males and eight females per group were treated for 11 consecutive days, and killed 2 h after the last dose. In each case ChE activity was determined for plasma, erythrocytes and brain.

Blood samples were collected from the retro-orbital sinus (for pups on PND 21 and adults), umbilical cord (fetuses on GD 20) or by decapitation (pups on PND 4 or 11). Blood samples were taken taken 3 h after dosing on GD 20, 4 h after dosing on PND 4, and 2 h after dosing on PNDs 11–21. Fetal blood samples from GD 20 were pooled for each litter. Plasma samples and erythrocyte haemolysates were stored at −80°C until analysis. Brains were removed immediately after sacrifice, weighed and frozen in liquid nitrogen. Fetal brains from GD 20 were pooled for each litter because the range-finding study showed no difference in ChE activity between the sexes. Cholinesterase activity was determined according to a modified Ellman method (EPA, 1996). Erythrocyte ChE activity was measured using 6,6'-dithiodinicotinic acid, plasma and brain activity was measured using DTNB as the colour component.

On GD 20 dams were sacrificed immediately after blood sampling, and their brains collected. The reproductive tract was examined for the following parameters: number of corpora lutea, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each horn. Fetuses were weighed and sexed, and sacrificed on a cool plate at 0°C. The brains of the fetuses were then sampled. The dams were thoroughly examined macroscopically and specimens of any abnormal tissue retained. All pups (except those culled on PND 4) were examined macroscopically. In order to gain experience in a specific method of whole body perfusion fixation of tissue, and in precise sectioning of brains from young adult rats, five offspring were killed on PND 61, perfused and their brains embedded, sectioned and subjected to examination by light microscopy.

There were no clinical signs that were considered to be related to treatment and no deaths at any dose. There was no adverse effect of treatment on weight gain of adult males and females, weight change of dams during gestation and lactation or macroscopic findings at necropsy. Litter data on GD 20 and body weight, weight gain and survival of offspring up to PND 11 were unaffected by treatment. There was no effect of direct treatment on the growth or survival of the offspring.

In trial A, on GD 20, plasma and erythrocyte ChE activity was statistically significantly inhibited at 3 mg/kg bw per day in dams (56% and 42% of control values respectively) and in fetuses (57% and 69% of control value respectively). Brain ChE activity was statistically significantly inhibited in dams at 0.5 and 3 mg/kg bw per day (90% and 40% of control value respectively) and in fetuses at 0.1, 0.5 and 3 mg/kg bw per day (88%, 90% and 67% of control value respectively).

Key findings in dams and fetuses for GD 20 from this study into the effects of dimethoate on cholinesterase activity are shown on the following page in Table 34.

Table 34. Effects of dimethoate on cholinesterase activity in dams and fetuses at GD 20

	Dose (mg/kg bw per day)			
Parameter	**Control**	**0.1**	**0.5**	**3**
Maternal cholinesterase (Groups 1–4)				
PChE (U/L)	1381	1216	1184	776**
PChE relative to control (%)	-	(12)	(14)	(44)
EChE (U/L)	1669	1563	1459	709**
EChE relative to control (%)	-	(6)	(13)	(58)
BChE 20 (U/kg)	12838	13044	11563*	5094**
BChE relative to control (%)		(−2)	(10)	(60)
Fetal cholinesterase (Groups 1–4)				
PChE (U/L)	258	257	239	147**
PChE relative to control (%)	-	(0)	(7)	(43)
EChE (U/L)	1213	1225	1181	834**
EChE relative to control (%)	-	(−1)	(3)	(31)
BChE (U/kg)	1781	1569*	1600*	1188**
BChE relative to control (%)	-	(12)	(10)	(33)

PChE Plasma cholinesterase EChE Erythrocyte cholinesterase BChE Brain cholinesterase

* $p < 0.05$ ** $p < 0.01$ compared to control

In pups at PND 4, plasma ChE activity was statistically significantly inhibited at 0.5 and 3 mg/kg bw per day in females (92% and 90% of control value respectively), while erythrocyte ChE activity was statistically significantly inhibited at 3 mg/kg bw per day in males only (83% of control value). Brain ChE activity was statistically significantly inhibited in males at 0.1, 0.5 and 3 mg/kg bw per day (90%, 92% and 87% of control value respectively).

Table 35. Effects of dimethoate on cholinesterase activity in the offspring at day 4 of age (Groups 1–4)

	Dose (mg/kg bw per day)			
Parameter	**Control**	**0.1**	**0.5**	**3**
PChE – male offspring (U/L)	612	607	588	566
PChE relative to control (%)		(1)	(4)	(8)
PChE– female offspring (U/L)	640	605	591*	576**
PChE relative to control (%)		(5)	(8)	(10)
EChE– male offspring (U/L)	1291	1403	1254	1071**
EChE relative to control (%)		(−9)	(3)	(17)
EChE– female offspring (U/L)	1260	1261	1352	1088
EChE relative to control (%)		(0)	(−7)	(14)
BChE– male offspring (U/kg)	3137	2817*	2889*	2744**
BChE relative to control (%)		(10)	(8)	(13)
BChE– female offspring (U/kg)	2823	2941	2650	2638
BChE relative to control (%)		(−4)	(6)	(7)

PChE Plasma cholinesterase EChE Erythrocyte cholinesterase BChE Brain cholinesterase

* $p < 0.05$ ** $p < 0.01$ compared to control

In pups on PND 21, plasma ChE activity was statistically significantly inhibited at 3 mg/kg bw per day in both sexes (61% of control value in males, 62% in females), while erythrocyte ChE activity was statistically significantly inhibited at 3 mg/kg bw per day in both sexes (41% of control value in

males, 35% in females) and at 0.5 mg/kg bw per day in females (77% of control value). Brain ChE activity was statistically significantly inhibited in males at 0.1, 0.5 and 3 mg/kg bw per day (96%, 87% and 55% of control value respectively) and in females at 0.5 and 3 mg/kg bw per day (88% and 58% of control value respectively). Offspring killed on PND 60 (that is, 39 days after the end of repeated dosing) showed complete recovery of ChE activity; the statistically significant inhibition of brain ChE activity in females at 0.5 and 3 mg/kg bw per day (96% and 96% of control value respectively) was considered to be not biologically significant.

Table 36. Effects of dimethoate on cholinesterase activity in the offspring on PNDs 21 and 60 (Groups 1–4)

	Dose (mg/kg bw per day)			
Parameter	**Control**	**0.1**	**0.5**	**3**
Offspring at 21 days of age				
PChE – male offspring (U/L)	506	535	478	307**
PChE relative to control (%)		(−6)	(6)	(39)
PChE – female offspring (U/L)	487	507	463	304**
PChE relative to control (%)		(−4)	(5)	(38)
EChE – male offspring (U/L)	1638	1659	1494	669**
EChE relative to control (%)		(−1)	(9)	(59)
EChE – female offspring (U/L)	1900	1619	1466*	663**
EChE relative to control (%)		(15)	(23)	(65)
BChE – male offspring (U/kg)	10 375	9944*	9044**	5675**
BChE relative to control (%)		(4)	(13)	(45)
BChE – female offspring (U/kg)	10 275	9906	9019**	5956**
BChE relative to control (%)		(4)	(12)	(42)
Offspring at 21 days of age				
PChE – male offspring (U/L)	373	369	340	337
PChE relative to control (%)		(1)	(9)	(10)
PChE – female offspring (U/L)	907	915	945	846
PChE relative to control (%)		(−1)	(−4)	(7)
EChE – male offspring (U/L)	1075	1100	1100	1038
EChE relative to control (%)		(−2)	(−2)	(3)
EChE – female offspring (U/L)	1109	1119	991	1044
EChE relative to control (%)		(−1)	(11)	(6)
BChE – male offspring (U/kg)	13 000	13 100	12 988	13 044
BChE relative to control (%)		(−1)	(0)	(0)
BChE – female offspring (U/kg)	13 275	12 950	12 738*	12 744*
BChE relative to control (%)		(2)	(4)	(4)

PChE Plasma cholinesterase EChE Erythrocyte cholinesterase BChE Brain cholinesterase

* $p < 0.05$ ** $p < 0.01$ compared to control

In trial B, with offspring treated once on PND 11, plasma and brain ChE activity was statistically significantly inhibited at 3 mg/kg bw per day in males (81% and 83% of control value). In females, inhibition of plasma, erythrocyte and brain ChE activities was seen at the same dose (82%, 74% and 82% of control value), differences attaining statistical significance for brain ChE activity only. The statistically significant inhibition of brain ChE activity in males at 0.5 mg/kg bw per day (95% of control value) was considered to be not biologically significant.

Table 37. Effects of dimethoate on cholinesterase activity in juvenile rats (single dose, day 11; Group 5)

	Dose (mg/kg bw per day)			
Parameter	**Control**	**0.1**	**0.5**	**3**
PChE – male offspring (U/L)	756	748	688	614**
PChE relative to control (%)		(1)	(9)	(19)
PChE – female offspring (U/L)	742	700	720	609
PChE relative to control (%)		(6)	(3)	(18)
EChE – male offspring (U/L)	1663	1634	1597	1544
EChE relative to control (%)		(2)	(4)	(7)
EChE – female offspring (U/L)	1997	1647	1894	1475
EChE relative to control (%)		(18)	(5)	(26)
BChE – male offspring (U/kg)	6475	6363	6144*	5375**
BChE relative to control (%)		(2)	(5)	(17)
BChE – female offspring (U/kg)	6256	6350	6125	5144**
BChE relative to control (%)		(−2)	(2)	(18)

PChE Plasma cholinesterase EChE Erythrocyte cholinesterase BChE Brain cholinesterase

* $p < 0.05$ ** $p < 0.01$ compared to control

In trial C, treatment of naive adult animals with a single dose of 3 mg/kg bw per day was associated with inhibition of plasma, erythrocyte and brain ChE activity in males (81%, 83% and 88% of controls) and in females (87%, 73% and 86% of controls); differences attained statistical significance for all parameters in males and for erythrocyte and brain ChE activity in females. The statistically significant inhibition of brain ChE activity in males at 0.5 mg/kg bw per day (96% of control value) was considered to be not biologically significant. Treatment of naive adults with 11 consecutive doses of dimethoate at 3 mg/kg bw per day was associated with inhibition of plasma, erythrocyte and brain ChE activity in males (63%, 42% and 53% of controls) and in females (79%, 37% and 42% of controls); differences attained statistical significance for all parameters, except plasma ChE activity in females. At 0.5mg/kg bw per day, there was a slight (not statistically significant) inhibition of plasma and erythrocyte ChE activity in males (88% and 83% of controls), while brain ChE activity was statistically significantly inhibited in males and females (90% and 87% of controls).

Table 38. Effects on cholinesterase in young adult rats after single or repeated (×11) administrations of dimethoate

	Dose (mg/kg bw/day)			
Parameter	**Control**	**0.1**	**0.5**	**3**
Single application				
PChE (U/L) – males	375	387	364	305*
PChE relative to control (%)		(−3)	(3)	(19)
PChE (U/L) – females	688	657	729	602
PChE relative to control (%)		(5)	(−6)	(13)
EChE (U/L) – males	1122	1247	1131 (−1)	928*
EChE relative to control (%)		(−11)		(17)
EChE (U/L) – females	1209	1128	1106 (9)	881**
EChE relative to control (%)		(7)		(27)
BChE (U/kg) – males	13 794	13 544	13 294*	12 131**
BChE relative to control (%)		(2)	(4)	(12)
BChE (U/kg) – females	14 150	13 625	13 850	12 106**
BChE relative to control (%)		(4)	(2)	(14)

Parameter	Dose (mg/kg bw/day)			
	Control	0.1	0.5	3
Repeated (×11) applications				
PChE (U/L) – males	343	327	302	215**
PChE relative to control (%)		(5)	(12)	(37)
PChE (U/L) – females	790	949	770	624
PChE relative to control (%)		(−20)	(3)	(21)
EChE (U/L) – males	1094	1169	903	456**
EChE relative to control (%)		(−7)	(17)	(58)
EChE (U/L) – females	1019	991	950	375**
EChE relative to control (%)		(3)	(7)	(63)
BChE (U/kg) – males	14 100	13 988	12 700*	7469**
BChE relative to control (%)		(1)	(10)	(47)
BChE (U/kg) – females	14 869	13 913	12 881**	6188**
BChE relative to control (%)		(6)	(13)	(58)

PChE Plasma cholinesterase EChE Erythrocyte cholinesterase BChE Brain cholinesterase

* $p < 0.05$ ** $p < 0.01$ compared to control

The NOAEL for dimethoate given as a single dose was 0.5 mg/kg bw per day on the basis of a statistically significant inhibition of brain ChE activity in preweaning rats (17–18%) and in young adults (12–14%) and a significant inhibition of erythrocyte ChE activity in preweaning females (26%) and in young adult females (27%) at 3 mg/kg bw per day.

The NOAEL for dimethoate given as repeated doses was 0.1 mg/kg bw per day on the basis of a slight, statistically significant, inhibition (10–13%) of brain ChE activity in pregnant rats, pups on PND 21 and young adult rats, and a statistically significant inhibition (23%) of erythrocyte ChE activity in female pups on PND 21 at 0.5 mg/kg bw per day. The slight, statistically significant depression (10–12%) of brain ChE activity at 0.1 and 0.5 mg/kg bw per day in fetuses and in male pups aged four days was considered to be of doubtful biological significance, since there was no dose–response relationship (Myers, 2001c; JMPR, 2003).

(e) Delayed polyneuropathy studies

The potential for delayed polyneuropathy of dimethoate has been investigated in a number of regulatory studies.

No behavioural or pathological evidence of delayed neuropathy was seen in a modern acute study in the hen at a dose level equivalent to the acute oral LD_{50}. There was some depression of neuropathy target esterase (NTE) activity. An older repeat dose study is considered to be inconclusive due to the limited histopathology and lack of NTE assessment, however no behavioural signs indicative of neuropathy were observed in this study.

Domestic hens (hybrid brown laying strain; birds aged ca 12 months, weight range 1935–2295 g) were used to investigate acute oral toxicity and acute delayed neurotoxic effects on adult hens after treatment with dimethoate. The birds were housed according to treatment groups in galvanized steel floor pens. Birds were offered diet and tap water ad libitum.

Study A

In a dose range-finding test using five groups of two birds each, oral dose levels of 12.5, 25, 50, 100 and 200 mg/kg bw were tested and birds were observed during a subsequent 14 day period. Surviving animals were sacrificed at the end of the observation period.

Single doses of 30, 45, 67.5, 101.25 and 151.88 mg/kg bw, selected on the basis of the dose range finding test were administered orally by gavage to hens (10 animals per group including an untreated control group randomly assigned). Water was used as a vehicle for the test compound and the

same volume of water given to control animals. Birds were subsequently observed after treatment for 14 consecutive days for any clinical signs or mortality. At the end of this observation period animals were sacrificed and not examined postmortem

Study B
Initially the protective efficiency of atropine was tested by treating five birds with 10 mg/kg bw atropine sulfate by intramuscular injection. Thereafter, birds were treated with dimethoate at twice the LD_{50} value and at two-hourly intervals after treatment birds received atropine until sacrifice. Clinical signs and mortality were recorded.

Neurotoxicity was assessed in hens after oral and subcutaneous administration of dimethoate in comparison with a negative (vehicle) and a positive control group. Positive control birds were dosed with tri-*o*-cresyl phosphate (TOCP) at 500 mg/kg bw using a 5% w/v solution in corn oil. Negative control birds were dosed with vehicle (water) only. Dimethoate at 55 mg/kg bw was administered subcutaneously or orally as a 1.1% or 0.55% solution in water, respectively. Subcutaneous administration was employed to investigate if significant differences in observed signs of toxicity or effects on enzyme activity (cholinesterase or NTE) exist between oral and subcutaneous routes of administration. The subcutaneous route is the most appropriate route to simulate the effects after dermal administration. After treatment, animals were observed daily for a period of up to 22 days for clinical health, mortality, locomotor ataxia, and the degree of ataxia was scored (scale from 0–8). Body weights were recorded once weekly. Brain ChE and spinal cord NTE were determined in three animals per group after 4 h and 48 h of treatment, whereas animals from the TOCP treatment group were just sacrificed after 48 h. Cholinesterase activity was determined in brain by the method of Ellman et al. (1961). Neuropathy target esterase activity (in brain and spinal cord) was analysed by the method of Johnson (1977). Tissues were rapidly frozen and stored at −20°C prior to analysis. The time of the first kill (4 h after dosing) was earlier than the 24 h recommended by the current OECD guideline. Taking the rapid oral absorption in hens into account, this deviation is not considered to affect the outcome and the validity of the study. At the end of the study all birds were sacrificed and selected birds were subjected to gross necropy and examined.

In study A in the dose range-finding study, both birds of the two highest dose levels died. On this basis dose levels were selected to determine acute oral toxicity.

Control birds remained in good health after dosing. Body weight in birds treated with dimethoate was reduced in the first week after dosing, followed by an increase in the second week, whereas a mean increase was observed in control animals. Clinical signs of toxicity were recorded in all groups dosed with dimethoate. These included subdued appearance and unsteadiness at all dose levels and inability to stand at 45 mg/kg bw and above. Four mortalities occurred at 45 mg/kg bw, six at 67.5 mg/kg bw and 10 at the two highest dose levels of 101.25 and 151.88 mg/kg bw.

In study B, animals dosed with dimethoate at twice the LD_{50} value and treated with atropine sulfate died within 24 hours of dosing. Hence atropine sulfate was not effective as a protective agent and it was not used in the main study.

No signs of cholinesterase-related toxicity were observed in birds dosed with TOCP and three birds developed signs of delayed locomotor ataxia. All of the dimethoate-treated birds revealed clinical signs of toxicity including subdued behaviour, unsteadiness, stumbling and squatting on the pen floor. Ten and 12 mortalities occurred in dimethoate-treated birds after subcutaneous and oral administration, respectively. No clinical signs of neurotoxicity were recorded in any of the control and dimethoate-treated animals.

During weeks 2–3, body weights in the control group were variable while those of the TOCP-treated groups showed a mean loss. Birds treated with dimethoate by the oral route showed a mean body weight loss during the first week, followed by mean body weight gains thereafter.

No abnormalities were detected in any of the birds during macroscopic examination. Control birds and birds dosed orally with dimethoate did not exhibit significant axonal degeneration (equal or less than Grade II changes), whereas the birds dosed with TOCP showed significant axonal degeneration characterized by Grade III changes.

Brain ChE activity was markedly reduced in groups treated with dimethoate giving a 61–90% inhibition relative to control values. This depression was greater 4 h after dosing than 48 h after dosing.

Brain NTE was slightly lower in dimethoate-treated animals compared to control birds (21–22% at 4 h and 31–33% at 48 h) but markedly lower in the concurrent positive control with TOCP (93% at 48 h). No effects were noted on spinal cord NTE in either group treated with dimethoate, whereas the TOCP group showed a 89% inhibition 48 h after treatment.

In view of the above, it is concluded that administration of dimethoate, either orally or subcutaneously at a dose level of 55 mg/kg bw (oral LD_{50} value) did not produce any clinical signs of delayed neurotoxicity in domestic hens. No clinical signs of ataxia, no significant depression of brain and spinal cord NTE activity nor significant histopathological findings were observed.

The study was GLP compliant (Redgrave et al., 1991).

In a study, adult Leghorn White hens, were treated by single and repeated oral doses of dimethoate (batch W-40403-1; purity 98.1%). Employing a single dose (17.5, 35.0 and 70.0 mg/kg bw) administration, an LD_{50} value of 50 mg/kg bw was determined. In a repeated dietary study with 0, 65, 130 and 260 ppm of dimethoate for 28 consecutive days, followed by four weeks of recovery, dimethoate had no adverse effect on nerve fibres or their myelin sheaths. Hence no indications of the induction of delayed neurotoxicity by dimethoate treatment were obtained.

The study was not GLP compliant (Levinskas & Shaffer, 1965).

(f) Endocrine-disrupting properties

The existing toxicological database comprising mammalian studies to characterize toxic effects relevant to humans, ecotoxicology studies to characterize toxic effects on wildlife, and newly submitted dedicated endocrine assays, have been considered in the area of the endocrine-disrupting potential of dimethoate. Consideration of the overall data package in this area has been undertaken and is summarized below.

Table 34. Results of tests for endocrine-disrupter properties

Sl. No.	Test	Method	Purity %	Result	Reference
1	Estrogenicity of organophosphorus and pyrethroid pesticides	Three in vitro methods [E-Screen assay, estrogen receptor (ER) competitive binding assay, and pS2 expression assay	> 90	Negative	Chen et al. (2002)
2	Estrogen receptor transcriptional activation in human cell line (HeLa-9903)	U.S. EPA OPPTS 890.1300 (2009) OECD 455 (2009)	98.0	Not an agonist of the human estrogen receptor alpha (hERα) in the HeLa-9903 model system.	Willoughby, 2011a
3	Androgen Receptor Binding (Rat Prostate Cytosol)	OPPTS 890.1150 (2009)	98.0	"Non-binder" at the AR	Willoughby, 2011b
4	Human recombinant aromatase assay	EPA for aromatase inhibition (OPPTS 890.1200)	98.0	A non-inhibitor of the aromatase enzyme	Wilga, 2011
5	H295R Steroidogenesis assay	US EPA OPPTS 890.1550 (2009)	98.0	Not considered to be positive using this steroidogenesis assay.	Wagner, 2011
6	Amphibian metamorphosis assay	U.S. EPA OPPTS Number 890.1100 OECD guideline 231	98.1	No apparent treatment-related effects	Schneider, Kendall & Krueger, 2012

Sl. No.	Test	Method	Purity %	Result	Reference
7	Fish short-term reproduction assay with the Fathead Minnow (*Pimephales promelas*)	OECD Guideline 229 U.S. EPA OPPTS 890.1350	98.1	Effects on egg production during the 21-day test; slight decrease in fertility controls, although fertility still averaged 95% in the high treatment group. Overall, the assay indicates that the findings at the high concentration were most likely due to general toxicity.	Schneider et al., 2012
8	A flow-through life cycle toxicity test with the saltwater Mysid (*Americamysis bahia*)	U.S. Environmental Protection Agency Series 850 – Ecological effects test guidelines, OPPTS number 850.1350: Mysid chronic toxicity test ASTM Standard E 1191-03a: Standard guide for conducting life-cycle toxicity tests with saltwater Mysids	98.1	Based on the effects on growth observed among males and females in the 1.2 and 2.2 mg a.i./L treatment groups, the LOEC for growth (dry weight) was 1.2 mg a.i./L and the NOEC was 0.57 mg a.i./L.	Claude et al., 2012
9	Early life-stage toxicity test with the Rainbow Trout (*Oncorhynchus mykiss*), Under flow-through conditions	U.S. EPA, Office of Chemical Safety and Pollution Prevention, Ecological effects test guideline OPPTS 850.1400 OECD guideline 210	98.1	Based on mean measured concentrations of dimethoate, the NOEC and LOEC values for rainbow trout hatch start, hatch completion, and egg hatchability were 9.92 and > 9.92 mg a.i./L. The NOEC and LOEC values for fry survival were 4.93 and 9.92 mg a.i./L, respectively. The NOEC and LOEC values for standard length and blotted wet weight were 1.25 and 2.49 mg a.i./L, respectively. The MATC for the most sensitive parameters (standard length and blotted wet weight) was 1.76 mg a.i./L.	Hicks, (2012)

(g) Studies on metabolites

In terms of residues of interest from a toxicological perspective, the key metabolite is omethoate. Other metabolites likely to be of interest include:

- dimethoate carboxylic acid,
- omethoate sulfoxide (not analytically identified; it is only an intermediate for phosphorylation of natural products/nucleophiles),
- dimethyl thiophosphate and dimethyl phosphate in food animals (goat and hen),
- there do not appear to be any significant metabolites in rotational crops,
- for dimethoate directly applied to crops, it looks like *O*-desmethyl-*N*-desmethyl omethoate is significant. Minor components include isodimethoate, dimethyl dithiophosphate, *O*-desmethyl omethoate carboxylic acid, *O*-desmethyl isodimethoate, dimethoate carboxylic acid, glucose conjugate of hydroxyl-dimethoate, and *O*-desmethyl omethoate.

Certain studies are available on metabolites of dimethoate. These metabolites are omethoate, and four plant metabolites, namely *O*-desmethyl omethoate, *O*-desmethyl omethoate carboxylic acid, *O*-desmethyl-*N*-desmethyl omethoate, and *O*-desmethyl isodimethoate. Omethoate is the oxygen analogue of dimethoate and a considerably more potent ChE inhibitor than dimethoate.

Absorption, distribution and excretion of omethoate

Absorption, distribution, excretion and metabolism of the insecticide omethoate were studied in Wistar rats, strain BOR:WISW (SPF Cpb). ^{14}C-omethoate (radiochemical purity 99.4%) was administered to male and female Wistar rats at dose levels of 0.5 or 10 mg/kg bw by i.v. or oral route. In addition, rats of both sexes were pretreated for 14 days with daily oral doses of 0.5 mg/kg bw using non-radiolabelled omethoate (purity 99.2%) followed by a single radiolabelled dose at the same rate after 24 h. Radioactivity was measured in the excreta and plasma at different sampling times, and in the organs and tissues at 48 h after administration (sacrifice). The results of the study concerning absorption, distribution and excretion (pharmacokinetic study) refer to the sum of parent compound and its radiolabelled metabolites. For the purpose of balance and comparison between dose group, all radioactivity values are expressed as percent of totally recovered radioactivity per animal or dose group.

Following oral administration of ^{14}C-omethoate at 10 mg/kg to male rats, only a little radioactivity (ca 0.14% of the administered radioactivity) was detected in the expired air in the subsequent 48 h.

The absorption rate after oral administration of 0.5 or 10 mg/kg bw respectively, was evaluated by the results of excretion rate in urine and residue amounts in the body excluding GI tract. More than 98% of the administered radioactivity was absorbed in the rat.

Following administration of ^{14}C-omethoate i.v. or orally to male and female rats at 0.5 mg/kg or 10 mg/kg. the radioactivity was rapidly excreted; about 88–98% of the administered radioactivity was eliminated in the excreta within 48 h of administration and total recovery was 89–98%. The radioactivity was excreted mainly with the urine: 85–96% of the radioactivity being found in the urine and 2–4% in faeces. The rate of renal excretion was very high, about 83–95% of the administered radioactivity was renally eliminated within 24 h of administration.

Very little radioactivity remained in the body at sacrifice. What remained in the GI tract of rats sacrificed 48 h after administration was 0.03–0.04% of the administered radioactivity. And the residual radioactivity in the body excluding GI tract was 0.24–0.42%.

The results from the pharmacokinetics investigations are summarized in Table 35. The pharmacokinetic parameter AUC was nearly identical in the i.v. and oral groups (low- and high-dose). The absorption was about 100% in all test groups. After oral administration, the maximum relative concentration in the plasma (T_{max}) was reached between 40 minutes and 1 hour, and the maximum plasma concentration (C_{max}) was in the range of 1.1–1.3. Significant differences in the elimination half-lives were detected between low-dose and high-dose groups (28 h and 13 h, respectively).

Table 35. Pharmacokinetic parameters from plasma curve analysis after administration to male and female rats (each value is calculated from the plasma curve of the average relative concentration)

	i.v. 0.5 mg/kg bw		Oral 0.5 mg/kg bw		Oral 0.5 mg/kg bw + pretreatment		Oral 10 mg/kg bw	
	Male	Female	Male	Female	Male	Female	Male	Female
Absorption (%)	-	-	100	101	100	100	98	102
AUC_{exp} [§] (mg×h/L)	6.116	5.064	4.795	5.123	4.268	4.552	5.436	4.920
$T_{½}$ (h)	25.0	28.4	24.6	21.6	26.6	26.2	15.2	12.8
C_{max} [#]	-	-	1.183	1.276	1.142	1.051	1.111	1.051
T_{max} [¶] (h)	-	-	0.65	1.00	0.67	0.67	1.00	0.67

[§] AUC_{exp} is the area under the blood concentration vs. time curve of experimental values calculated by data from the measured first time point to the last time point

[#] $C_{max} = P_{max}$ which is the maximum level of the plasma concentration after oral administration

[¶] T_{max} is the time of the maximum level of the plasma concentration after oral administration. T_{max} was evaluated from experimental time points

In organs and tissues, the relative concentration *P* (radioactivity measured per g of plasma or tissue divided by radioactivity administered per g bodyweight) in the thyroid was the highest in all test groups; 0.34–0.59 in low-dose groups and 0.16–0.19 in high-dose groups. Compared with plasma concentration in each test, the concentration in the thyroid was greater by 112–197 times (low-dose groups) and 65–70 times (high-dose groups) than in the plasma.

Relative concentrations in liver, kidney, testes, spleen and lungs were also higher than that of plasma; *P* values were 0.05–0.1 in liver and kidney and 0.004–0.01 in testes, spleen and lungs. These were 1.5–4 times higher than the *P* value for the plasma. (Hoshino, 1990)

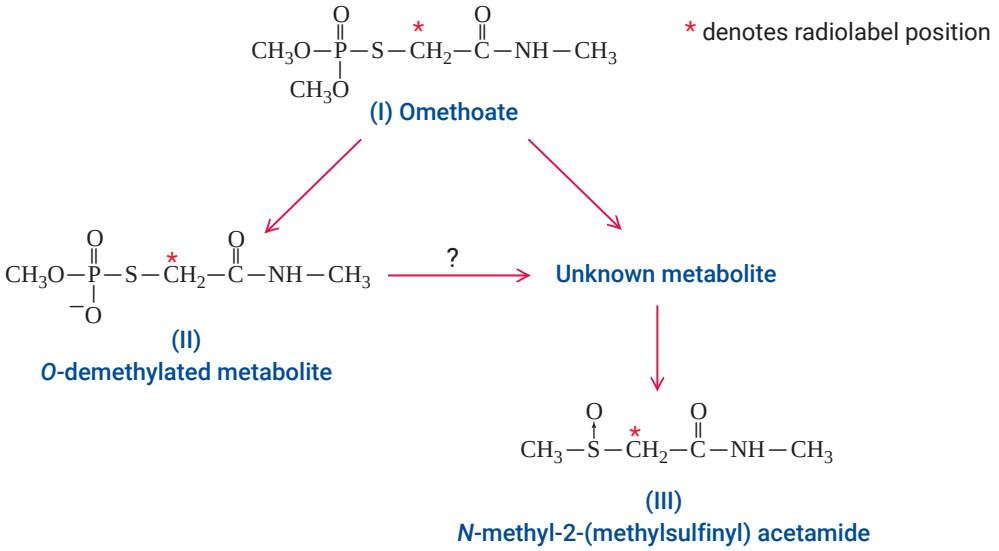
Biotransformation of omethoate

The metabolic pattern in urine and extracts of faeces was studied. In the case of urine, the samples collected from 0–24 h were combined (representative of native urine) and investigated by TLC and HPLC. The metabolic pattern of omethoate was very similar in all test groups; the main radioactive compound was the parent omethoate and 26–62% of the administered radioactivity was detected as omethoate. Two major metabolites were detected and these were identified as *N*-methyl-2-(methylsulphinyl) acetamide (16–36%) and the *O*-desmethyl metabolite of omethoate (free form, 4–9%). Much more omethoate was detected in females than in males, on the other hand more *N*-methyl-2-(methylsulphinyl) acetamide was observed in males. In comparison between low-dose and high-dose groups, more omethoate was found in high-dose groups. Some minor metabolites were observed and these amounted to less than 10% each. The structure of these minor metabolites could not be identified.

Faeces (high-dose group only) were extracted with 70% aqueous acetonitrile yielding ca 63–77% of the radioactivity in the extracts. They were concentrated and the metabolic pattern of faeces extracts was investigated by TLC. The metabolic pattern in faeces extracts was different from that of native urine and the main radioactive compound was the *O*-desmethyl metabolite (0.7–2.6% of the administered radioactivity). Omethoate and *N*-methyl-2-(methylsulphinyl) acetamide were also identified in the faeces extracts, but these were very minor in amount, only 0.1% and 0.3% of the administered radioactivity respectively.

Omethoate itself is water soluble, excreted mainly with urine and was not metabolized excessively. The main metabolic pathway of omethoate was hydrolysis of the phosphoric acid structure and desmethylation; *N*-methyl-2-(methylsulphinyl) acetamide was produced as the main metabolite in rat (Hoshino, 1990).

Figure 3. Proposed metabolic pathway of omethoate (redrawn from Hoshino, 1990)



To summarize, 72–85% of the administered radioactivity was identified as omethoate and its metabolites in excreta of the high-dose group and 46–62% was the parent compound omethoate.

The study was GLP compliant (Hoshino, 1990).

3. Toxicological studies on omethoate

3.1 Acute toxicity of omethoate; oral, dermal and inhalation

Table 36. Summary of acute toxicity studies with omethoate

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/m³ air)	Reference
Rat	Wistar albino	M + F	Oral	94	M – 27.3 [25.5–29.6] F – 25.6 [23.8–27.6]	Flucke, 1978
Rat	WISW (SPF-Cpb)	M + F	Oral	96–97	M – 22 [18.5–27.8] F – 28 [26.3–31.2]	Krötlinger, 1989a
Rat	WISW (SPF-Cpb)	M + F	Dermal	96	M – 232 [178–346] F – 145 [approx.]	Krötlinger, 1989b
Rat	WISW (SPF-Cpb)	M + F	Inhalation 4 h	97.4	287 (MMAD-1.40 µm)	Pauluhn, 1989

3.2 Short-term studies on toxicity of omethoate

(a) Oral administration

Rat

In a 28-day dietary toxicity study, weanling albino rats (135 males and 135 females) of Carworth Farms, Nelson Colony strain (CFN), about one month of age with body weights of males 64–93 g and females 60–90 g were used in this study and randomly assigned to treatment groups. Dietary levels of 0; 0.2; 0.4; 0.8, 1.6 and 8 ppm (equivalent to 0; 0.02; 0.04; 0.08; 0.16 in males, 0.8 mg/kg bw per day in females) Omethoate were fed for 28 consecutive days to 20 rats/sex per dose.

Five animals/sex per group were sacrificed after 3, 7, 14 and 28 days of treatment to determine plasma, erythrocyte and brain ChE activity. Visceral organs were observed for gross lesions, but no detailed autopsy observations were recorded.

Additional groups of 10 female and 10 male rats were fed a diet containing 8.0 ppm omethoate for 25 days to assess systemic toxicity of the compound. These animals were sacrificed at the end of their treatment phase, blood and brain were taken for ChE determinations and tissues were preserved for detailed microscopic examination including myelin staining. Cholinesterase activity in blood and brain was determined.

In general, appearance and behaviour of animals at all dietary levels of omethoate were normal and comparable to controls. No signs of systemic toxicity, which could be associated with treatment for 25 or 28 days were noted in any of the experimental animals. Food consumption and body weight gain were unaffected. Two males, one each from the 0.2 and 0.8 ppm group, died during the study, but their deaths were unrelated to treatment.

Dietary levels of 0.8 ppm omethoate or less had no toxicologically relevant effect on erythrocyte, plasma or brain ChE activity at days 3, 7 and 28. At 0.4 and 0.8 ppm, some deviations were noted after 14 days of treatment, especially regarding erythrocyte ChE activity. At this time point the erythrocyte activity was unusually low in all groups compared to all other time points. Since no significant inhibition was noted at any other time point during the 28 day treatment period, the findings at 14 days were not considered indicative of an adverse effect at these dose levels.

A marked reduction in ChE activity was observed in all compartments in animals treated with 8.0 ppm. A less marked, but also significant, reduction of ChE activity was observed after feeding of 1.6 ppm in erythrocytes of both sexes (23% inhibition after 28 days treatment) and in the brain of females (10% inhibition after 28 days treatment).

The feeding of 8.0 ppm omethoate produced no significant gross pathology. No morphological changes were seen in liver, kidney, adrenal, heart, brain, spinal cord or sciatic nerve, which were examined microscopically from five animals of each sex. Brain, spinal cord and sciatic nerve showed no myelin damage.

In view of the above, based on ChE inhibition in erythrocytes and brain at 1.6 ppm, a NOAEL in rats of 0.8 ppm (equivalent to ca 0.08 mg/kg bw per day) was identified after feeding rats omethoate for 28 days in the diet.

The study was not GLP compliant (Fogleman & Levinskas, 1963).

In a 28-day dose range-finding feeding study, omethoate (batch 24/67; purity 82%, possible impurity 10% trimethyl thiolphosphate) was administered to male and female Wistar rats (10 rats/sex per concentration; average weight of females and males at initiation of the study was 161 and 234 g, respectively. Omethoate was administered at dietary concentrations of 0, 2.5 and 15 ppm (equal to 0, 0.23 and 1.24 mg/kg bw per day for males, 0, 0.26 and 1.40 mg/kg bw per day for females). No treatment-related mortalities were noted during the study.

The activity of ChE was markedly inhibited at 15 ppm. The inhibition of ChE in blood and erythrocytes ranged throughout the study from 62.4% to 76.7% and 77.9% to 81.4%, respectively. Effects on plasma ChE activity were less pronounced (16% to 49.2% inhibition).

At 2.5 ppm the only effect noted was an inhibition of ChE activity in erythrocytes (clearly exceeding the trigger value of 20% with figures of 35.1–50.3% and 14–47.1% for females and males, respectively) and whole blood. The main effect noted at concentrations of 2.5 and 15 ppm was the inhibition of ChE in whole blood and erythrocytes. Hence no NOAEL can be identified from this study.

Based on the findings reported in this study, the dose levels for the 90 day toxicity study were selected as 0, 0.5, 1, 2 and 4 ppm.

The study was not GLP compliant (Loser, 1968a).

In a 90-day feeding study, omethoate (93.3%) was administered to male and female Wistar rats,(average body weight 150 g for females, 225 g for males; 15 rats/sex per concentration) at concentrations of 0, 0.5, 1, 2 and 4 ppm (equal to 0, 0.04, 0.08, 0.17 and 0.34 mg/kg bw per day for males, 0, 0.05, 0.10, 0.19 and 0.36 mg/kg bw per day for females).

Parameters evaluated included body weight (measured weekly), food consumption (measured daily), clinical signs, haematology, clinical chemistry, urinalysis and plasma, effects on ChE in plasma

and erythrocytes (measured in five animals per group at weeks 1, 4 and 13), absolute and relative organ weights, and gross and microscopic pathology (heart, kidney, gonads, uterus, liver, spleen, brain, thyroid, adrenals and lung).

No treatment-related mortalities were observed for the entire treatment period. Besides the high-dose group, where typical symptoms of ChE inhibition were noted, no clinical signs were observed throughout the study.

Body weight and food consumption were unaffected by the treatment with omethoate. No treatment-related effects were noted on absolute or relative organ weights. Gross and microscopic pathology did not reveal any treatment-related findings. No effects were noted in any treatment group with regard to haematology, clinical chemistry or urinalysis, besides of effects on ChE activity.

Biologically and toxicologically relevant inhibition of erythrocyte ChE (> 20%) was noted at 2 ppm in males (28.8–46.8% inhibition compared to control) and females (21.2–26.5% inhibition compared to control). Even more pronounced inhibition (up to 59.1% in males in week 1) was observed at 4 ppm, which explains the observed clinical signs at this dose level. The minor variations in ChE activity (up to 15% inhibition) observed at 0.5 and 1 ppm are not considered biologically relevant.

Only minor effects on plasma ChE activity were noted up to the highest test dose in this bioassay. Besides, at week 1 in the female 2 and 4 ppm groups the trigger value of 20% was not exceeded. Furthermore, no dose dependency was observed with regard to ChE inhibition in plasma.

A NOAEL of 1 ppm for males (equal to 0.08 mg/kg bw per day) and females (equal to 0.1 mg/kg bw per day) was identified based on a biologically relevant inhibition of ChE in erythrocytes in both sexes at 2 ppm.

The study was not GLP compliant (Loser, 1968b).

In a 32 week study, omethoate (batch 234808038; purity 96.5–96.9%) was administered daily to groups of 20 male and 20 female Wistar rats (strain Bor:WISW (SPF Cpb)) at concentrations of 0, 100 and 300 ppb (equal to 0, 9.3 and 27.1 µg/kg bw per day in males and 0, 10.9, 32.2 µg/kg bw per day in females) for ad libitum ingestion in the drinking water (adjusted to pH 3) over a period of 32 weeks.

No test substance-related mortalities were observed in the control nor in the 100 and 300 ppb dose groups of male and female animals. All animals survived until scheduled necropsy or AChE activity determinations.

Inspection of animals revealed no substance-related effects on appearance and behaviour, condition of the coat, posture, respiration and excretory products, up to and including the highest dose tested. Also, daily food and water intake rates were not different from untreated controls.

There were no substance-related effects observable on body weight development during the study and determination of both absolute and relative organ weights revealed no differences in either sex when compared to untreated control animals. On macroscopic examination of the organs no findings related to substance treatment were detectable. No histopathological examination of the organs was performed.

The activity of AChE in plasma, erythrocytes and in the brain was not adversely affected by substance treatment up to and including the 300 ppb dose group. In males plasma AChE activity was slightly increased in the 300 ppb dose group and erythrocyte AChE activity was slightly increased in both the 100 and 300 ppb dose group. Brain AChE activity was reduced by 17% in the 300 ppb dose group of males. In female animals a slight decrease in AChE activity in plasma, erythrocyte and brain was observable in the 100 ppb dose group only, while with the 300 ppb dose group corresponding activities were increased (plasma and brain) or comparable to those of the untreated controls (erythrocytes). Since no clear dose–response relationship was detectable, it is concluded that treatment of male and female rats with substance concentrations up to, and including, 300 ppb does not produce toxicologically relevant effects on ChE in the rat strain used.

Based on the results obtained after investigating the most sensitive parameter (AChE activities in plasma, erythrocytes and brain), and due to the absence of effects on body and organ weights the NOAEL of this study was 300 ppb (equal to 27.1 and 32.2 µg/kg bw per day in male and female rats, respectively), the highest dose tested.

The study was GLP compliant (Schladt, 1994).

Dog

Study 1

In a 90-day gavage study, omethoate (batch 234808038; purity 96.6%), was administered to male and female Beagle dogs (4 dogs/sex per dose) at 0 or 0.0125 mg/kg bw per day. Parameters evaluated included body weight, body weight gain, food and water consumption, clinical signs (including reflex tests, body temperature and pulse rate), haematology, clinical chemistry, urinalysis, ChE activity in plasma and erythrocytes (at weeks −2, 0, 1, 4, 7, and 11) and in brain (week 13), ophthalmology, organ weights and gross pathology

No mortalities occurred throughout the treatment period. No clinical signs were noted besides diarrhoea in one control and one treated dog and vomiting in a single treated dog. These findings are not considered toxicologically relevant, since doses of up to 0.625 mg/kg bw per day were tolerated without such findings in a 12-month study in dogs (Hoffmann & Schilde, 1984). No pathological findings were observed in the reflex tests. Monitoring of body temperature and pulse rates did not reveal any treatment-related findings. Food and water intake were unaffected in males. In females a slightly lower food intake compared to control was noted. This deviation is not considered to be an indication for an adverse effect, since significantly higher doses (up to 0.625 mg/kg bw/day) were tolerated without any effect on this parameter in a 12-month study in dogs (Hoffmann & Schilde, 1984).

No effects on body weight gain were noted. Ophthalmoscopy did not show any effects after treatment with omethoate. Haematology, clinical chemistry and urinalysis were not affected by the treatment. No effects were noted with regard to absolute and relative organ weights and at gross pathology.

The investigations on the most sensitive parameter for omethoate toxicity, the inhibition of ChE (determined according to Ellmann, 1961), did also not reveal any indication of an adverse effect at the tested dose. In males at the beginning of the study slightly lower activities compared to control were observed with regard to plasma and erythrocyte ChE activity, whereas towards the end of the study slightly higher activities were noted. In females higher activities of ChE in erythrocytes were noted throughout the study in the treated group compared to the control, revealing a statistically significant difference at week −2, 0, 7 and 11. As the higher activity was already observed before treatment had commenced this observation is not due to the treatment with omethoate, but due to interindividual variations in ChE activity. Practically no effect was noted on ChE activity in plasma at any time point. Cholinesterase activity in the brain was not affected in either sex. At no time point was there noted a biologically relevant (> 20%) or statistically significant inhibition compared to the control. As no effects for any investigated parameter were noted, the tested dose of 0.0125 mg/kg bw per day represents the NOEL for this 90-day gavage study in dogs.

No effects even with regard to the most sensitive parameter, the AChE activity, were noted at the single test dose of 0.0125 mg/kg bw per day in this 90-day gavage study in female and male dogs. Consequently, 0.0125 mg/kg bw per day represents the NOAEL for a subchronic treatment of dogs with omethoate.

The study was GLP compliant (Ruf & Mager, 1991).

Study 2

In another 90 day repeated feeding study, groups of pure-bred Beagle dogs, four male (age 6 months) and four female (age 8 months) were fed for 14 weeks either a control diet or a diet containing omethoate at 0 0.4, 0.8 or 1.6 ppm (equal to 0, 0.016, 0.032 and 0.063 mg/kg bw per day for males and 0, 017, 0.034, 0.069 mg/kg bw per day for females). Omethoate was from batch AC 989-39, purity: > 95%, with no impurities seen by TLC. Diet was prepared fresh weekly and the test substance admixed on a weight basis.

Cholinesterase activity in blood (erythrocytes and plasma) was measured in blood samples taken pretreatment (twice) and after 1, 3, 7 and 12 weeks of treatment. Cholinesterase activity was determined by a modified Michel's electrometric methodology.

All dogs survived the feeding period of 14 weeks with omethoate and the animals showed no signs of toxicity related to treatment. Food consumption and body weight gain of males was higher than females, but no dose-related effect on these two parameters could be observed at dietary levels up to 1.6 ppm.

No evidence of an effect related to treatment with omethoate could be found on haematocrit, haemoglobin or total and differential leukocyte count. Determination of the plasma levels of alkaline phosphatase activity, glucose and urea nitrogen showed a slight decrease in mean urea nitrogen after 12 weeks of treatment with 1.6 ppm omethoate in the diet. This value is only marginally below the lower 95% confidence interval and was considered to be of no clinical significance. Therefore, diets containing up to 1.6 ppm omethoate have no adverse effect upon clinical chemical parameters.

The mean individual plasma and erythrocyte ChE activity values did not indicate that omethoate treatment up to 1.6 ppm might have an inhibitory effect in female or male dogs. Therefore it is concluded that up to the dietary concentrations of 1.6 ppm no inhibition of blood ChE activity was induced in female or male dogs.

No effects were observed on any parameter investigated in a 90-day feeding study in dogs up to the highest dose group of 1.6 ppm in the diet. Consequently 1.6 ppm omethoate in the diet (equal to 0.063 mg/kg bw per day in males, 0.069 mg/kg bw per day in females), the highest dose tested, represents the NOAEL in this study.

The study was not GLP compliant (Hutchison et al., 1968).

Study 3

In a one-year gavage study omethoate (purity 97.1%, dissolved in Lewatit water adjusted with HCl to pH 3–4) was administered in a volume of 10 mL/kg bw via stomach tube to male and female Beagle dogs (six dogs/sex per dose) at 0, 0.025, 0.125 and 0.625 mg/kg bw per day. At the time of randomization (one week before treatment, week −1) the animals were 30–32 weeks old and weighed 6.7–9.7 kg. The test doses were selected on results from a pilot study, showing no effect on ChE activity at 0.025 mg/kg bw per day and clear inhibition at 0.625 mg/kg bw per day. The animals were treated continuously seven days per week for 364 or 365 days. Parameters evaluated included appearance and behaviour (daily) clinical signs including body temperature, pulse rate, reflex tests, body weight (weekly), food consumption (daily), ophthalmology, haematology, clinical chemistry, urinalysis, ChE activity (according to the modified method of Ellman, 1961) in erythrocytes and plasma (weeks −1, 0, 2, 5, 8, 12, 25, 39 and 51) and in the brain at week 52, organ weights (absolute and relative), gross and microscopic pathology.

No mortalities occurred throughout the entire study. No treatment-related effects were noted with regard to appearance and behaviour of the animals. Feed and water intake, body weights and nutritional status were unaffected up to the highest test dose. The reflex tests, body temperatures and pulse rates, as well as the ophthalmoscopic examinations did not reveal any treatment-related findings. No treatment-related effects were observed with regard to haematology, clinical chemistry and urinalysis. Absolute and relative organ weights were unaffected by omethoate. At necropsy neither gross pathological nor histopathological findings related to omethoate administration were noted.

The only treatment-related effect noted in this study, was a biologically relevant inhibition of ChE at some test doses.

Effects on plasma ChE activity, as a marker of exposure, were noted for the two higher test doses, exceeding 20% reduction compared to the concurrent control at several time points for the highest test dose.

Slightly decreased activity, close to the biological variations of 13.2% and 10.0% as observed for females and males at week −1 (pretreatment) were noted at 0.125 mg/kg bw per day. In males at week 0 an inhibition slightly above 20% (−25%) was observed when compared to the concurrent control group. In contrast, only a deviation of 7% is obtained if the activity is compared to the pretreatment value for the same group.

No effects were noted at 0.025 mg/kg bw per day, except for in week 0 where an inhibition of plasma ChE slightly above 20% (−22.3%) in males was noted. However, this variation is not considered to be of any toxicological relevance taking into account that almost no deviation (−2%) is noted compared to the activities recorded at week −1 (before treatment) and controls have a remarkably high activity just at day 0.

In general, the pretreatment (starting) values of plasma ChE for the mid- and low -dose groups in females and males were clearly lower than the concurrent control. Consequently, lower activities were observed for these groups throughout the study, causing a "pseudo" inhibitory effect by comparing activities to the concurrent control group.

By comparing the figures obtained at the different time points with the pretreatment ChE activities in these groups, no relevant effects are noted for the mid- and low-dose groups.

Table 37. Effects of omethoate treatment on plasma cholinesterase activity (PChE) in dogs

Dose	Week	−1	0	2	5	8	12	25	39	51
		Plasma cholinesterase activity (kU/L) [% inhibition given in brackets]								
Males										
0		1668	1898	1683	1732	1698	1795	1772	1972	1810
0.025 mg/kg bw/day		1502	1475 [22.3]	1468 [12.8]	1462 [15.6]	1473 [13.3]	1557 [13.3]	1605 [9.4]	1773 [10.1]	1588 [12.3]
0.125 mg/kg bw/day		1525	1423 [25.0]	1437 [14.6]	1410 [18.6]	1362 [19.8]	1487 [17.2]	1443 [18.6]	1608 [18.5]	1508 [16.7]
0.625 mg/kg bw/day		1602	1390 [26.8]	1262 [25.0]	1177 [32.0]	1205 [29.0]	1272 [29.1]	1238 [30.1]	1407 [28.7]	1333 [26.4]
Females										
0		1642	1702	1648	1675	1652	1682	1910	1942	1957
0.025 mg/kg bw/day		1587 [3.4]	1497 [12.0]	1555 [5.6]	1553 [7.3]	1570 [5.0]	1635 [2.8]	1772 [7.2]	1958 [+0.8]	1828 [6.6]
0.125 mg/kg bw/day		1482 [9.7]	1368 [19.6]	1393 [15.5]	1407 [16.0]	1397 [15.4]	1537 [8.6]	1552 [18.7]	1743 [10.3]	1560 [20.3]
0.625 mg/kg bw/day		1707 [4.0]	1423 [16.4]	1213 [26.4]	1218 [27.3]	1203 [27.2]	1245 [26.0]	1388 [27.3]	1563 [19.5]	1382 [29.4]

+ This value is a % increase relative to untreated controls

Inhibition of ChE in erythrocytes, a marker for potentially adverse effects, was observed in male and female dogs throughout the entire test period. In general, more pronounced erythrocyte ChE inhibition was noted in male than in female dogs after omethoate administration.

At 0.625 mg/kg bw per day a quite constant biologically relevant inhibition of up to 48.7% was noted for both sexes from week 2 onwards for the remaining study period.

At 0.125 mg/kg bw per day the trigger value of 20% erythrocyte ChE inhibition was reached or slightly exceeded from week 2 to 37 in male dogs indicating a biologically relevant effect at this dose level. In females the inhibition was less pronounced reaching 20% only at week 20. No clear time course for the inhibitory effects was noted and no indications were found that the effects may increase with prolonged exposure.

At 0.025 mg/kg bw per day no effects on erythrocyte ChE were noted in female dogs. In males at weeks 2, 5, 8 and 12 somewhat higher variations in erythrocyte ChE inhibition (up to a maximum of 20% inhibition observed only in week 12) were noted compared to all other time points, where only marginal effects were observed. Again no relation was seen between treatment time and effect (there was less inhibition at week 0 and from week 25 onwards).

Taking into account the higher than usual activity in control animals in comparison with the concurrent control at other time points, it is concluded that no relevant ChE inhibition is evident at any time point.

Consequently the observed variations at 0.025 mg/kg bw per day was not considered a biologically relevant effect of omethoate treatment.

Table 38. Effects of omethoate administration on erythrocyte cholinesterase activity in dogs

Dose	Week	−1	0	2	5	8	12	25	39	51
		Erythrocyte cholinesterase activity (kU/L) [% inhibition given in brackets]								
Males										
0		1.972	1.872	2.007	2.422	2.068	2.253	1.782	1.825	2.062
0.025 mg/kg bw/day		1.960	1.622 [13.4]	1.693 [16]	1.958 [19]	1.703 [18]	1.795 [20]	1.678 [6]	1.685 [8]	1.943 [6]
0.125 mg/kg bw/day		1.955	1.667 [11.0]	1.520 [24]	1.773 [27]	1.495 [28]	1.625 [28]	1.423 [20]	1.517 [17]	2.002 [3]
0.625 mg/kg bw/day		1.983	1.545 [17.5]	1.218 [40.0]	1.242 [48.7]	1.178 [43.0]	1.297 [42.4]	1.133 [36.4]	1.290 [29.3]	1.388 [32.7]
Females										
0		1.787	1.683	1.798	2.005	1.863	1.998	1.875	1.697	2.262
0.025 mg/kg bw/day		1.797	1.590 [5.5]	1.835 [+2.1]	1.975 [1.5]	1.788 [4.0]	1.907 [4.6]	1.747 [6.8]	1.812 [+6.8]	2.090 [7.6]
0.125 mg/kg bw/day		1.808	1.662 [1.2]	1.573 [12.5]	1.685 [16.0]	1.490 [20.0]	1.697 [15.1]	1.542 [17.8]	1.638 [3.5]	1.820 [19.5]
0.625 mg/kg bw/day		1.882	1.318 [21.7]	1.343 [25.3]	1.263 [37.0]	1.183 [36.5]	1.302 [34.8]	1.225 [34.7]	1.332 [21.5]	1.355 [40.1]

+ These values are % increases relative to untreated controls

The effect on brain ChE activity was investigated immediately after necropsy.

Once again, male dogs were more sensitive to omethoate treatment than females. At 0.625 mg/kg bw per day a marked reduction of brain ChE activity was noted (39% and 30% inhibition for males and females, respectively).

At 0.125 mg/kg bw, no effects were noted in female dogs, but a borderline result for biologically relevant brain ChE activity inhibition was noted for male dogs (the trigger value of 20% inhibition was just reached).

No effects were noted at 0.025 mg/kg bw per day.

Table 39. Effects of omethoate treatment on brain cholinesterase activity in dogs

	Brain cholinesterase activity (kU/L) [% inhibition given in brackets]	
Dose	**Males**	**Females**
0	0.605	0.620
0.025 mg/kg bw/day	0.582 [3.8]	0.603 [2.7]
0.125 mg/kg bw/day	0.482 [20]	0.627 [+1.1]
0.625 mg/kg bw/day	0.370 [39]	0.435 [30]

+ This value is a % increase relative to untreated controls

The NOAEL in this one-year gavage study in dogs was 0.025 mg/kg bw per day for males and 0.125 mg/kg bw per day for females. This NOAEL is based on slight effects (reaching or slightly exceeding the trigger value of 20% inhibition) on ChE activity in erythrocytes and brain in males at 0.125 mg/kg bw per day and clear effects (30 to 40% inhibition) on brain and erythrocyte ChE activity in females at 0.625 mg/kg bw per day.

The study was not GLP compliant (Hoffmann & Schilde, 1984).

(b) Dermal application

Rabbit

Groups of six male and six female New Zealand White rabbits (weight 2.4–2.9 kg) were dosed with dermal application of omethoate (batch Eg. 1/76; purity 94%) at levels of 0 (vehicle control), 2, 5 and 20 mg/kg bw per day. The vehicle used was deionised water. The test substance was applied in a volume of 0.5 mL/kg bw evenly to an area of 25 cm² of the clipped back of each animal and left there uncovered for 7 h. Thereafter, the test sites were washed with soap and water. For half the animals in each group, the test areas were abraded before dose application. The applications were made daily for five days per week for three weeks (15 applications/animal in total). Rabbits were immobilized in restraining devices during the 7 h exposure time. Rabbits were examined daily for dermal reactions and clinical signs and weighed at the start and the end of each week of the study. Blood (taken from the ear vein) and urine samples were taken at initiation and termination of the study for haematological and clinical chemistry assays including plasma and erythrocyte ChE determinations as well as for urinalysis. In addition, ChE activity was also determined after the eighth treatment.

At termination, animals were subjected to a gross postmortem examination, a selection of eight tissues weighed (heart, lungs, liver, spleen, kidneys, adrenals, testes or ovaries and thyroid) and a complete set of tissues examined microscopically in the control and top dose groups. Brain AChE was determined at necropsy in all groups.

Acetylcholinesterase activity in blood, plasma, and brain were determined according to the Ellmann method.

No animal died during the study.

No effects on appearance and behaviour due to omethoate treatment were noted, besides clinical signs of slight muscle spasms for the first three treatments in the high-dose group with abraded skin. No other effects were noted.

The effects were noted on AChE activity in erythrocytes, plasma and brain.

The NOAEL in the 21-day dermal toxicity study in rabbits was 2.5 mg/kg bw per day, based on biologically relevant effects on AChE activity in erythrocytes and brain at 20 mg/kg bw per day.

The study was not GLP-compliant (Flucke & Luckhaus, 1979).

3.3 Long-term studies on toxicity and carcinogenicity of omethoate

Mouse

In an oncogenicity study in mice (strain B6C3F_1), omethoate (batch 234808038; purity 96.5–97.4%) was administered daily to groups of 50 male and 50 female mice, including 10 animals per sex and dose for an interim kill after one year. Omethoate concentrations were 0, 0.5, 4 and 32 ppm (equal to 0, 0.10, 0.82 and 6.48 mg/kg bw per day in males, 0, 0.11, 0.80 and 6.61 mg/kg bw per day in females) for ad libitum ingestion in the drinking water (adjusted to pH 3) for a period of 24 months.

Acetylcholinesterase activities were measured in plasma and erythrocytes during weeks 52/53 and 104 as well as on 10 randomly selected animals per sex and dose during week 10 and on 10 males per dose during week 105. Acetylcholinesterase activity in the brain was determined on interim sacrifice and after termination of the study. For determination of the plasma and brain AChE activity the modified Ellmann method (1961) was followed. A complete examination of clinical chemical parameters was performed during weeks 51/52/53, 79 and 103/104/105. Haematology was determined during weeks 51/52 and 103. An interim necropsy was performed after 12 months and after 24 months with all surviving animals and organs/tissues subjected to both thorough gross pathological and histopathological examination including determination of organ weights. In addition a complete necropsy was performed on all mice that died or were killed in extremis in moribund condition. The primary objective of the study was to investigate the neoplastic potential of the substance.

The most prominent substance-related clinical sign of toxicity was characterized by tremor in males and females of the high-dose group only, which was first observed during week 2 and disappeared

after week 8. Loss of hair in females of the low- and high-dose groups, poor general condition of males in the mid- and high-dose groups as well as dose-related signs of emaciation in both sexes of mice could be attributed to treatment. Other observable effects showed no dose–response relationship or occurred sporadically in one sex of animals and are therefore considered not toxicologically relevant.

The cumulative water intake per animal was comparable for all dose groups. Water intake related to body weight was reduced by 13 and 10% in the mid- and high-dose groups of female animals, respectively, while corresponding water intake rates for males differed only marginally from controls.

Food intake rates per animal were reduced in males of the high-dose group by ca 15% while in females no influence was noted. Food uptake rates related to kg body weight were decreased in the mid- and high-dose group of males by 10 and 17% as well as in the mid- and high-dose group of females by 16 and 13%, respectively. This finding is not associated with a toxicologically adverse effect.

Body weights of male mice were statistically significantly lowered in the high-dose group up to and including week 12. Thereafter, body weights normalized and were intermittently increased when compared to controls. In the low- and mid-dose groups of males occasional body weight depressions were observable in a statistically significant manner. Since the degree of body weight reduction did not exceed 7% and no clear dose–response relationship was observable no toxicological adverse effect was inferred.

In the low-dose group of females occasional body weight increases were seen throughout the study. In the mid-dose group statistically significant body weight depressions were obvious from week 3 through 10, while from week 24 through 88 significant increases in body weights (max. 18%) were noted. In females of the high-dose group statistically significant body weight depressions were observed during the first four study weeks. During weeks 7 through 68 body weights of females of the high-dose group were increased and the maximum difference was reached during weeks 28 through 48 (18–22%). Thereafter body weights returned to levels of control animals until termination of the study.

There were no treatment-related higher mortality rates notable during and on termination of the study in both sexes of mice. By the end of week 65 higher mortalities were seen in the low-dose group of females and by the end of week 78 higher mortalities were observed in the mid-dose group of males and in the low-dose group of females. Since these deaths were not causally related to a dose–response relationship this finding is neither considered treatment-related nor toxicologically relevant.

Examinations performed during weeks 51/52 and 103 revealed several changes in haematological parameters mainly in the mid- and high-dose groups of male and female animals, respectively. A clear substance-related lowering of erythrocyte count and haematocrit were detectable in male animals of the high-dose group both after 51/52 and at 103 weeks. Haemoglobin concentration was also decreased in the high-dose males after 103 weeks. Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration (MCHC) were statistically significantly increased in male animals of the mid- and high-dose groups after 51/52 weeks and in the high-dose group of males after 103 weeks (MCHC only). In females the thrombocyte count was significantly increased in the high-dose group after 51/52 weeks and in the mid-dose group after 103 weeks. The thromboplastin time was significantly decreased in female animals of the mid- and high-dose groups after 51/52 weeks only, while during the second examination after 103 weeks no differences to controls were evident in any of the treatment groups. A marginal increase in haemoglobin concentration was noted in the low- and high-dose females after 51/52 weeks with no detectable dose–response relationship.

After examination of white blood cells, the monocyte count was statistically significantly increased in all treatment groups of male mice after 51 weeks and in the high-dose group only after 79 weeks. This increase was also observable in females of all treatment groups during examination after 51 weeks and reached statistical significance in the mid- and high-dose groups only. In addition, segmented neutrophils were significantly decreased after 79 weeks and significantly increased after 103 weeks in males, which is not considered to be of toxicological relevance due to both an inverse correlation at two investigation time points and a missing dose–response relationship. The above-mentioned effects and all other deviations, not explicitly described, were not considered to be of toxicological relevance since they were either isolated findings in single animals or they did not follow a clear dose–response relationship. In addition, all values lie in any case within the range of historical controls.

In male animals a significant decrease in apartate aminotransferase (ASAT) in the mid-dose group and of the alkaline phosphatase (ALP) in the high-dose group was observable after 104 weeks. ASAT was significantly increased in low-dose females after 104 weeks, an effect which is not considered toxicologically relevant in light of the missing dose–response relationship and since the higher values reported are caused by two individuals. Urea was statistically significantly lowered in high-dose males and females after 52 weeks and both in mid- and high-dose males after 104 weeks. In the case of male animals the differences seen as compared to controls after 104 weeks were regarded as not relevant since one control animal showed an exceptionally high value after 104 weeks. Creatinine was lowered in mid- and high-dose males after 104 weeks but was in the control range after 52 weeks for all dose groups. This effect is again attributed to one control animal which showed extremely high values after 104 weeks. Triglycerides were statistically significantly lowered in mid- and high-dose females after 104 weeks and comparable to controls after 52 weeks in all other treatment groups. The increased glucose concentrations in the low- and high-dose females after 52 weeks are not of toxicological relevant since they are caused by single animals in these dose groups.

Plasma AChE activity was lowered in the high-dose group of males after 52/53, 59 and 104/105 weeks and in the mid-dose group after 52/53 and 104/105 weeks. In females plasma AChEactivity was decreased at all time points in the high-dose group, decreased in the mid-dose group after 52/53 and 59 weeks and decreased in the low-dose group only after 59 weeks. In males and females erythrocyte AChE activity remained statistically significantly lowered in the high-dose groups at all time points (up to 93% inhibition) and was decreased also in the mid-dose groups after 52/53 and 59 weeks (up to 57% inhibition) as well as in the low-dose group after 52/53 weeks (up to 25% inhibition) only. Depression of brain acetylchloiesterase activity by a maximum of 53% and 84% was observable in the mid- and high-dose groups respectively, apparent at interim sacrifice and on termination of the study.

Investigation of organ weights at interim necropsy revealed statistically significantly increased spleen weights in male animals of the mid- and high-dose groups. Absolute and relative spleen weights were increased by 22% and 13% in the mid-dose group and increased by 15% and 9% in the high-dose group, respectively (not shown). Since higher spleen weights were accompanied by body weight increases in the corresponding treatment groups of males, no toxicological relevance is inferred. In female animals relative weight of the lung was lowered in the mid- and high-dose groups at interim sacrifice as was the relative weight of the liver and the kidneys in the mid-dose group only.

At terminal sacrifice absolute weight of kidneys was statistically significantly increased in the high-dose males, while absolute liver weights were decreased, thereby showing a dose–response relationship. Relative weights of the following organs were reduced in a statistically significant manner in male animals at terminal sacrifice: brain in the mid- and high-dose groups, heart in the mid-dose group, lung in the low- and mid-dose groups, liver in all treatment groups, testes in the mid- and high-dose groups. In females relative brain weight was reduced in the mid-dose group only, relative heart weight was lowered in the high-dose group only and relative kidney weights were decreased in the mid- and high-dose groups.

None of these findings are considered to be of toxicological relevance since changes were either minor and, in most cases, did not exceed 10% compared to controls, or a clear dose–response relationship was not detectable. The significant reduction of absolute and relative liver weights can be associated with treatment but does not represent an adverse effect because both clinical chemistry and histopathology did not reveal any indication of an impairment in organ function.

Histopathological examination of animals scheduled for interim necropsy did not show any features related to treatment so any findings were considered to be of spontaneous origin.

At terminal sacrifice the only histopathological finding was characterized as ovarian atrophy with statistical significance starting in the mid-dose group. It was shown that 31% of control animals, 43% of the animals of the low-dose group, 83% of the animals of the mid-dose group and 70% of the animals of the high-dose group were so affected. Since no clear dose–response relationship was shown between mid- and high-dose groups the observation is interpreted to be age- and not substance-related. All other organs and tissues examined showed no differences to control animals and were considered to be within the historical range.

After performing histopathological examinations on animals scheduled for interim necropsy, no increase in the incidence of spontaneous tumours was observed in treated animals, nor the formation of tumours other than those normally spontaneously occurring. There were also no treatment-related influences detectable on the number of benign and malignant tumours among groups.

On terminal necropsy it was found that male animals of the high-dose group only showed a slightly increased incidence of tumours of the Harderian gland, while in females no differences were seen compared to untreated controls. Furthermore, male animals of the high-dose group showed higher incidences in the formation of bronchioalveolar adenomas of the lungs while in the low- and mid-dose groups incidences were lower than in controls. In females, higher tumour rates were not observed in the lungs among treated groups. While the incidences of hepatocellular carcinoma were lower in the mid- and high-dose groups of males when comparison to untreated controls was made, it was shown that in mid- and high-dose females the incidence in the development of hepatocellular adenoma and carcinoma was higher when compared to controls. In all other organs and tissues examined treatment-related increases were found in neither the number nor type of tumours. In addition, treatment did not lead to an acceleration of tumour formation. Tumours found only in one dose group and/or in one sex were regarded as incidental findings and not related to treatment.

In view of the above, in a 24-month chronic toxicity/carcinogenicity study in male and female mice with omethoate, no higher incidence of tumour formation was detectable. Clear dose- and substance-related effects were seen on the activity of the AChE in plasma, erythrocytes and brain. The inhibition of the corresponding activities exceeded 20% in the mid- and high-dose groups of both sexes of mice. In females and males of the low-dose group AChE activities were demonstrated to be slightly above the trigger value of 20%. This may be considered a borderline effect and a first indication of a potentially adverse reaction to omethoate administration. Thus, the LOAEL in this chronic toxicity study conducted in mice can be expected to be 0.5 ppm in drinking water (equal to 0.10 and 0.11 mg/kg bw per day in male and female mice, respectively) based on inhibition of erythrocyte AChE at the lowest dose tested. Hence no NOAEL can be deduced. The NOAEL for carcinogenicity for mice is more than 32 ppm (equal to 6.48 mg/kg bw per day in males, 6.61 mg/kg bw per day in females) the highest dose tested.

The study was GLP compliant (Schladt, 2001).

Rat

Omethoate (batch 234808038; purity 96.5–97.4%) was administered daily to groups of 50 male and 50 female Wistar rats, each group including 10 animals per sex and dose for an interim kill after one year (strain Bor:WISW). Omethoate was given at concentrations of 0, 0.5, 4 and 32 ppm (equal to 0, 0.04, 0.30 and 2.92 mg/kg bw in males, 0, 0.05, 0.44 and 3.93 mg/kg bw in females) for ad libitum ingestion in the drinking water (adjusted to pH 3) for a period of 24 months. Dose levels were selected on the basis of the results obtained in a preceding seven-week drinking water study in male and female mice where doses of 0, 25, 50 and 100 ppm were tested. Experimental animals were inspected twice daily for clinical signs and anomalies. Detailed examination of individual animals was performed once weekly with emphasis on appearance, general behaviour, exterior of the body, bodily orifices, posture, respiration and excretory products. Body weights were determined weekly, water consumption was determined once a week until week 13 and monthly thereafter for the calculation of test substance intake and food intake rates. Acetylcholinesterase activities were measured in plasma and erythrocytes during weeks 26, 52/53, 78 and 105 as well as on ten randomly selected 0 and 0.5 ppm group animals per sex and dose during week 28. Acetylcholinesterase activity in the brain was determined on interim sacrifice and after termination of the study. A complete examination of clinical chemical parameters was performed during weeks 26, 52, 78 and 105. Haematology was determined during weeks 26/27, 52, 78 and 105. An interim necropsy was performed after 12 months and after 24 months with all surviving animals and organs/tissues subjected to both thorough gross pathological and histopathological examination including determination of organ weights. In addition a complete necropsy was performed on all mice that died or were killed in extremis in moribund condition. The primary objective of the study was to investigate the neoplastic potential of the substance.

The most obvious effects after inspection of treated animals were primarily evident in the high-dose group. Substance-related signs were characterized by loss of hair in females and emaciation in

both males and females. With the exception of one male, tremor was observable in males and females of the high-dose group until week 7. An increased incidence of ocular opacity was evident in females of the mid- and high-dose groups as well as in males of just the high-dose group. Other observable effects showed no clear dose–response relationship and are therefore not considered toxicologically relevant.

Body weights in male animals were significantly depressed in the low- and mid-dose groups until week 8 which was considered a minor effect since body weight reductions did not exceed 10% and returned to control levels again by weeks 12 and 24, respectively. In the high-dose group of males, however, body weights were reduced throughout the study (mean reduction of 11% compared to untreated controls) and did not reach the levels of control animals. In males, pronounced body weight depression was notable during week 1 (23%). In the high-dose group, body weights of female animals were depressed by 19% during week 1 and remained lowered until week 36. Thereafter levels of control animals were reached.Haematological parameters are shown in following table:

Table 40. Haematology and differential blood count parameter changes in male and female rats at different time points after chronic administration of omethoate in drinking water over 24 months

Dose (ppm)	Haematology							Differential blood count		
	LEUCO (10^9/L)	ERY (10^{12}/L)	Hb (g/L)	Ht (L/L)	MCV (fl)	MCH (pg)	MCHC (g/L_{ERY})	LYM (%)	EOS (%)	MONO (%)
Week 26/27										
Males										
0	9.2	9.21	155	0.495	54	16.8	313	87.1	0.8	1.6
0.5	8.2	9.21	157	0.501	54	17.1	314	88.3	0.6	1.9
4	8.3	9.42	154	0.493	52*	16.3*	312	84.7	0.4	2.7
32	9.5	8.95*	147**	0.461**	52**	16.4	318*	86.5	0.8	2.1
Females										
0	6.1	8.27	147	0.471	57	17.8	311	87	1.2	1.9
0.5	6.0	8.40	149	0.474	56	17.7	314	84.1	1.0	2.7
4	6.4	8.42	146	0.467	56	17.3	312	86.8	1.0	2.4
32	7.3	8.04	145	0.452*	56	18.0	320**	80.5*	0.9	2.8
Week 52										
Males										
0	10.4	9.20	161	0.472	51	17.5	342	81.8	0.6	5.0
0.5	9.0*	9.06	157	0.471	52	17.4	333	83.3	0.4	4.1
4	7.5**	9.58	157	0.485	51	16.4**	324**	85.1	0.6	3.2*
32	10.0	9.21	150**	0.451*	49**	16.3**	333	83.4	0.8	2.0**
Females										
0	5.7	8.03	142	0.452	56	17.7	314	84.3	0.8	3.1
0.5	5.8	7.96	142	0.450	57	17.8	315	79.6	1.0	3.1
4	5.5	7.82	137*	0.433**	55	17.5	316	84.2	0.7	1.9
32	6.6	7.87	143	0.440	56	18.1	324**	79.9	0.0**	3.9
Week 78										
Males										
0	9.4	9.17	156	0.478	52	17.0	327	76.7	0.8	5.1
0.5	8.1*	9.09	159	0.485	53	17.5	328	79.6	0.8	6.0
4	7.9*	9.53	158	0.490	52	16.6	322	82.1	0.3	5.1
32	9.1	9.18	151	0.453*	49**	16.4*	333	82.6	0.1*	4.2

Dose (ppm)	Haematology							Differential blood count		
	LEUCO (10^9/L)	ERY (10^{12}/L)	Hb (g/L)	Ht (L/L)	MCV (fl)	MCH (pg)	MCHC (g/L_{ERY})	LYM (%)	EOS (%)	MONO (%)
Females										
0	7.7	8.21	150	0.481	59	18.3	311	76.7	0.5	2.4
0.5	6.5	8.17	151	0.482	59	18.5	313	82.4	0.0	1.1
4	6.2	8.42	149	0.478	57	17.8	313	83.5	0.2	0.4**
32	6.8	7.77	145	0.454	58	18.6	319*	75.4	0.9	1.4
Week 105										
Males										
0	8.2	9.22	155	0.491	53	16.9	317	70.0	0.8	5.4
0.5	8.1	8.83	151	0.477	54	17.1	317	68.8	0.8	6.1
4	6.7	9.03	149	0.469	52	16.5	317	75.4	1.1	5.6
32	6.7*	8.53	142**	0.438**	52*	16.8	324*	70.7	0.8	5.8
Females										
0	6.7	8.50	149	0.474	56	17.6	315	68.5	0.0	5.8
0.5	6.0	8.53	149	0.469	55	17.5	318	71.1	0.7*	4.8
4	6.0	8.56	147	0.459	54	17.2	319	68.3	0.4	6.5
32	6.8	8.24	145	0.448	54	17.6	324*	66.9	0.4	4.2

LEUCO Leukocytes ERY Erythrocytes Hb: Haemoglobin Ht: Heamatocrit MVC Mean corpuscular volume
MCH mean corpuscular haemoglobin MCHC mean corpuscular haemoglobin concentration
LYM lymphocytes EOS eosinophils MONO monocytes
* $p < 0.05$ ** $p < 0.01$

Several substance-related changes in haematological parameters were found: decrease in haemoglobin (Hb) concentration, heamatocrit (Ht), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) primarily in the high-dose group of male animals. Changes in other haematological parameters, such as thrombocyte count, occurred either at a single time point or were not associated with a dose–response relationship. In females MCHC was statistically significantly increased at all time points and Ht was lowered in the mid-dose group after 52 weeks and in the high-dose group after 26/27 weeks.

There was a statistically significant decrease in lymphocyte count in high-dose females after 26/27 weeks and a decrease in the monocyte count in mid- and high-dose males after 52 weeks as well as a decrease in eosinophils in the high-dose males after 78 weeks. All other significant findings did not show a dose–response relationship and were thus considered not toxicologically relevant.

The details of clinical chemistry parameters are given in the Table 41.

Table 41. Clinical chemical parameters determined in male and female rats at different time points after chronic administration of omethoate in drinking water over a period of 24 months

Dose (ppm)	Clinical chemistry				
	AST (U/L)	ALT (U/L)	Cholesterol (mmol/L)	TG (mmol/L)	Creatinine (mmol/L)
Week 26					
Males					
0	29.9	40.2	2.61	1.58	46
0.5	33.7	43.6	2.51	1.62	47
4	31.9	40.2	2.42	1.21	50
32	37.2**	45.6	2.56	1.25	47

Dose (ppm)	Clinical chemistry				
	AST (U/L)	ALT (U/L)	Cholesterol (mmol/L)	TG (mmol/L)	Creatinine (mmol/L)
Females					
0	38.7	44.5	2.46	1.45	49
0.5	47.0	64.4	2.26	0.95*	47
4	52.1	50.6	2.55	1.03	49
32	49.8	51.9	2.43	0.71**	52*
Week 52					
Males					
0	41.1	56.3	3.28	1.86	47
0.5	43.0	61.8	3.20	2.19	46
4	45.3	56.0	2.87*	1.37*	46
32	45.3	58.1	3.29	1.44	45
Females					
0	65.4	65.4	2.72	1.84	52
0.5	60.1	73.8	2.43	1.39	47
4	62.5	65.9	3.06	1.47	44
32	81.1	93.6*	3.00	1.25	45
Week 78					
Males					
0	41.5	50.3	4.32	2.10	44
0.5	38.2	48.9	4.67	2.59	46
4	34.6*	46.8	4.20	1.57**	45
32	42.2	56.2	4.04	1.62**	44
Females					
0	83.9	62.2	2.97	2.05	48
0.5	69.2	64.6	2.60	1.66	44**
4	83.1	73.8	3.51	2.11	44**
32	95.7	90.7	3.47	2.15	46
Week 105					
Males					
0	39.8	43.9	5.13	2.56	46
0.5	44.2	45.9	5.99	2.78	60
4	40.5	49.4	4.31	2.07	45
32	39.6	52.7*	4.48*	1.79*	46
Females					
0	107.7	76.4	3.16	1.77	59
0.5	78.0	62.2	2.51	1.42	47*
4	82.9	71.0	2.95	1.60	48
32	77.8	76.3	3.00	1.42	45**

AST Aspartate aminotransferase ALT Alanine aminotransferase TG Triglycerides

* $p < 0.05$ ** $p < 0.01$

The above mentioned effects, and all other not explicitly described deviations on clinical chemical parameters, were only marginally changed and always lay in the range of the historical controls or were evident in one sex only without a recognizable dose–response relationship.

Regarding urine parameters, the only change reaching statistical significance was a decrease in protein excretion in male animals in the high-dose group after 25, 77 and 104 weeks and in the mid-dose group after 51 and 104 weeks, respectively. This result is not considered toxicologically relevant since these decreases were always associated with a lowered urinary volume and the excretion of protein with the urine was lower in treated animals when compared with concurrent controls.

Plasma AChE activity was lowered in the high-dose group of males after 26, 52, 78 and 105 weeks and in the mid-dose group after 26, 52 and 104 weeks, while erythrocyte AChE activity was statistically significantly lowered in all treatment groups at all time points with an inhibition at or slightly above the trigger of 20%. Brain AChE activity was statistically significantly reduced in male animals of the mid- and high-dose groups both at interim sacrifice and on termination of the study, while in the low-dose group an increase by 18.2% was evident at interim sacrifice and only a marginal and toxicologically irrelevant decrease by 5% was noted on termination of the study. In females plasma AChE activity was decreased at all time points in the high-dose group only while erythrocyte AChE activity remained statistically significantly lowered in the mid- and high-dose groups at all time points studied. The inhibition of the erythrocyte AChE activity in females of the low-dose group remained below the trigger of 20% after 26, 28, 52, 78 and 105 weeks and is thus considered not toxicologically relevant. Brain AChE activity was significantly reduced in females of the mid- and high-dose groups at interim sacrifice and on termination of the study, whereas in the low-dose group only a marginal and toxicologically irrelevant inhibition of at most 9%, or no inhibition, respectively, was apparent at interim and terminal sacrifice (see Table 42).

Table 42. Acetylcholinesterase activities in plasma, erythrocytes and brain of male and female rats after chronic administration of Omethoate in drinking water over a period of 24 months

		Plasma ChE				Erythrocyte ChE				Brain ChE			
		male		female		male		female		male		female	
Week	ppm	kU/L	I (%)	kU/L	I (%)	kU/L	I (%)	kU/L	I (%)	kU/L	I (%)	kU/L	I (%)
26	0	0.49	-	1.58	-	0.76	-	0.79	-				
	0.5	0.46	6	1.67	−5.7	0.48**	37	0.64**	19				
	4	0.37**	24	1.39	12	0.18**	76	0.26**	67				
	32	0.20**	59	0.49**	69	0.06**	92	0.06**	92				
28	0	0.48	-	1.93	-	0.88	-	0.90	-				
	0.5	0.48	-	1.73	10	0.62**	30	0.80	11				
52	0	0.61	-	1.64	-	0.74	-	0.77	-	1.43	-	2.78	-
	0.5	0.58	5	1.68	5	0.58**	22	0.63*	18	1.69	−18.2	2.54**	9
	4	0.46**	25	1.45	25	0.18**	76	0.13**	83	0.96**	33	1.57**	44
	32	0.22**	64	0.58**	65	0.04**	95	0.04**	95	0.45**	69	0.54**	81
78	0	0.76	-	1.61	-	1.09	-	1.09	-				
	0.5	0.67	12	1.74	−8.1	0.86**	21	0.98	10				
	4	0.67	12	1.59	1	0.34**	69	0.30**	72				
	32	0.30**	61	0.52**	68	0.06**	94	0.50**	95				
105	0	1.00	-	1.93	-	0.81	-	0.73	-	2.02	-	1.86	-
	0.5	0.82	18	2.05	−6.2	0.65**	20	0.63	14	1.91	5	1.87	−0.5
	4	0.79*	21	1.71	11	0.21**	74	0.21**	71	1.28**	37	0.98**	47
	32	0.32**	68	0.56**	71	0.03**	96	0.02**	97	0.51**	75	0.34**	82

* $p < 0.05$ ** $p < 0.01$

Source: Schladt, 1995

Ophthalmoscopic examinations after one year did not reveal any substance-related changes in the examined high-dose groups of male and female animals compared to untreated controls. On termination of the study control, mid- and high-dose groups were subject to ophthalmoscopic examinations. While in the 4 ppm group no changes were seen with respect to untreated control animals there was an increase in the incidence of lens opacity and vascularization of the cornea in males of the high-dose group and higher incidences of vascularization of the cornea in high-dose females. Since these effects are commonly seen in older rats and are also detectable in the concurrent controls this has been regarded as a premature ageing process rather than primary damage to the lens of the eye.

Investigation of organ weights revealed a statistically significant increase of absolute adrenal weight in females of the mid- and high-dose groups and of relative adrenal weight in females of the high-dose group at terminal sacrifice. In male animals, absolute weight of the heart, lung, liver, spleen and kidneys were lowered primarily in the high-dose group at interim and terminal sacrifice. This effect is attributable to a reduced body weight development in males of this dose group. In males, a statistically significant increase in the relative brain weight was seen in the mid- and high-dose groups and a slight but statistically significant increase in the relative adrenal weight in the low- and high-dose groups. A statistically significant increase in the relative testes weight was obvious in the high-dose group only. In treated females no changes in relative organ weights could be seen at interim sacrifice when comparison to controls was made. In treated males relative brain and heart weights were statistically significantly increased in the mid- and high-dose groups at terminal sacrifice, while relative kidney weight was increased in the low-dose group only. In treated females, a statistically significant increase in relative adrenal weight was observable in the high-dose group and a statistically significant decrease in relative kidney weight in the low-dose group only.

After gross pathological examinations at terminal necropsy the main findings observed in male and female rats were substance-related increases in lens opacities in the mid- and high-dose groups. No other effects were seen in the other organs examined.

Histopathological examination of the eyes of control and treated animals at interim sacrifice revealed increased incidences of moderate to severe retinal degeneration in high-dose animals of both sexes. In addition, males of the high-dose group showed increased vacuolation of the epididymal epithelia cells. No further treatment-related effects were observed in other organs examined.

At terminal sacrifice retinal degeneration in males was comparable in control and high-dose animals. However, the degree of severity was more pronounced in the high-dose group which was not the case with high-dose females. In males of the high-dose group increased incidences of lens mineralization were also observable. Effects on the eyes of female animals were characterized by higher rates of keratitis and choroidal proliferation in the high-dose group only.

Vacuolation of the lacrimal glands was markedly increased in high-dose males only and comparable to controls in all other treatment groups of male and female animals. In just the high-dose groups, severe incidences of vacuolation of the epididymal epithelia cells and an increase in the formation of hyperplasia of the mammary glands were observable in males and females, respectively (see Table 43).

Table 43. Non-neoplastic lesion in male and female rats at terminal sacrifice after administration of omethoate in drinking water over a period of 24 months

Remarkable incidences of non-neoplastic lesions at terminal sacrifice								
Sex	**Male**				**Female**			
Dose (ppm)	**0**	**0.5**	**4**	**32**	**0**	**0.5**	**4**	**32**
Eyes								
No. of animals examined	50	49	50	49	50	50	50	50
Retinal degeneration								
No. of animals affected	41	37	36	45	40	36	44	41
slight	5	5	6	1	1	8	2	2
moderate	10	6	3	4	7	4	5	1
severe	26	26	27	40	32	24	37	38

Remarkable incidences of non-neoplastic lesions at terminal sacrifice								
Sex	**Male**				**Female**			
Dose (ppm)	**0**	**0.5**	**4**	**32**	**0**	**0.5**	**4**	**32**
Lens mineralization	2	4	2	9	5	3	11	7
Keratitis	1	0	0	1	0	0	0	5
Choroid proliferation	2	1	3	1	1	2	0	6
Lacrimal glands								
No. of animals examined	50	45	47	48	46	50	50	49
Unilateral vacuolation	17	16	9	48	28	26	29	32
Epididymides								
No. of animals examined	50	50	50	50				
Vacuoles	4	0	10	50				
Mammary glands								
No. of animals examined	35	25	27	23	47	49	49	47
Hyperplasia	1	2	1	1	13	21	23	30

Source: Schladt, 1995

Histopathology performed at interim sacrifice revealed only a few tumours in the various organs examined and no substance-related increase could be inferred. When histopathological evaluation of neoplastic changes was performed at terminal sacrifice no increased incidences of benign or malignant tumours in the various organs and tissues examined were detectable across dose groups, nor could a dose–response relationship be derived. Also, the latency period for the development of tumours was not influenced by treatment with the test substance.

In view of the above, no carcinogenic potential of the test substance could be identified from this combined chronic toxicity/oncogenicity study conducted in male and female rats with omethoate for a period of 24 months. Acetylcholinesterase activities in mid- and high-dose animals of both sexes were significantly reduced by substance treatment, thereby showing that the maximum tolerated dose (MTD)was reached under the conditions of the study. Activities of AChE remained below the trigger value of 20% in females in the 0.5 ppm dose group. In contrast, a significant inhibition of erythrocyte AChE activity exceeding this trigger value could already be observed in the 0.5 ppm group of males during the first half of the study, but returned to about 20% by the end of the study, which may be considered a borderline effect with respect to toxicological relevance of AChE inhibition. In view of the fact that inhibition of AChE activities was below the trigger value of 20% in the 0.5 ppm dose group of female rats and only slightly exceeded this trigger value in the 0.5 ppm dose group of males, 0.5 ppm can be considered close to the NOAEL. Hence a NOAEL for toxicity of 0.5 ppm (equal to 0.04 and 0.05 mg/kg bw per day in male and female rats, respectively) was identified based on inhibition of AChE activities at the LOAEL of 4 ppm (equal to 0.30 mg/kg bw in males and 0.44 mg/kg bw in females). The NOAEL for carcinogenicity was 32 ppm (equal to 2.92 mg/kg bw in males and 3.93 mg/kg bw in females), the highest dose tested.

The study was GLP compliant (Schladt, 1995).

3.4 Genotoxicity of omethoate

Omethoate was evaluated for its mutagenic and genotoxic potential in a battery of in vitro and in vivo assays.

In vitro tests to investigate mutagenic and cytogenetic effects were performed in bacterial and mammalian cells. In bacterial test systems omethoate was positive (Ames test; tester strains TA1535 and TA100 with and without S9 mix) at very high doses of 12 500 and 12 400 µg/plate, which by far exceed the recommended maximum test concentrations. But in mammalian cell test systems controversial findings were noted with regard to point mutations.

Indications of a mutagenic potential for omethoate were noted in the hypoxanthine–guanine phosphoribosyl transferase (HPRT) assay in Chinese hamster ovary (CHO) cells but at severely cytotoxic concentrations only and with no clear dose-dependency. The mouse lymphoma assay did not reveal any effects indicative of a mutagenic potential neither in the presence nor absence of S9 mix. Genotoxic effects were noted in the unscheduled DNA synthesis (UDS) assay, in vitro, in rat hepatocytes and in the cytogenetic assay (measuring sister chromatid exchange (SCE) induction in CHO cells).

Positive findings in a somatic cell assay in vivo were noted for the mouse spot test, indicating a potential for omethoate to induce point mutations in vivo under the described test conditions. In contrast, neither the micronucleus test in mice, the SCE test in hamsters nor the UDS ex vivo test in rats revealed any indication of omethoate being an in vivo mutagenic or genotoxic agent, even when tested at clearly systemically toxic doses. In addition, no indication was given from the Dominant lethal test in male mice, that omethoate might be a germ cell mutagen.

Besides the positive result in the mouse spot test, no indication for omethoate being mutagenic or genotoxic in vivo was noted in any of the frequently used and very sensitive mutagenicity studies in somatic cells covering several species and modes of actions. Furthermore, in the study in germ cells too, no indication that omethoate was an in vivo mutagen was found and no clear evidence was found that omethoate induces point mutations in mammalian test systems in vitro. Effects in mammalian cells, if noted at all, were observed at severely toxic doses only.

The findings of mutagenicity studies are summarized in Table 44.

Table 44. Summary of genotoxicity studies

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537	20–12 500 µg/plate (±S9)	96	Positive (± S9)	Herbold,. 1988a
Sister chromatid exchange	Chinese hamster ovary cells with and without S9 mix	25–3000 µg/mL (−S9) 166.7–5000 µg/mL (+S9)	96	Positive (± S9)	Taalman, 1988
Gene mutation	L5178Y mouse lymphoma cells (TK)	500–2500 µg/mL (−S9) 500–5000 µg/mL (+S9)	96.9	Negative (± S9)	Bootman, & Rees, 1982
Forward mutation assay in mammalian cells (HPRT test)	Chinese hamster ovary cells with and without S9 mix	500–6000 µg/mL (−S9) 3000–6000 µg/mL (+S9)	97.4	Positive (± S9)	Lehn, 1989
Unscheduled DNA synthesis in mammalian cells	Rat hepatocytes;	25.6–1030 µg/mL	97.4	Positive	Cifone, 1989
In vivo					
Micronucleus test	NMRI mouse, male and female (oral gavage)	22.5 mg/kg bw	96	Negative	Herbold, 1988b
Sister chromatid exchange (SCE) test	Chinese hamsters	0, 5, 10 and 20 mg/kg bw	96.7	Negative	Herbold, 1990a
Mouse spot test[§]	Mouse Somatic cell	0, 4, 8 and 16 mg/kg bw	96.7	Positive	Herbold, 1990b
Unscheduled DNA synthesis (UDS)	rat hepatocytes	0, 3, 10 and 30 mg/kg bw	96.6	Negative	Benford, 1989
Dominant lethal test	NMRI mice (oral administration)	0, 10 and 20 mg/kg bw	96.9	Negative	Herbold, 1991

[§] Details of the mouse spot test given in Table 45

Table 45. Results obtained with omethoate in the mouse spot test

Groups and doses	F_1 animals without spots		F_1 animals showing mid-ventral spots		F_1 animals showing relevant spots	
	Number	%	Number	%	Number	%
Vehicle control	339	97.7	6	1.7	2	0.6
Omethoate 4 mg/kg bw	334	94.6	7	2.0	12*	3.4
Omethoate 8 mg/kg bw	295	92.2	12	3.8	13*	4.1
Omethoate 16 mg/kg bw	300	87.5	24*	7.0	19*	5.5
1-Ethyl-1-nitrosourea 40 mg/kg bw	284	73	22**	5.7	83**	21.3

* significantly different to control ($p \leq 0.05$ in χ^2 test) ** significantly different to control ($p \leq 0.01$ in χ^2 test)

3.5 Reproductive and developmental toxicity of omethoate

(a) Multigeneration studies

In a dose range-finding study for the two-generation reproduction study, four groups of 10 male and 10 female rats each (Wistar HAN, outbred) received omethoate (batch 234808038; purity 96.6%) via their drinking water at doses of 0, 10, 30 and 90→50 ppm (equal to average 0, 0.84–1.72, 2.62–4.98 and 4.54–8.46→11.48–12.93 mg/kg bw per day for males, 0, 1.30–1.99, 4.28–6.3, and 6.6–10.2→13.27–13.3 mg/kg bw per day for females) during a three week prepairing period, as well as during mating, gestation and lactation. Parental animals were paired one male to one female for a maximum period of 20 days. A subgroup of an additional five animals/sex per dose received the test substance for 22 days for the determination of plasma, erythrocyte and brain AChE activities.

Due to severe clinical findings (ruffled fur, exophthalmia, tremor, ataxia, lateral and dorsolateral recumbency, stiff gait, sedation, squealing spasm and grinding of teeth), the nominal concentration of test article in the drinking water was reduced from 90 to 50 ppm on day 10 of the prepairing period. After reducing the dose, clinical signs of reaction to treatment were less severe.

At 50 ppm, reduced food and water consumption were noted in the parent females during the lactation period.

At 30 ppm, restlessness was noted in parent animals of both sexes. Reduced food consumption was noted in the females during the lactation period. Retardation of body weight gain was noted in the males during the pre-pairing period and in the females during the first week of treatment.

At 10 ppm, no clinical signs of reaction to treatment were observed. Females displayed reduced food consumption during the lactation period and a slight retardation of body weight gain was noted in the males during the pre-pairing period.

The reproduction data indicated reduced fertility, reduced mean number of implantation sites and increased postnatal loss in the 90→50 ppm and 30 ppm groups. Additionally, an increased mean precoital time was noted at 90→50 ppm which was considered to be due the parental toxicity noted at that dose level.

At 10 ppm there was no effect on the reproduction parameters of the parent animals.

In all groups(10, 30, 90→50 ppm) dose-related retardation of body weight gain of the F1 pups was evident during lactation as well as during the rearing period (postpartum days 21–28). Additionally during the rearing period, pups of all dose groups showed reduced food and water consumption.

Inhibition of ChE activity, in particular of erythrocytes and brain, was evident at all dose levels in both parent females and the subgroup animals of both sexes.

Based on these results, dosages of 0.5, 3 and 18 ppm of omethoate in the drinking water were used for the rat two-generation reproduction study.

The study was GLP compliant (Dotti, Kinder & Luetkemeier, 1994).

In a two-generation reproduction study, four groups of 25 male and 25 female rats each (Wistar HAN, outbred) received omethoate (batch 234808038; purity 96.7% at start, 96.6% at first re-analysis and 96.9% at second re-analysis) via the drinking water (adjusted to pH 3 with hydrochloric acid) at doses of 0, 0.5, 3.0 and 18 ppm (equal to average 0, 0.08, 0.57, 3.16 mg/kg bw per day for males , 0, 0.12, 0.72, 4.35 mg/kg bw per day for females) during a 70 day pre-pairing period and then throughout pairing, gestation and lactation for breeding the F_1 generation.

No substance-related mortalities were observed until scheduled necropsy in any dose group of either sexe of F0 generation animals throughout the entire treatment period.

Among the 18 ppm group, reduced water consumption was evident in both sexes of both the F0 and F1 generations. Reduced food consumption was noted in the F0 and F1 parent females during the lactation period. Retardation of body weight gain was noted in the animals of the F0 generation, in the females of the F1 generation and in both F1 and F2 pups. A reduced mean number of implantations per dam and increased postnatal loss were evident in both F0 and F1 generations. In the F1 generation, increased mean precoital time, increased number of nonpregnant females, increased postimplantation loss and lower litter size were noted. Inhibition of plasma and/or of erythrocyte and brain ChE activity was noted in the F0 parent animals and F1 females. Inhibition of brain ChE activity was also evident in the female F1 pups and for both sexes among the F2 pups. Additionally, the F0 and F1 parent males showed an increase in the severity of epithelial vacuolation in the epididymis. However, correlation of this finding with the reproductive function of the animals were not evident.

At the 3 ppm dose, inhibition of erythrocyte and brain ChE activity was noted in the parent animals of both F0 and F1 generations. A slight inhibition of brain ChE activity noted in the female pups of the F2 generation.

At 0.5 ppm, the inhibition of erythrocyte ChE activity noted in the females of the F0 generation was considered not to be toxicologically relevant (below the trigger value of 20%). Slight inhibition of erythrocyte and brain or just brain ChE activity was noted in the females and males, respectively, of the F1 generation.

Table 46. Acetylcholinesterase (ChE) activity in plasma, erythrocytes and brain in male and female rats of the F0 generation at scheduled necropsy

	Plama ChE		Erythrocyte ChE		Brain ChE	
Dose (ppm)	Activity (µmol-SH/mL)	Inhibition (%)	Activity (µmol-SH/mL)	Inhibition (%)	Activity (µmol-SH/mL)	Inhibition (%)
Males						
0	0.65	-	1.73	-	4.61	-
0.5	0.60	7	1.60	8	4.56	1
3	0.60	7	0.91**	47	3.80**	18
18	0.49**	25	0.31**	82	2.83**	39
Females						
0	1.13	-	1.72	-	4.11	-
0.5	1.03	9	1.38**	20	4.00	3
3	1.07	6	0.99**	42	3.26**	21
18	0.91	20	0.25**	85	2.70**	34

* $p < 0.05$ ** $p < 0.01$

Source: Dotti et.al.,1992

Table 47. Acetylcholinesterase (ChE) activity in plasma, erythrocytes and brain in male and female rats of the F1 generation at scheduled necropsy

	Plama ChE		Erythrocyte ChE		Brain ChE	
Dose (ppm)	Activity (µmol-SH/mL)	Inhibition (%)	Activity (µmol-SH/mL)	Inhibition (%)	Activity (µmol-SH/mL)	Inhibition (%)
Males						
0	0.67	–	1.84	–	7.55	–
0.5	0.75*	13	1.70	8	6.84*	9
3	0.73	−9	1.11**	40	5.32**	30
18	0.64	5	0.34**	82	3.35**	56
Females						
0	2.29	–	1.48	–	6.75	–
0.5	2.29	0	1.33*	10	5.68**	16
3	2.33	−2	0.60**	59	4.72**	30
18	1.42**	38	0.35**	76	3.04**	55

* $p < 0.05$ ** $p < 0.01$

Source: Dotti et.al.,1992

Based on the above results, the NOAEL for parental toxicity was 0.5 ppm (equal to average 0.08 mg/kg bw per day for males and 0.12 mg/kg bw per day for females) based on inhibition of ChE activities above the trigger value of 20% at 3 ppm (equal to average 0.57 mg/kg bw per day for males and 0.72 mg/kg bw per day for females). The NOAEL for reproductive toxicity was 3 ppm (equal to average 0.72 mg/kg bw per day) for female parental animals based on reduced fertility and impairment of reproductive performance in parental generation females at 18 ppm (equal to average 4.35 mg/kg bw per day for females).

The NOAEL for developmental toxicity was also 3 ppm (equal to average 0.57 mg/kg bw per day for males and 0.72 mg/kg bw per day for females) based on depressed body weights and retarded body weight gain in pups at 18 ppm (equal to average 3.16 mg/kg bw per day for males and 4.35 mg/kg bw per day for females).

The study was GLP compliant and a QA statement was attached (Dotti et al.,1992).

(b) Developmental toxicity

Rat

Omethoate (batch 234808038; purity 96.6%) was administered daily by gavage to four groups of 25 pregnant female Wistar rats each (strain Bor:WISW) from GD 6 to GD 15 at doses of 0, 0.3, 1.0 and 3.0 mg/kg bw per day in demineralized water (adjusted to pH 3–4) containing 0.5% of the emulsifier Cremophor® EL. General condition, behaviour and appearance was checked twice daily from GD 0 to GD 20 with special emphasis on alterations in excretory products. Body weights of dams were determined on GD 0, each day from GD 6 to 15 and on GD 20. Food consumption was determined for the periods day 0–6, day 6–11, day 11–16 and day 16–20. On GD 20, animals were sacrificed and subjected to gross pathological examination during caesarean section. Fetuses were weighed, sexed and examined for external, skeletal and soft tissue anomalies and developmental variations. Litter parameters such as number of corpora lutea, number of implantations, number and distribution of live young, embryonic and fetal deaths were recorded. Half of the fetuses were used for the examination of visceral malformations employing the modified Wilson technique while for the examination of abdominal and thoracic organ defects the Dawson technique was used.

Substance treatment during GDs 6–15 had no impact on general condition, behaviour, appearance or mortality rates in animals treated with doses up to and including 1 mg/kg bw per day. Also, food and water intake as well as excretory products were comparable to those of the control group. In the highest

dose group of 3 mg/kg bw per day, substance-related signs of toxicity in dams were characterized by tremor, isolated cases of exophthalmos, sunken flanks, bristling fur and a blood-smeared muzzle during treatment. Isolated animals from this dose group demonstrated alterations in appearance and amount of stool, as well as an increase in water intake coupled with an elevated urine yield. One dam of this high dose group died on GD 11.

During treatment on GDs 6–16 food consumption was statistically significantly reduced in the 3 mg/kg bw per day group but was comparable to control levels from GDs 16–20. This depression of food consumption was apparent in all animals of the 3.0 mg/kg bw per day dose group.

Mean and corrected body weight development in dams treated with 0.3 and 1.0 mg/kg bw/day was not different from control animals and only a few individual animals in the control as well as in the low- and mid-dose groups showed comparably slightly lower body weight gains. In the high-dose group, however, body weight gains were statistically significantly reduced from start of treatment until termination of the study on GD 20.

The mean number of corpora luteae and the percentage of inseminated animals with implantations did not differ from controls in statistically significant manner in any treatment group and was lay within the range of historical controls.

Investigations performed on intrauterine development showed that gestation rate, external appearance of placentas, number of fetuses, resorption rates, fetal sex ratio and numbers of minor skeletal system deviations, as well as type and number of malformations, were comparable to controls and no treatment-related effects were noted up to and including the highest dose tested. The weight of fetuses and placentas did not differ from controls up to and including doses of 1 mg/kg bw per day, while in the 3 mg/kg bw per day dose group fetal weights were slightly, and placental weights significantly, lower than those of control animals. The effects on the intrauterine development in the 3 mg/kg bw per day dose group were correlated to maternal toxicity observed in dams of this group.

After administration of omethoate at doses of 0, 0.3, 1.0 and 3.0 mg/kg bw per day to pregnant Wistar rats it was shown that at doses up to and including 1.0 mg/kg bw per day the test substance causes neither maternal toxicity or embryo/fetal toxicity, nor increased incidences of malformations in the offspring. Also, no adverse effects on fertility or gestation rates were observable in any of the treatment groups. In the highest dose group (3 mg/kg bw per day) the test substance induced clinical signs of toxicity (tremor, depressed food consumption during treatment, reduction in body weight gain during entire treatment and gestation period) and mortality in dams. At necropsy no substance-related findings different from controls were made in treated animals. In addition, substance-related depressions in placental weights and a reduction in the mean fetal weight were evident in the 3 mg/kg bw per day dose group. At this dose level clear signs of maternal toxicity were already observed.

The NOAEL for both maternal and developmental toxicity was 1.0 mg/kg bw per day based on test substance-induced clinical signs of toxicity (tremor, depressed food consumption during treatment, reduction in body weight gain during entire treatment and gestation period) and mortality in dams; depressions in placental weights and a reduction in the mean fetal weight in the highest dose group of 3 mg/kg bw per day.

The study was GLP compliant and a QA statement was attached (Holzum, 1990a).

Rabbit

Study 1

Omethoate (batch 234808038; purity 96.6–96.7%) was administered daily by gavage to four groups of 15 pregnant female Himalayan rabbits (strain CHBB:HM) from GDs 6–18 of gestation at doses of 0, 0.2, 1.0 and 5.0 mg/kg bw per day in demineralized water (adjusted to pH 3–4 with 0.1 N hydrochloric acid) containing 0.5% of the emulsifier Cremophor® EL. An additional five dams per dose group served as satellite groups for the determination of ChE activities.

General condition, behaviour and appearance of all rabbits were checked twice daily from GD 0 to GD 29 with special emphasis on alteration in excretory products. Body weights of dams were determined on GD 0, each day from GD 6 to GD 18 and on GD 29. Food consumption and water intake of animals was determined during inspections by visual estimation. On GD 29 the main

group of animals was sacrificed and subjected to gross pathological examination during caesarean section, and brain weights were determined of animals from the satellite group on GD 19. Plasma and erythrocyte ChE activity was determined on GDs 6, 7, 14, 19 and brain AChE on GD 19 only after sacrifice. Fetuses were weighed, sexed and examined for external, skeletal and soft tissue anomalies and developmental variations. Litter parameters such as number of corpora lutea, number of implantations, number and distribution of live young, embryonic and fetal deaths were recorded. For the determination of visceral malformations the modified Wilson technique was applied while for the examination of lesions of the abdominal and thoracic organs as well as deviations of the skeletal system the Dawson technique was used.

There was no impact on general condition, behaviour, appearance and mortality rates of animals treated with doses up to and including 1 mg/kg bw per day. Also, food and water intake as well as excretory products remained unaffected by treatment and were comparable to those of untreated controls. In the highest dose group (5 mg/kg bw per day) substance-related signs of toxicity in dams were characterized by tremor, increased heart rate and in isolated animals ataxia and prostration was evident. No substance-related mortalities were observed during treatment or thereafter.

Mean body weight gains were unchanged in animals treated up to and including doses of 1 mg/kg bw per day in the periods GD 6–18 and GD 0–29. In the 5 mg/kg bw per day dose group, body weight gain was statistically significantly reduced compared to untreated controls during the treatment phase, but lay within the range of historical controls during the overall period from GD 0–29. In light of the clinical symptoms evident in this dose group, a treatment-related effect on body weight development cannot be completely excluded after treatment with 5 mg/kg bw per day.

In the low-dose group (0.2 mg/kg bw per day) AchE activity in the erythrocytes and brain was not influenced by treatment with omethoate at any time point. In addition, in the 1 mg/kg bw per day group no effect on the plasma AChE was evident, either. Starting at day 14 post coitum (PCD 14) statistically significant depressions of erythrocyte AChE were observable in the 1 and 5 mg/kg bw per day dose groups while plasma AChE was depressed in a statistically significant manner in the 5 mg/kg bw per day dose group on PCDs 7, 14 and 19. Brain ChE was statistically significantly reduced in the 1 and 5 mg/kg bw per day dose groups on PCD 19.

No significant gross pathological findings were observed at necropsy of the animals.

The percentage of inseminated animals with implantations as well as the mean number of corpora lutea in all treatment groups did not differ from controls in a statistically significant manner and lay within the range of historical controls. For this strain of rabbits the fertility index of historical controls varies from 65% to 100%.

There was no effect on the intrauterine development, gestation rate (historical control range 85.7–100%), external appearance or weight of placentas, mean fetal weight, mean number of low-weight fetuses, ratio of male to female fetuses. The numbers of minor skeletal system deviations were comparable to controls at all doses. The resorption rate was statistically significantly increased at doses of 1 and 5 mg/kg bw per day which is paralleled by a significantly lowered number of fetuses in the 5 mg/kg bw per day dose group. A treatment-related increase in the number of malformation (arthrogryposis, epignathus) at 1 mg/dose cannot be excluded. Since the elevated malformation rate was determined by an increase in the number of spontaneous malformations, it is presumed that a secondary toxic effect, rather than a genuinely teratogenic effect, was the cause of the elevated malformation rate. In these dose groups increased incidences were noted of arthrogryposis of the front extremities and in the 1 mg/kg bw/day group one case of epignathus was observed. Arthrogryposis is known to occur spontaneously in this strain of rabbits and the higher incidence which exceeds the range of historical controls is considered to be of secondary nature. All these effects were observed at dose levels that were already maternally toxic and no clear dose–response relationship was discernible, either.

Table 48. Fetal effects

	Intrauterine development			
Dose (mg/kg bw per day)	**0**	**0.2**	**1.0**	**5.0**
Number of litters per group	15	15	12	14
Number of fetuses per group	84	89	77	70
Number of fetuses per dam	5.6	5.9	6.4	5.0
Mean fetal weight (g)	43.4	42.9	41.6	43.9
Mean placental weight (g)	5.0	4.85	4.42*	4.95
Sex ratio (male : female)	1 : 0.84	1 : 0.99	1 : 0.78	1 : 0.96
Number of runts	0.2	0.6	0.25	0.43
Number of fetuses with malformations/group	0	0	4	2
Number of fetuses with malformations/dam	0	0	0.33	0.14
Fetuses with malformations per group (%)	0	0	5.2	2.9
Number of litters with malformations	0	0	3	2
Litters with malformations per group (%)	0	0	25	14.3
Arthrogryposis	0	0	3	2
Epignatus	0	0	1	0

* $p < 0.05$ ** $p < 0.01$
Source: Holzum, 1990b

In view of he above it can be concluded that after administration of omethoate at doses of 0, 0.2, 1.0 and 5.0 mg/kg bw per day to pregnant Himalayan rabbits it has been shown that at 0.2 mg/kg bw per day the test substance neither caused maternal toxicity or embryo/fetal toxicity, nor increased incidences of malformations in the offspring. The fertility and gestation indices were comparable to historical controls and no pathological changes were visible up to and including the highest dose tested. Starting at a dose of 1 mg/kg bw per day inhibition of erythrocyte and brain AChE activity was evident in dams. In the 5 mg/kg bw per day dose group clinical signs of toxicity (tremor, increased heart rate; ataxia and prostration in isolated animals) and a retarded body weight development was observed during the treatment period. Higher resorption rates were seen in the 1 and 5 mg/kg bw per day group resulting in a lower number of fetuses in the 5 mg/kg bw per day group. In addition, malformation rates were increased in the 1 and 5 mg/kg bw per day groups without showing a clear dose–response relationship. Since these malformations occur spontaneously in the strain of rabbits used and were seen at doses already causing maternal toxicity, a secondary effect is likely to be responsible for this effect.

On the basis of the findings the NOAEL for both maternal and developmental toxicity was 0.2 mg/kg bw per day based on inhibition of erythrocyte and brain ChE activity and increased malformation rates at 1 mg/kg bw per day and above.

The study was GLP compliant and a QA statement was attached (Holzum, 1990b).

Study 2
In another study, the objective was to detect adverse effects of omethoate (batch D2014 HRM-OMT-07-2; purity 96.6%) on pregnant Hra:(NZW)SPF female rabbits and development of the embryo and fetus consequent to exposure of the female from the anticipated time of implantation (GD 6) until just prior to parturition (GD 28). In addition, ChE activity in brain and erythrocytes was measured.

Female rabbits in four groups were administered omethoate formulations at 0, 0.20, 1.0 and 4.0 mg/kg bw per day once daily on presumed GDs 6 –28.

Doses were adjusted based on the most recently recorded body weight and administered at approximately the same time each day. The following parameters and end-points were evaluated in this study: viability, clinical signs, food consumption, body weight, body weight change, gross necropsy observations, organ weights, brain and erythrocyte ChE activity, and fetal examinations including external abnormalities, sex, body weight, skeletal and visceral examinations.

At 4.0 mg/kg bw per day, there was an increase or a statistically significant increase in the total number of rabbits that were either found dead or had to be euthanized due to adverse clinical signs during the study. There was also one rabbit in the control group that aborted and was subsequently euthanized. There were statistically significant increases in the number of rabbits at 4.0 mg/kg bw per day observed with ungroomed coat, tachypnea and fasiculations. There was also an increase in the number of rabbits at this dose with soft or liquid faeces, scant faeces, ataxia, decreased motor activity, low carriage, tremors, excess salivation (moderate or extreme), hunched posture and thin body condition. There was a statistically significant decrease in body weight observed at 4.0 mg/kg bw per day on GD 28. There was also a reduction or statistically significant reduction in body weight gain observed during the dose period and the overall study period in this dose group. Additionally in this group, statistically significant body weight losses were observed on GDs 24–28 and GDs 28–29.

Although not statistically significant, uterine weights in the 4.0 mg/kg bw per day dose group were slightly reduced (91% of concurrent control value) compared with the control group. Corrected body weights (body weight on GD 29 minus the gravid uterine weight) were slightly reduced (94% of concurrent control value) in this group, achieving statistical significance. At 4.0 mg/kg bw per day, there was a statistically significant reduction in food consumption value for the dose period when compared with concurrent controls. There were no test substance-related macroscopic observations in the rabbits at scheduled euthanasia at any dose. Pregnancy occurred in 22 (100%), 20 (90.9%), 22 (100%) and 21 (95.4%) rabbits in Groups 1 through 4, respectively. Due to the previously described early euthanasia of one control group rabbit, three rabbits that were found dead and two rabbits that were euthanized early in the 4.0 mg/kg bw per day dose group, caesarean-sectioning observations on GD 29 were based on 21, 20, 22 and 16 pregnant rabbits in the 0 (control), 0.20, 1.0 and 4.0 mg/kg bw per day dose groups, respectively. A statistically significant reduction in fetal body weights (total, male and female) occurred in the highest dose group in comparison with the controls. Related to these reduced fetal body weights in the 4.0 mg/kg bw per day dose group, delays in fetal ossification occurred, including statistically significant increases in the number of fetuses observed with a large anterior fontenelle, large posterior fontenelle, large frontal suture and large nasal suture. This dose group also had a statistically significant decrease in the average numbers of ossification sites per fetus for the hyoid. The gestating female rabbits had a statistically significant (dose-related) reduction in erythrocyte ChE activity at 1.0 and 4.0 mg/kg bw per day on GDs 14 or 15, and again at scheduled euthanasia. Brain ChE activity was statistically significantly reduced in all dose groups, in a dose-related manner, at scheduled euthanasia.

There was a statistically significant reduction in erythrocyte ChE activity in the 1.0 and 4.0 mg/kg bw per day dose groups at GDs 14 or 15 and again at scheduled euthanasia. Brain ChE activity was statistically significantly reduced, in a dose-related manner in all dose groups at scheduled euthanasia. The reduction in brain ChE activity observed in the 0.20 mg/kg bw per day dose group was considered minor (5.1% inhibition compared with controls; less than the trigger value of 20%) and not toxicologically relevant. In the 4.0 mg/kg bw per day dose group there was an increased incidence in the number of rabbits that were either found dead or euthanized due to adverse clinical signs, reductions in body weight gain and/or body weight losses and food consumption values.

Table 49. Fetal ossification site per fetus per litter; number of cases N, with % in brackets

	Dose (mg/kg bw/day)	0	0.20	1.0	4.0
Hyoid	Mean ± SD	1.0 ± 0.02	1.0 ± 0.00	1.0 ± 0.00	0.96 ± 0.06**
Delayed ossification					
Skull anterior fontanelle large	Litter incidence *N* (%)	0 (0.0)	0 (0.0)	3 (13.6)	6 (37.5)**
	Fetal incidence *N* (%)	0 (0.0)	0 (0.0)	8 (4.1)	16 (10.7)**
Skull posterior fontanelle large	Litter incidence *N* (%)	0 (0.0)	0 (0.0)	3 (13.6)	1 (6.2)
	Fetal incidence *N* (%)	0 (0.0)	0 (0.0)	8 (4.1)	5 (3.3) **
Skull frontal suture large	Litter incidence *N* (%)	0 (0.0)	0 (0.0)	3 (13.6)	1 (6.2)
	Fetal incidence *N* (%)	0 (0.0)	0 (0.0)	8 (4.1)	5 (3.3) **
Skull nasal suture large	Litter incidence *N* (%)	0 (0.0)	0 (0.0)	3 (13.6)	1 (6.2)
	Fetal incidence *N* (%)	0 (0.0)	0 (0.0)	8 (4.1)	5 (3.3) **

** $p < 0.01$

In view of the above, the maternal NOAEL for omethoate was 0.20 mg/kg bw per day based on the statistically significant reduction in erythrocyte ChE activity in the 1.0 and 4.0 mg/kg bw per day dose groups at GDs 14 or 15 and again at scheduled euthanasia. The developmental NOAEL was 1.0 mg/kg bw per day based on reductions in gravid uterine weights, fetal body weights and delayed skeletal ossification occurring in the 4.0 mg/kg bw per day dose group. Omethoate is not a selective developmental toxicant.

The study was GLP-compliant and a QA statement was attached (Barnett, 2015b).

3.6 Special studies on omethoate

(a) Acute neurotoxicity

Omethoate (purity 96.5%) was administered to groups of 25 male and 25 females Wistar rats (strain CrlGlxHan: WI) as a single oral gavage dose at a level of 0 (control), 0.2, 0.25, 0.35 and 5 mg/kg bw. Animals were 49 (± 1) days old on the day of test substance administration.

An examination of FOB and motor activity measurements revealed clear substance-related effects at 5 mg/kg bw on the day of test substance administration in both sexes, which included tremors, frequent chewing, lack of pupil reaction, reduction of motor activity. These signs are typical of ChE inhibitors and were reversible within a week after treatment. At 0.35 mg/kg bw few animals showed changes in respiration, impairment of co-ordination or effects on pupil reflex in parallel with a biologically relevant inhibition of brain ChE. At 0.25 mg/kg bw no effects besides some changes with regard to pupil reflex were noted. The changes in pupil reflex were noted in few animals at 0.25 and 0.35 mg/kg bw, but without any dose relationship. In parallel, no biologically relevant ChE inhibition or other relevant clinical signs, like effects on respiration or impairment of coordination as observed in 0.35 mg/kg bw, were noted at 0.25 mg/kg bw. The variability and lack of reliability of such pupil effects is further demonstrated if the findings of the pretest are taken into account. Under identical test conditions, no such effects on pupil reflex were noted at a 20-times higher test dose of 5 mg/kg bw. By contrast, with other toxicologically relevant findings (like ChE inhibition or other clinical signs of toxicity, such as effects on respiration) the findings in the pretests agree well with the main study. At 0.2 mg/kg bw only one male animal showed a "reduced exploration of the area" which was assessed as being incidental and not treatment-related.

Regarding the clinical pathology findings, the administration of 5 mg/kg bw produced marked decreases in serum ChE (54% and 41% inhibition of males and females respectively), erythrocyte ChE (77% and 75% inhibition of males and females respectively) and brain ChE (81% and 80% inhibition

of males and females respectively) 2.5 h after treatment. At this time interval a slight but biologically relevant inhibition (27%) of ChE activity was also seen in the brain of the female rats that had received 0.35 mg/kg bw. No effects of biological concern were seen in the ChE determinations of the lower dose groups although statistical differences from the control group were noted. In both sexes the erythrocyte and brain ChE activities were more inhibited than was serum ChE. Fifteen days after test substance administration ChE examination revealed no toxicologically relevant changes in any dose group of either sex. Thus, all findings seen at the 2–5 hour time interval were fully reversible within a treatment-free period of 15 days.

No test substance-related changes were apparent in mean absolute or relative organ weights. Neurophysiologically there were no treatment-related findings. The single occurrence of an "axonal degeneration" in the peripheral nerves of control and treated top-dose animals was regarded as incidental or spontaneous in nature.

In conclusion, administration of 5 mg/kg bw omethoate caused abnormal clinical signs and a marked inhibition of ChE activities in the serum, erythrocytes and brain of male and female animals. At 0.35 mg/kg bw a few animals showed abnormal clinical signs with regard to respiration, co-ordination, and pupil reflex. At this dose level, a slight but biologically relevant decrease of brain ChE activity was found in females. Since no toxicologically relevant effects were found at 0.2 or 0.25 mg/kg bw the NOAEL in this study was 0.25 mg/kg bw, based on changes in respiration, impairment of co-ordination and effects on pupil reflex and inhibition of brain ChE activity at 0.35 mg/kg bw.

The study was GLP-compliant and a QA statement was attached (Mellert et al., 2003).

(b) Delayed polyneuropathy studies

Groups of 15 Leghorn hens (eight months old) were gavaged with a single dose of 140 mg/kg bw of omethoate (in demineralized water, batch 234808038; purity 96.7%). Antidotes atropine sulfate and 2-pyridine aldoxime methyl chloride (PAM) were also administered as protection. A second dose of omethoate was administered 21 days later. A group of six hens acted as vehicle controls and a further group of five hens acted as positive controls, receiving 400 mg/kg bw TOCP. Appearance and behaviour were examined in detail at least daily for 21 days after each dose. Forced motor activity was employed twice weekly. Body weights were measured weekly. Gross necropsy was performed on decedents. At termination, survivors were perfused prior to fixation of neural tissues for histopathological examination.

The study was performed according to the 1984 version of the OECD guideline. The more recent (1995) guideline would not require the second dose after 21 days, but would require measurement of NTE inhibition. The stability and accuracy of test substance concentrations in the dosing solutions were acceptable. The conduct and reporting of the study are otherwise considered to be acceptable.

There were two deaths in the treatment group, one on the day of the first treatment and the other nine days after the second treatment. Body weights of omethoate-treated birds were reduced throughout the study period. Signs of toxicity (apathy, staggering gait, diarrhoea, dry/flaccid comb, lying on side or prostrated, panting, ruffled feathers and spasms) were noted in all birds; one bird also showed laboured breathing and increased salivation. Recovery was apparent from three days after each treatment and all birds were free of symptoms eight days after the first treatment and 16 days after the second treatment. There were no signs of delayed neurotoxicity. In the positive control group, ataxia progressing to paresis was observed from day 8. All birds were sacrificed moribund on day 18. There were no gross necropsy findings in the birds sacrificed at termination. Findings in decedents included severely distended oedematous lungs, pale spleen, distended crop, white film on liver and heart, pale and enlarged kidneys, ulcerous foci in the glandular stomach and reddening of the duodenal mucosa. There were no histopathological findings in the neural tissues of omethoate-treated birds. Positive control group birds showed findings of axonal degeneration.

The results of this study do not provide any behavioural or histopathological evidence of delayed neurotoxicity, though there was no measurement of ChE activity or NTE inhibition as would be expected in a modern study. However, enzyme studies with human and hen autopsy tissue suggest omethoate does not cause delayed neuropathy in man. No inhibtion of NTE was found in human and hens at $4 \times LD_{50}$ (Lotti et al., 1981).

The study was GLP compliant and a QA statement was attached (Bomann & Sykes, 1993).

(c) Studies on cholinesterase activity

A dose range-finding study was undertaken with the objective of determining the effect of acute dosing of omethoate on erythrocyte and brain ChE activity in female rat pups (Part A) and female adult rats (Part B), and to provide information for selecting the dose level to be tested in a time-of-peak-effect study.

The study design is shown below in Table 50.

Table 50. Experimental design for dose range-finding study of omethoate ChE effects

Group No.	Dose material	Dose level (mg/kg bw)	Dose concentration (mg/mL)	Dose volume (mL/kg)	No. of neonatal female pups (Part A)	No. of adult female rats (Part B)
1	Reverse osmosis deionized water	0(Vehicle)	0	5	5	5
2	Omethoate[a]	0.25	0.05	5	5	5
3	Omethoate[a]	0.5	0.1	5	5	5

[a] The test substance was considered 96.5% pure for the purpose of dose calculations

Omethoate (batch -676-BSe-74B; purity 96.5%) was orally administered once by gavage to five female rat pups (strain Crl:CD(SD)) per group on PND 11 and to five female adult rats per group at dose levels of 0, 0.25, or 0.5 mg/kg bw. The vehicle was deionized water, the dosing volume was 5 mL/kg bw. The following parameters and end-points were evaluated for Parts A and B of this study: viability, clinical signs, body weights and body weight changes and ChE activity.

For Part A of the study, female pups were euthanized on PND 11 by an intraperitoneal (i.p.) injection of sodium pentobarbital approximately 2.5 h after dose administration. Blood was collected via cardiac puncture immediately following the euthanizing i.p. injection. The brain from each pup was excised and weighed. Red blood cell (RBC) and brain samples were analyzed for ChE activity at the testing facility. All pups were discarded without further evaluation.

For Part B of the study, on day 1 of study (SD 1), adult female rats were anesthetized under isoflurane/oxygen approximately 2.5 h after dose administration, and following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised and weighed. Red blood cell and brain samples were analyzed for ChE activity at the testing facility. All rats were discarded without further evaluation.

There were no mortalities and no clinical signs of toxicity in either pups or adult female rats. Brain ChE activity was statistically significantly decreased in the female pups after an acute dose of 0.25 and 0.5 mg/kg bw of omethoate (by 19% and 28%, respectively) compared with the controls. Erythrocyte ChE activity was decreased by 39% at 0.25 mg/kg bw and by 24% at 0.5 mg/kg bw in female pups compared with controls, however because of the high degree of variability with this parameter (which is likely due to problems with the sample preparation and processing) no definitive conclusions can be made from the questionable results. Erythrocyte ChE inhibition was also observed in the female adult rats after an acute dose of 0.5 mg/kg bw of omethoate (23% inhibition for brain and RBC; statistically significant for brain only) compared with controls. The dose level of 0.6 mg/kg bw would be used to establish the time-to-peak-effect for pups and adult rats after an acute dose of omethoate.The study was neither GLP-compliant, nor was QA statement attached (Barnett, 2012a).

A study was undertaken to determine the time-to-peak ChE depression in Crl:CD(SD) neonatal pups and adult rats after acute dosing with omethoate. The purpose of Subset 1 was to measure ChE activity in adult rats at various time points to determine the time-to-peak ChE depression following a single administration. The purpose of Subset 2 was to measure ChE activity in neonatal rat pups at various time points to determine the time-to-peak ChE depression following a single administration on PND 11.

The study design is provided below in Tables 51 and 52.

Table 51. Experimental design – adult rats (Subset 1)

Group	Dose material	Dose level (mg/kg bw)	Dose concentration (mg/mL)	Dose volume (mL/kg)	Number of rats	
					Male	Female
1	RO deionized water	0 (Vehicle)	0	5	24[b]	24[b]
2	Omethoate[a]	0.6	0.12	5	48[c]	48[c]

RO Reverse osmosis

[a] The test substance was considered 96.5% pure for the purpose of dose calculations.

[b] Eight rats/sex per time point; three time points (approximately 0.5, 2 and 4 h post dose)

[c] Eight rats/sex per time point; six time points (approximately 0.5, 1, 2, 3, 4, and 8 h post dose)

Table 52. Experimental design – neonatal pups (Subset 2)

Group	Dose material	Dose Level (mg/kg bw)	Dose Concentration (mg/mL)	Dose Volume (mL/kg)	No. of Neonatal Pups	
					Male	Female
1	RO deionized water	0 (Vehicle)	0	5	24[b]	24[b]
2	Omethoate[a]	0.6	0.12	5	48[c]	48[c]

RO Reverse osmosis

[a] The test substance was considered 96.5% pure for the purpose of dose calculations

[b] Eight pups/sex per time point; three time points (approximately 0.5, 2 and 4 h post dose)

[c] Eight pups/sex per time point; six time points (approximately 0.5, 1, 2, 3, 4, and 8 h post dose)

Adult rats (Subset 1) were administered the test substance, omethoate, and/or the vehicle (deionized water) once by oral gavage. The dose volume was 5 mL/kg and doses were based on body weights recorded prior to dose administration. Pups at PND 11 (Subset 2) were administered the test substance, omethoate, and/or the vehicle (deionized water) once by oral gavage on PND 11. The dose volume was 5 mL/kg and doses were based on body weights recorded prior to dose administration. The following parameters and end-points were evaluated in this study: viability, clinical signs, body weights, and ChE activity.

On day 1 of study (SD 1), adult male and female rats were anesthetized under isoflurane/oxygen prior to blood sampling at the appropriate time points as follows. Blood samples were collected from eight rats/sex per time point from the 0 mg/kg bw (vehicle) dose group at the 0.5, 2, and 4 h time points and eight rats/sex per time point from the 0.6 mg/kg bw dose group at the 0.5, 1, 2, 3, 4, and 8 h time points. Following blood collection from the vena cava, the rats were euthanized by an injection of sodium pentobarbital into the inferior vena cava. The brain from each rat was excised and weighed. Red blood cell and brain samples were analyzed for ChE activity at the testing facility. All rats were discarded without further evaluation.

On PND 11, neonatal male and female pups were euthanized by an i.p. injection of sodium pentobarbital prior to blood sampling at the appropriate time points as follows. Blood samples were collected from eight pups/sex per time point from the 0 mg/kg bw (vehicle) dose group at the 0.5, 2, and 4 h time points and eight pups/sex per time point from the 0.6 mg/kg bw dose group at the 0.5, 1, 2, 3, 4, and 8 h time points. Blood was collected via cardiac puncture immediately following the i.p. injection of sodium pentobarbital. The brain from each pup was excised and weighed. Red blood cell and brain samples were analyzed for ChE activity at the testing facility. All pups were discarded without further evaluation.

There were no mortalities or test substance-related adverse clinical signs observed in either the PND 11 pups or the adult rats.

Decreases or statistically significant decreases in erythrocyte and brain ChE inhibition were observed in the 0.6 mg/kg bw male and female PND 11 pups and adult rats at all time points evaluated when compared with the vehicle control group values.

In the adult male rats, the highest degree of erythrocyte ChE inhibition (32.8% and 34.2%, respectively) and brain ChE inhibition (24.0% and 24.3%, respectively) was observed between 3 h and 4 h post dose. In the adult female rats, the highest degrees of erythrocyte ChE inhibition (a decrease of between 25.2% and a 28.7%) and brain ChE inhibition (decrease of between 20.1% and 21.2%) were observed between 2 hand 4 h post dose.

In the PND 11 pups, the greatest amount of erythrocyte ChE inhibition (56.3% and 55.8% in male and female pups respectively) and brain ChE inhibition (39.7% and 33.6% in male and female pups respectively) was observed at the 4 h post dose time point.

After discussion with Environmental Protection Agency (EPA) of the USA, scientists it was agreed that the time-to-peak-effect for omethoate was three hours for adult male and female rats and four hours for the PND 11 pups.

The study was not GLP-compliant (Barnett, 2012b).

To compare the effects of omethoate when administered as an acute gavage dose at the time of peak effect on ChE inhibition, Male and female PND 11 neonatal pups and male and female adult rats (Crl:CD(SD) were administered a single dose of 0 (vehicle), 0.1, 0.3, 0.6 or 0.9 mg/kg bw omethoate (purity 96.5%). Erythrocyte and brain ChE activity were measured at the predetermined time of peak effect; 3 h following dose administration for the adults and 4 h for the pups. All male and female PND 11 pups and adult rats survived until scheduled sacrifice, and there were no adverse clinical signs observed after dose administration. Therefore, the NOEL for systemic toxicity was considered to be greater than 0.9 mg/kg bw in male and female adult and neonatal rats. For adult male and female rats, erythrocyte ChE activity was statistically significantly reduced in the 0.3, 0.6 and 0.9 mg/kg bw dose groups compared with the vehicle controls. For PND 11 male and female pups, erythrocyte ChE activity was statistically significantly reduced in the 0.3, 0.6 and 0.9 mg/kg bw dose groups compared with the vehicle control group. No effect on erythrocyte ChE activity was observed in the male and female adults or neonatal rats at 0.1 mg/kg bw compared with the vehicle control group. For adult rats, brain ChE activity was statistically significantly reduced in all dose groups for males, and in the 0.3, 0.6 and 0.9 mg/kg bw dose groups for the females when compared with the vehicle controls. For PND 11 pups, brain ChE activity was statistically significantly reduced in all dose groups for males and females compared with the vehicle controls. The statistically significant decreases in brain ChE activity observed in the adult male rats, male and female pups at 0.1 mg/kg bw were not considered to be biologically important because all decreases were by less than 10% (range 5.4%–7.4%) of the vehicle control group values. The bench mark dose (BMD) estimates for adults and PND 11 pups were compared, and the statistical significance of the ratio of BMDs tested. The ratio of adult to PND 11 pup BMDs for both brain and RBC ChE activity ranged from 1.07 to 1.32. These ratios were not statistically different from unity, indicating that the pup and adult responses were similar. The relative sensitivity of adults compared to PND 11 pups can be estimated from the ratio of the BMDs for adults and pups. For RBC activities, the ratio can be estimated using either the BMD_{10} or the BMD_{20}, while. For brain activities the ratio is estimated using the BMD_{10}. The ratios estimated for RBC ChE activity were 1.26 (BMD_{10} for the male rats), 1.08 (BMD_{10} for the female rats), 1.32 (BMD_{20} for the male rats), and 1.07 (BMD_{20} for the female rats). The ratio estimates for brain ChE activity were 1.13 for male rats and 1.25 for female rats. The BMDs for PND 11 pups and adults were not statistically different for either brain or RBC ChE inhibition. Therefore, these data indicate that a relative sensitivity factor is not necessary for omethoate.

Table 53. Omethoate adult RBC cholinesterase activity

Group	Dose level (mg/kg bw)	Mean RBC ChE activity (U/mL ± SD) [*n*]	Percent decrease compared with controls
		Male rats	
1	0 (RO deionized water)	1.220 ± 0.219 [15]	-
2	0.1	1.148 ± 0.174 [15]	5.9%
3	0.3	0.992 ± 0.138 [15]**	18.7%
4	0.6	0.811 ± 0.135 [15]**	33.5%
5	0.9	0.772 ± 0.118 [15]**	36.7%

Group	Dose level (mg/kg bw)	Mean RBC ChE activity (U/mL ± SD) [*n*]	Percent decrease compared with controls
		Female Rats	
1	0 (RO deionized water)	1.233 ± 0.113 [15]	-
2	0.1	1.180 ± 0.095 [15]	4.3%
3	0.3	1.020 ± 0.137 [15]**	17.3%
4	0.6	0.811 ± 0.114 [15]**	34.2%
5	0.9	0.807 ± 0.127 [15]**	34.5%

n The number of rats evaluated for cholinesterase activity SD Standard deviation RO Reverse osmosis

** Significantly different from the vehicle control substance group value at $p \leq 0.01$

Table 54. Omethoate adult brain cholinesterase activity

Group	Dose level (mg/kg bw)	Mean brain ChE activity (U/mL ± SD) [*n*]	Percent decrease compared with controls
		Male Rats	
1	0 (RO deionized water)	14.675 ± 0.935 [15]	-
2	0.1	13.585 ± 0.462 [15]**	7.4%
3	0.3	12.141 ± 0.600 [15]**	17.3%
4	0.6	10.746 ± 0.707 [15]**	26.8%
5	0.9	10.154 ± 0.846 [15]**	30.8%
		Female Rats	
1	0 (RO deionized water)	14.495 ± 0.591 [15]	-
2	0.1	14.117 ± 0.512 [15]	2.6%
3	0.3	12.666 ± 0.710 [15]**	12.6%
4	0.6	11.080 ± 0.782 [15]**	23.6%
5	0.9	10.454 ± 0.716 [15]**	27.9%

n The number of rats evaluated for cholinesterase activity SD Standard deviation RO Reverse osmosis

** Significantly different from the vehicle control substance group value at $p \leq 0.01$

Table 55. Omethoate PND 11 pups RBC cholinesterase activity

Group	Dose level (mg/kg bw)	Mean RBC ChE activity (U/mL ± SD) [*n*]	Percent decrease compared with controls
		Male pups	
1	0 (RO deionized water)	1.514 ± 0.218 [14]	-
2	0.1	1.453 ± 0.175 [15]	4.0%
3	0.3	1.183 ± 0.135 [15]**	21.9%
4	0.6	0.874 ± 0.168 [14]**	42.3%
5	0.9	0.774 ± 0.197 [15]**	48.9%
		Female pups	
1	0 (RO deionized water)	1.554 ± 0.182 [15]	-
2	0.1	1.478 ± 0.189 [14]	4.9%
3	0.3	1.273 ± 0.198 [15]**	18.1%
4	0.6	0.972 ± 0.199 [15]**	37.5%
5	0.9	0.830 ± 0.204 [15]**	46.6%

n The number of rats evaluated for cholinesterase activity SD Standard deviation RO Reverse osmosis

** Significantly different from the vehicle control substance group value at $p \leq 0.01$

Table 56. Omethoate PND 11 pups brain cholinesterase activity

Group	Dose level (mg/kg bw)	Mean brain ChE activity (U/mL ± SD) [*n*]	Percent decrease compared with controls
		Male pups	
1	0 0 (RO deionized water)	6.614 ± 0.454 [15]	-
2	0.1	6.156 ± 0.389 [15]*	6.9%
3	0.3	5.283 ± 0.328 [15]**	20.1%
4	0.6	4.489 ± 0.804 [15]**	32.1%
5	0.9	3.729 ± 0.691 [15]**	43.6%
		Female pups	
1	0 0 (RO deionized water)	6.570 ± 0.329 [15]	-
2	0.1	6.213 ± 0.354 [15]*	5.4%
3	0.3	5.391 ± 0.268 [15]**	17.9%
4	0.6	4.480 ± 0.599 [15]**	31.8%
5	0.9	3.707 ± 0.506 [15]**	43.6%

n The number of rats evaluated for cholinesterase activity

SD Standard deviation RO Reverse osmosis

* Significantly different from the vehicle control substance group value ($p \leq 0.05$).

** Significantly different from the vehicle control substance group value ($p \leq 0.01$).

Table 57. BMD results for RBC cholinesterase activity for adults and pups for a single oral gavage dose of omethoate

Age	Sex	*A* (unit/mL)	P_B	BMD_{10} (mg/kg bw)	BMD_{20} (mg/kg bw)
Adults	Male	1.230	0.5	0.133	0.304
	Female	1.251	0.5	0.149	0.316
Pups	Male	1.553	0.3	0.106	0.231
	Female	1.573	0.1	0.138	0.295

A defines the cholinesterase inhibition without exposure

P_B defines the asymptotic limit for cholinesterase inhibition at a high dose

Table 58. BMD results for brain cholinesterase activity for adults and pups for a single oral gavage dose of omethoate

Age	Sex	*A* (unit/mL)	P_B	BMD_{10} (mg/kg bw)
Adults	Male	14.615	0.6	0.163
	Female	14.665	0.6	0.200
Pups	Male	6.612	0.3	0.144
	Female	6.593	0.1	0.160

A defines the cholinesterase inhibition without exposure

P_B defines the asymptotic limit for cholinesterase inhibition at a high dose

Table 59. BMD estimates and the estimated ratios

ChE	BMD	Gender	Pups	Adults	Ratio adult:pup BMD	95th percentile CL for BMD ratio[§]
Compartment						
Red blood cell	BMD_{10} (mg/kg bw)	Males	0.106	0.133	1.26	0.90–1.7
		Females	0.138	0.149	1.08	0.83–1.4
	BMD_{20} (mg/kg bw)	Males	0.231	0.304	1.32	0.93–1.8
		Females	0.295	0.316	1.07	0.84–1.4
Brain	BMD_{10} (mg/kg bw)	Males	0.144	0.163	1.13	0.93–1.4
		Females	0.160	0.200	1.25	1.0–1.5

[§] 95th percentile confidence interval in for the ratio of adult:pup BMD values

The study was GLP-compliant and a QA statement was attached (Barnett, 2012c).

(d) Toxicity and anticholinesterase activity of omethoate and other metabolites

The toxicity and anticholinesterase activity of dimethoate, omethoate, isodimethoate and four other plant metabolites of dimethoate (*O*-desmethyl omethoate carboxylic acid, *O*-desmethyl-*N*-desmethyl omethoate, *O*-desmethyl isodimethoate and isodimethoate) were investigated in two studies performed in 2000.

Groups of male CD rats were gavaged with a single dose of test material (in distilled water) as detailed in Table 60 and observed for 14 days. Fasting blood samples were taken from the orbital sinus 2.5 h and 24 h after dosing and erythrocyte ChE activities measured using an automated procedure based on the method of Ellman et al. (1961). Gross necropsy was performed on all animals.

No deaths occurred in any group. There were no clinical signs of toxicity observed during the course of study that were related to treatment

Table 60. Toxicity of dimethoate metabolites: study design and Percentage of ChE inhibition figures when compared to predose values

Group	Compound	Purity	Dose level (mg/kg bw)	Cholinesterase inhibition 2.5 h post dose	*p* value[a]	Cholinesterase inhibition 24 h post dose	*p* value[b]
1	Dimethoate	99.5%	30	53%	0.0001	40%	0.0021
2	Omethoate	96.3%	5	74%	0.0000[§]	34%	0.0009
3	*O*-desmethyl omethoate (Metabolite I)	98.6%	30	19%	0.0799	16%	0.1393
4	*O*-desmethyl omethoate carboxylic acid (Metabolite II)	89.5%	30	25%	0.0220	21%	0.0512
5	*O*-desmethyl-*N*-desmethyl omethoate (Metabolite III)	96.4%	30	–2%	0.8647	9%	0.3627
6	*O*-desmethyl-isodimethoate (Metabolite IV)	98.2%	30	28%	0.0133	20%	0.0698

[§] *p* value of 0.000000000806568, reported as 0.0000

[a] Statistical analysis performed on difference between predose and 2.5 h post dose

[b] Statistical analysis performed on difference between predose and 24 h post dose

The results of this study indicate that omethoate is a considerably more potent ChE inhibitor than dimethoate. Findings also indicate that o-demethylation of omethoate markedly reduces anticholinesterase activity. Activity is further reduced (or abolished) by *N*-demethylation. *O*-desmethyl omethoate and its carboxylic acid derivative were shown to have similar activity. *O*-demethylation of isodimethoate was similarly shown to markedly reduce anticholinesterase activity. The metabolites *O*-desmethyl omethoate carboxylic acid, *O*-desmethyl-*N*-desmethyl omethoate, and *O*-desmethyl isodimethoate seemed to have, if at all, a markedly lower potential to inhibit ChE than dimethoate or omethoate as well.

The study was GLP compliant and aQA statement was attached (Brennan, 2001).

Another study was performed to identify the acute single dose of dimethoate and the plant metabolites *O*-desmethyl-omethoate carboxylic acid (Metabolite XX) and des-*O*-methyl isodimethoate (metabolite XII) that causes approximately 50% inhibition of RBC and/or brain AChE activity in the rat. This was done as a prerequisite for subsequent studies to identify the time-to-peak-effect on AChE activity after a single oral dose of each test substance.

Sixty-eight male and sixty-eight female rats (Crl:CD(SD); 60 days old on receipt) were assigned to 13 dosage groups (eight rats per sex in Group 1 and five rats per sex in each of groups 2 through 13) as shown in Table 61.

Table 61. Dosing scheme

Dosage group	Number of rats per sex	Dosage (mg/kg)	Concentration (mg/mL)	Dosage volume (mL/kg)
Control				
1	8	0	0	5
Dimethoate technical				
2	5	5	1	5
3	5	10	2	5
4	5	20	4	5
5	5	30	6	5
***O*-Desmethyl omethoate carboxylic acid, di-sodium salt (Metabolite XX)**				
6	5	10	2	5
7	5	20	4	5
8	5	30	6	5
9	5	40	8	5
Des-*O*-methyl iso**dimethoate, sodium salt (Metabolite XII)**				
10	5	10	2	5
11	5	20	4	5
12	5	30	6	5
13	5	40	8	5

Animals received single oral doses by gavage. Control animals received vehicle only. The dose volume was 5 mL/kg for all groups.

Animals were checked for viability twice daily and for clinical symptoms and general appearance once daily from the predose period until prior to sacrifice. Body weights were recorded prior to dose administration. Whole blood samples (2–3 mL) were collected into EDTA-coated tubes from the inferior vena cava of the anaesthetized animals 2.5 h post dose. After blood sample collection, the brains were excised and weights recorded. Blood samples were stored mixed on cold packs and the brains were stored in saline on iced water until assayed.

Analysis of erythrocyte and brain AChE levels was performed within two hours of sample collection according to the modified Ellman procedure (Ellman et al., 1961).

After sample collection, the animals were discarded without further evaluation.

No mortality occurred throughout this study.Three male (one at 10 mg/kg bw and two at 20 mg/kg bw) and three female rats (two at 10 mg/kg bw and one at 20 mg/kg bw) dosed with dimethoate were observed with miosis prior to sacrifice. No additional clinical signs were observed.

In the rats administered dimethoate, test substance-related inhibition of brain ChE was observed at 10, 20 and 30 mg/kg bw in males and in all treated groups for female rats. Erythrocyte ChE activity was inhibited at all dose levels, in both sexes.

For male rats the inhibition of the brain ChE ranged from 10.8 to 71.6% of control while the inhibition of erythrocyte ChE ranged from 27.8% to 73.0% of control. While at dose levels of 5 and 10 mg/kg bw the percentage inhibition is higher for erythrocyte ChE than for brain ChE, there is no such difference at higher dose levels. In female rats, brain and erythrocyte ChE was inhibited to the same extent, with inhibitions ranging from 26.1 to 66.5%.

For the two plant Metabolites XX and XII, no ChE inhibition was observed even at 40 mg/kg bw, the highest dose level tested this study.

Table 62. Male brain and RBC cholinesterase levels and percent inhibition 2.5 h after dosing

Test compound	Dose (mg/kg bw)	Brain cholinesterase		RBC cholinesterase	
		Level (Unit/g ± SD) [*n*]	Inhibition (%)	Level (Unit/g ± SD) [*n*]	Inhibition (%)
Control	0	13.407 ± 1.224 [8]	-	1.139 ± 0.083 [8]	-
Dimethoate	5	11.953 ± 0.500 [4]§	10.8	0.822 ± 0.164 [5]	27.8
	10	7.703 ± 1.822 [5]	42.5	0.470 ±0.105 [5]	58.7
	20	3.802 ± 0.439 [5]	71.6	0.354± 0.015 [3]§	68.9
	30	4.513 ± 0.111 [5]	66.3	0.308 ± 0.048 [4]§	73.0
Metabolite XX (*O*-desmethyl-omethoate carboxylic acid)	10	14.623 ± 0.546 [5]	0 (−9.1)	1.172 ± 0.137 [5]	0 (−2.9)
	20	15.031 ± 0.261 [5]	0 (−12.1)	1.242 ± 0.198 [5]	0 (−9.0)
	30	15.133 ± 0.705 [5]	0 (−12.9)	1.411 ± 0.090 [5]	0 (−23.9)
	40	14.264 ± 0.673 [4]§	0 (−6.4)	1.140 ± 0.014 [5]	0 (−0.1)
Metabolite XII (des-*O*-methyl isodimethoate, sodium salt)	10	19.856 ± 1.047 [5]	0 (−48.1)	1.226 ± 0.225 [5]	0 (−7.6)
	20	14.559 ±1.234 [5]	0 (−8.6)	1.054 ± 0.158 [5]	7.5
	30	13.458 ± 0.875 [5]	0 (−0.4)	1.129 ± 0.085 [5]	0.9
	40	13.982 ± 1.460 [5]	0 (−4.3)	1.052 ± 0.341 [5]	7.6

n Number of adult rats evaluated for cholinesterase levels

SD Standard deviation

§ Samples were excluded from summerization as sample results did not meet the acceptability criteria

Table 63. Female brain and RBC cholinesterase levels and percent inhibition 2.5 h after dosing

Test Compound	Dose (mg/kg bw)	Brain cholinesterase		RBC cholinesterase	
		Level (Unit/g ± SD) [*n*]	Inhibition (%)	Level (Unit/g ± SD) [*n*]	Inhibition (%)
Control	0	12.964 ± 0.736 [7]§	–	1.236 ± 0.132 [8]	–
Dimethoate	5	9.583 ± 0.863 [5]	26.1	0.812 ± 0.156 [5]	34.3
	10	5.190 ± 1.452 [5]	60.0	0.472 ± 0.131 [4]	61.8
	20	4.399 ±0.646 [5]	66.1	0.450 ± 0.023 [4]	63.6
	30	4.512 ± 0.502 [5]	65.2	0.414 ± 0.037 [4]	66.5
Metabolite XX (*O*-desmethyl-omethoate carboxylic acid)	10	15.508 ± 1.784 [5]	0 (−19.6)	1.301 ± 0.063 [5]	0 (−5.3)
	20	15.143 ± 2.611 [5]	0 (−16.8)	1.449 ± 0.103 [5]	0 (−17.2)
	30	15.281 ± 1.119 [5]	0 (−17.9)	1.303 ± 0.082 [5]	0 (−5.4)
	40	13.979 ± 0.697 [5]	0 (−7.8)	1.283 ± 0.147 [5]	0 (−3.8)
Metabolite XII (des-*O*-methyl isodimethoate, sodium salt)	10	16.146 ± 1.449 [5]	0 (−24.5)	1.311 ± 0.270 [5]	0 (−6.1)
	20	13.682 ± 0.912 [5]	0 (−5.5)	1.119 ± 0.148 [5]	9.5
	30	13.033 ± 0.989 [5]	0 (−0.5)	1.180 ± 0.229 [5]	4.5
	40	13.171 ± 1.359 [5]	0 (−1.6)	1.385± 0.175 [5]	0 (−12.1)

n Number of adult rats evaluated for cholinesterase levels

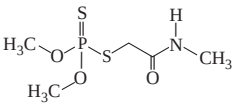
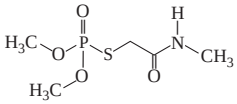
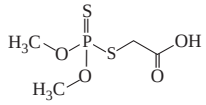
SD Standard deviation

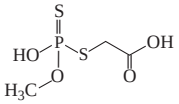
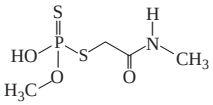
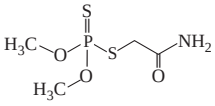
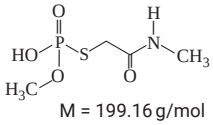
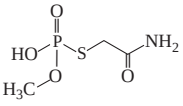
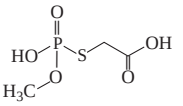
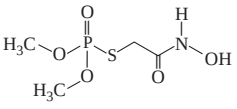
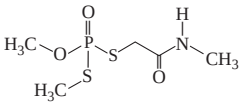
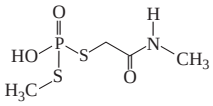
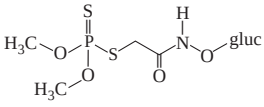
§ Samples were excluded from summerization as sample results did not meet the acceptability criteria

Treatment with the two plant metabolites *O*-desmethyl omethoate carboxylic acid (metabolite XX) and des-*O*-methyl isodimethoate (metabolite XII) did not cause cholinesterase inhibition up to 40 mg/kg bw, the highest dose level tested this study.

The study was not GLP-compliant (Barnett, 2009).

Table 64. Dimethoate metabolites in brief

Name	IUPAC name	Structure	FAO monographer comments	WHO panel comments
Dimethoate	*O,O*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate		Parent compound	All toxicological information available
Omethoate	*O,O*-dimethyl *S*-methylcarbamoylmethyl phosphorothioate		Is itself a pesticide, plant and animal metabolite (including rats), and has an extensive data package.	All toxicological information available
Dimethoate carboxylic acid	[(dimethoxyphosphorothioyl) sulfanyl] acetic acid	 M = 216.21 g/mol	Plant and animal metabolite, found in rats	A rat metabolite covered under toxicity data of dimethoate

Name	IUPAC name	Structure	FAO monographer comments	WHO panel comments
O-Desmethyl dimethoate carboxylic acid	{[Hydroxy(methoxy) phosphorothioyl] sulfanyl}acetic acid		Plant metabolite only in non-edible portions; not found in rat	
O-Desmethyl dimethoate	*O*-methyl *S*-methylcarbamoylmethyl phosphorodithioate	 M = 215.22 g/mol	Metabolite in potatoes and sugar beet; not found in rats	
N-desmethyl dimethoate	*O,O*-dimethyl *S*-carbamoylmethyl phosphorodithioate		Low level plant metabolite; not found in rats	
O-Desmethyl omethoate	*O*-methyl *S*-methylcarbamoylmethyl phosphorothioate	 M = 199.16 g/mol	Metabolite in olives and potatoes; not found in rats	ChE inhibition is 16% in comparision to dimethoate (40%) and omethoate (34%)
O-Desmethyl *N*-desmethyl omethoate	*O*-methyl *S*-carbamoylmethyl phosphorothioate	 M = 185.13 g/mol	Metabolite in potatoes, olives, and wheat; not found in rats	ChE inhibition is 20% in comparision to dimethoate (40%) and omethoate (34%)
O-desmethyl omethoate carboxylic acid	{[hydroxy(methoxy)phosphoryl] sulfanyl}acetic acid	 M = 186.12 g/mol	Plant (potato, wheat) metabolite; not found in rats	ChE inhibition is 21% in comparision to dimethoate (40%) and omethoate (34%)
Hydroxy omethoate	*O,O*-dimethyl *S*-hydroxymethylcarbamoylmethyl phosphorothioate		Low level plant metabolite; not found in rats	
Isodimethoate	*O,S*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate		Low level metabolite in plants; not found in rats.	
O-Desmethyl isodimethoate	*S*-methyl *S*-methylcarbamoylmethyl phosphorodithioate	 M = 215.22 g/mol	Plant (potato, olive, wheat) metabolite; not found in rats	
Hydroxy dimethoate (and its glucose conjugate)	*O,O*-dimethyl *S*-hydroxymethylcarbamoylmethyl phosphorodithioate		Potato metabolite; not found in rats	

4. Observations in humans

4.1 Medical surveillance on manufacturing plant personnel

The medical surveillance data on manufacturing personnel does not reveal any indications of negative health effects caused by the exposure to dimethoate during manufacturing or formulation (Stephansen, 2016).

Review of literature for dimethoate and omethoate

A literature review report (LRR) for the active substance dimethoate was performed. Additional search criteria regarding medical data were applied on all references resulting from the search executed for the LRR to identify literature relevant to effects on humans. A total of 264 references met the search criteria; of these 80 were screened at abstract level and two publications were found to be potentially relevant. An executive summary of each publication is provided in this section.

A literature search was also made for omethoate poisoning. Only two papers could be located.

A 74-year-old man accidentally ingested an unknown amount of dimethoate in a bottle of beer. The patient developed cholinergic syndrome immediately, accompanied by bradycardia and hypotension. Atropine 0.5 mg/hour was administered for the first 10 days according to the cholinergic symptoms. Subsequently, the dose was reduced to 0.25 mg/hour for three days. Measurements indicated that plasma concentrations of dimethoate were low initially and declined in the course of the five days with a half-life of 30.4 h, following first-order kinetics. From the total amount of dimethoate eliminated an apparent half-life of 23.8 h was calculated in urine. Because no sample was stored on admission, initial plasma concentration could not be measured and therefore no distribution half-life could be calculated. Excretion was complete after five days, while the butyrylcholinesterase (BChE) activity recovered only very slowly. Its activity was 10% of normal value to begin with and fell below the level of detection thereafter, but then steadily recovered. Mechanical ventilation was markedly longer necessary as dimethoate could be detected but ventilation was stopped before BChE activity reached 50% of its normal value. The patient recovered and was discharged after a total of three weeks in hospital (Hoffman & Papendorf, 2006).

Three proven cases of severe dimethoate poisoning with cholinergic features were reported. Clinically, all three patients had peripheral vasodilatation and profound hypotension on presentation, which progressed despite treatment with atropine, i.v. fluids, pralidoxime chloride, and inotropes. All patients died 2.5–32 h after admission. Continuous cardiac monitoring and quantification of troponin T provided little evidence of a primary cardiotoxic effect due to dimethoate. In conclusion, dimethoate self-poisoning caused a syndrome characterized by marked hypotension with progression to distributive shock and death, despite standard treatments. A lack of cardiotoxicity until just before death suggests that the mechanism is of organophosphorus-induced low systemic vascular resistance (SVR). (Davies et al., 2006)

As reported in a published paper, two victims of poisoning with organophosphorus insecticides, one with fenthion the other with omethoate, were admitted to an Intensive Care Unit a few hours after ingesting the insecticides. They received appropriate treatment for organophosphorous poisoning (gastric lavage, activated charcoal, atropine and pralidoxime) and supportive care. Both patients survived. The blood levels of the victim of omethoate poisoning was determined on admission (omethoate 1.6 µg/ml) and during the hospitalization, and proved to be sizeable. A slow elimination rate of the poisons already distributed in the body was indicated for both pesticides. The patient with omethoate poisoning remained clinically well (Glasgow Coma Scale: 15) and was discharged three days later, apparently in good condition (Tsatsakis, Manousakis & Anastasaki, 1998).

Suicidal Poisoning

Cases of accidental and suicidal poisoning have been reported with dimethoate. On the basis of a number of cases, the oral lethal dose of dimethoate for human beings was estimated to be in the order of 50–500 mg/kg bw (Gosselin et al., 1984, cited in WHO, 1989). This estimate suggests that humans are not significantly more sensitive than rats to the acute toxicity of dimethoate.

A 34-year-old female attempted suicide by ingesting 10 g of dimethoate. Half an hour after admission to hospital, the serum dimethoate level was 2.340 mg/L. Combined haemoperfusion

and haemodialysis were performed and, after 18 h dimethoate was no longer detected in the serum (Negler, 1981).

A severe case of poisoning after ingestion of approximately 20 g of dimethoate was reported. On admission, the 52-year-old man was comatose with an undetectable plasma ChE activity (< 200 U/L). He was admitted two hours after ingestion and received an injection of 20 mg atropine every 20 minutes. Two haemoperfusions with charcoal and amberlite were performed and atropine was given by infusion up to day 12. Twenty-five days after admission, he was discharged, fully recovered (Köppel, Forycki & Ibe, 1986).

A patient died on the ninth day after dimethoate poisoning with an atypical central neurological disorder. The neuropathological findings, which were similar to those observed in severe forms of Wernicke's encephalopathy, included severe haemorrhagic necrosis of the walls of the ventricles. The authors suggest that the increased level of acetylcholine in the brain led to thiamine depletion and Wernicke's encephalopathy (De Reuck, Colardyn & Willems, 1979).

A 20-year-old male attempted suicide by injecting subcutaneously 10 mL of Sistemin 40 (containing 40% dimethoate) and was admitted to hospital 16 h later. General weakness, muscular fibrillations and a marked inhibition of erythrocyte and serum ChE activity were the prominent signs of intoxication. Treatment consisted of atropine, oxime HI-6 and diazepam combined with symptomatic therapy. Cholinesterase activity decreased over the following three days. In contrast to the marked general improvement of the patient, recovery of ChE activity was very slow. The patient was discharged 24 days after the poisoning with no notable conequences. (Jovanovic, Randjelovic, & Joksovic, 1990)

A 46-year-old man exhibited symptoms of severe cholinergic intoxication after suicidal ingestion of 20 g of dimethoate and was treated successfully by infusion of high-dose atropine (0.5–1.0 mg/kg bw/hour) in combination with charcoal haemoperfusion. Artificial respiration was maintained for 12 days and the patient was discharged on the eighteenth day. Before haemoperfusion, the concentration of dimethoate in plasma was 13.3 mg/L with a half-life of approximately 9 h. Cholinergic signs continued during haemoperfusion and treatment with atropine was continued on the basis of bronchopulmonary hypersecretion. Pupil size was not considered to be a reliable indication of intoxication in this case (Rahn et al., 1988).

A male patient aged 32 was admitted to the hospital about six hours after drinking an unknown volume of a preparation containing 37% dimethoate. At the time of admission the patient was unconscious, in respiratory failure and exhibited pronounced muscarinic and nicotinic effects. Following intubation, artificial ventilation was started and gastric lavage performed. Atropine (27 mg) and Toxobin (750 mg) were given as antidotes. Clinical chemistry revealed ethanol (100 mg/dL) and undetectable AChE activity; the serum dimethoate concentration was 3090 ng/mL. Ten hours after ingestion and in the absence of an improvement in the patient's condition, haemoperfusion was carried out. After four hours of haemoperfusion the condition of the patient improved distinctly; he regained consciousness, hypersecretion stopped and the pupils dilated. Respiratory failure persisted; artificial ventilation was maintained for three days and the patient was administered small doses of atropine (Pach, Groszek & Bogwsz, 1987).

A 57-year-old male was admitted to hospital approximately 1.5 h after ingestion of an unknown quantity of a product containing dimethoate. On admission he was semicomatose, with pupils contracted to 1 mm in diameter. Immediate treatment included mechanical ventilation, gastric lavage, intravenous administration of atropine sulfate and charcoal haemoperfusion. The patient recovered and was discharged from hospital three days after admission. Serum ChE activity (normal range 175–440 IU) was 130 IU at 2.5 h, 95 IU at 8.5 h, 46 IU at 1 day, 60 IU at 2 days and 101 IU at 3 days after ingestion. (Kojima *et al*., 1990)

A 68 year old male attempted suicide by drinking 85 g of Cygon 2E (contains 23.4% dimethoate). The individual was immediately admitted to hospital, responded to standard treatment (ipecac, activated charcoal, 2-PAM and atropine) and was transferred from intensive care to general care 24 h after admission. Within 8 h of transfer, the patient relapsed and required 5 mg of atropine every 10 minutes for 24 h before starting on atropine drip (0.5–2.4 mg/kg bw per hour) for five weeks. The individual received a total atropine dose of 30 g. Although serum AChE activities gradually increased over this period, they were not found to be helpful in determining when the atropine drip could be removed. Control of hypersecretion

was found to be the best monitoring parameter. The patient recovered completely with the exception of a detectable sensorineural hearing deficit, a slight, non-specific personality change and minimal spastic rigidity thought to be secondary to several anoxic episodes (Le Blanc, Benson & Gilg, 1986*)*.

A 68-year-old man was admitted to hospital after a suicidal ingestion of dimethoate. Despite temporary improvement, the patient's condition progressively deteriorated with the development of adult respiratory distress syndrome and acute renal failure. Further investigations indicated non-cardiogenic pulmonary oedema and acute tubular necrosis. Despite vigorous organ-specific support, the patient died on the 12th day following admission. Autopsy confirmed adult respiratory distress syndrome and acute tubular necrosis. (Betrosian et al., 1995)

A 51-year-old man was taken promptly to a hospital where gastric lavage was performed, following a suicidal attempt after drinking a large amount of dimethoate. The patient vomited occasionally, but his condition appeared to improve. The following day, the patient suddenly collapsed, lost consciousness and exhibited pinpoint pupils and dyspnoea. Emergency treatment was started, but the patient died within a few minutes (Hayes & Laws, 1991).

A case of suicide with the insecticide omethoate was reported. An 18-year-old apprentice gardener had ingested an unknown amount of omethoate. His body was found in his room lying in the storage space under his bed. The autopsy first showed multiple superficial incisions in the skin of his wrists, furthermore hemorrhagic pulmonary oedema, dilation of the right cardiac ventricle and oedema of the brain. The gastric mucosa was swollen and showed a dark brownish colour. An intensive, chemical-like smell arose from the corpse and organs. Toxicological analysis detected omethoate in cardiac blood (208 µg/mL), urine (225 µg/mL) and bile (524 µg/mL), in the liver (341 µg/mL) and kidneys (505 µg/mL). In the gastric content the level was 48.223 mg/ml. The amount of active AChE in peripheral blood serum was reduced to less than 0.2% of the normal level. The author has stated that to the best of their knowledge no case of a fatal suicide by ingestion of omethoate had been reported in literature (Pavlic, 2002).

Accidental poisoning.

A 28-year-old farmer was hospitalized with profound weakness, faintness and somnolence, attempts to vomit, chills and prostration on the day after spraying olive trees with dimethoate ('Rogor'); he had reportedly worn protective clothing. On admission to hospital, weak pulse, miosis, vomiting, sweating and pronounced inhibition of ChE activity were noted. Recovery followed treatment with large doses of atropine (20 mg on day 2, 12 mg on day 3 and 5 mg per day then until day 9), prednisone, analgesics and penicillin.

Thirty firemen exposed to dimethoate by inhalation as a result of a manufacturing plant accident developed symptoms of intoxication. Peripheral blood lymphocytes from these workers were examined for the frequency of sister chromatid exchanges two months after the accident. Frequencies of 9.2 ± 0.2 (exposed) and 8.5 ± 0.2 (unexposed) were found ($p < 0.05$). Dicentric chromosomes and a low frequency of chromatid breaks were also noted in two exposed workers. It is not certain to what the firemen were exposed in addition to dimethoate (Larripa et al., 1983).

A case of poisoning in a woman working in agriculture and exposed to dimethoate in the field two days after spraying was reported. Within 3–3.5 h of beginning work, the woman noted an unpleasant odour recognised as dimethoate. She developed a headache, dry cough, dyspnoea, nausea and vomiting and was admitted to hospital in a somnolent state with muscular fibrillations. The patient recovered after treatment with saline solution, glucose, caffeine, atropine and insulin (Asatryan, 1971).

A 16-year-old boy developed weakness, nausea, headache and severe depression after working for two weeks picking hops previously treated with dimethoate. The depression was characterized by psychomotor retardation, an inability to concentrate, suicidal thoughts, guilt, apathy and anxiety. Laboratory examination revealed moderate inhibition of ChE activity, parameters indicating hepatitis and elevated cadmium levels. Recovery was said to coincide with the normalization of ChE activity in 2.5 months. No treatment for poisoning by an organophosphorus compound was indicated or administered (Masiak & Olajossy, 1973).

A 24-year-old male experienced abdominal pain, nausea, vomiting, increased salivation, generalized muscle weakness, excessive lacrimation and muscle fasciculations 4 h after cleaning spray equipment used to apply a mixture of dimethoate and triphenyltin hydroxide, without wearing gloves or a mask. Physical examination on admission to hospital 12–16 h following exposure revealed blood pressure of 137/70, a pulse of 112 and diffuse epigastric tenderness. A diagnosis of anticholinesterase toxicity and acute pancreatitis was made after serum amylase and lipase levels were found to be elevated. Following a period of clinical improvement over several days the nausea, vomiting and abdominal pain worsened. Cholinesterase activities returned to normal levels eight weeks following exposure. Eating continued to result in hyperamylasaemia and exacerbation of abdominal pain. The patient was fed parenterally and was able to tolerate an elementary dietary formula at 10 weeks. At three months following exposure, he was placed on a low fat diet. Although a normal diet was resumed at four months, anorexia, mild hyperamylasemia, abdominal pain and nausea recurred. The patient was reported to be asymptomatic with normal serum amylase values at seven months and no further complications occurred (Marsh, Vukov & Conradi, 1988).

4.3 Direct observation

(a) Volunteer studies

The study protocol, which is reported to be in accordance with an International Conference for Harmonisation (ICH) guideline for good clinical practice, was approved by the laboratory's Medical Ethics Committee. In this study, a single dose of dimethoate in water (ca 0.03 mg/kg bw dimethoate) was taken orally by each of six healthy adult males. Dimethoate carboxylic acid, the main metabolite identified in urine was collected up to 72 h post dose and analysed by liquid chromatography–mass spectrometry, (LC-MS), where it accounted for on average 43% of the administered dose. This compared with much lower urinary levels of dimethoate and omethoate, each of which accounted for less than 1% of the administered dose. Of the administered dose, 67% was accounted for in urine (Meuling & Roza, 2004).

A series of investigations with human subjects is briefly summarized in a published paper. Cholinesterase activity was measured manometrically according to methods described by Fenwick et al. (1957) and Edson et al. (1962). An absence of toxic effects (including ChE inhibition) was reported in five males orally administered a single dose of dimethoate (purity ca 93%) at a dose level of 0.25 mg/kg bw. A similar lack of effects was reported in 20 adults orally administered dimethoate at approximately 0.04 mg/kg bw per day for four weeks. An absence of ChE inhibition is also reported for individual subjects orally administered dimethoate at 0.13 or 0.26 mg/kg bw per day for 13 days (Sanderson & Edson, 1964).

A further (briefly reported) study investigated the effect of oral doses of dimethoate on ChE activity in human volunteers. A total of 36 male and female adult volunteers were given repeated oral doses of dimethoate (administered as an aqueous solution) on five days per week for up to 57 days**.** Missed doses were compensated for by administering higher doses on the preceding or following day. Venous blood was taken twice pretest and once- or twice-weekly during dosing. Whole blood ChE activity was measured by the electrometric method of Michel; activities in red cells and plasma were also determined separately. A toxicologically relevant reduction in whole blood ChE activity was seen at ca 0.6 mg/kg bw. The study's authors report that ChE activity at 0.6 mg/kg bw per day showed a slow downward trend by day 20 and that this continued to the end of the test on day 57. The same effect was reported at the higher dose levels, but at an earlier stage and faster rate. A NOAEL of 0.202 mg/kg bw per day can be determined for the study, based on the toxicologically relevant inhibition of whole blood ChE activity at 0.431 mg/kg bw per day (Edson, Jones & Watson, 1967).

A significant increase in the incidence of chromosomal aberrations is reported in smokers occupationally exposed to a number of pesticides including dimethoate by Rupa, Reddy & Reddi (1989).

In another study, an increased frequency of sister chromatid exchange is reported in the peripheral blood lymphocytes of male pesticide applicators in India. Applicators had been exposed to a number of pesticides including dimethoate (Rupa et al., 1991).

(b) Epidemiological studies

Reiss (2015) reviewed nonoccupational epidemiologic studies relevant to dimethoate. Biomonitoring was used to classify exposure in populations that are generally nonoccupationally exposed. Most of these studies measured biomarkers in pregnant women and assessed outcomes in children, using a biomarker related to organophosphates in general (for example dialkylphosphates, DAPs) rather than to dimethoate specifically. These epidemiologic studies found inconsistent associations between neurodevelopment or infant anthropometric measurements and organophosphates generally. The biomarkers used in most of the nonoccupational studies have substantial limitations that could lead to exposure misclassification. For example:

- most exposure to the biomarkers in the nonoccupational studies is from direct ingestion of preformed metabolites and not organophosphate or dimethoate exposure,
- only one or two biomarker measurements are made during pregnancy, which may not accurately represent long-term exposure.

Overall, the collective nonoccupational epidemiologic data for dimethoate do not provide any convincing evidence of adverse human health effects resulting from dimethoate exposure. Moreover, the epidemiologic data have substantial methodological problems that limit their validity and interpretability (Reiss, 2015).

COMMENTS

Biochemical aspects

Oral absorption of dimethoate in rats was extensive (≥ 90% based on urinary excretion) after dosing with 10 mg/kg bw (low) and 100 mg/kg bw (high). Excretion of the radioactivity was rapid, with 52–72% of the dose excreted in urine (predominant route of excretion) within the first six hours of treatment, and 80–90% within 24 h. In general, concentrations in tissues were the highest at 0.5 h after dosing, though maximum concentrations were occasionally reached at two hours in males. In all cases, concentrations in tissues declined rapidly after reaching the maximum concentration, with only low levels present 48 hours after dosing. Highest concentrations were found in kidneys and liver, with low levels in brain and fat. There was clear evidence of radioactivity in the bone marrow following oral dosing.

At least eight metabolites and unchanged dimethoate were isolated in urine after oral dosing. Dimethoate is mainly metabolized via initial cleavage of the C–N bond, yielding dimethoate carboxylic acid (29–46% of parent excreted through urine), dimethyldithiophosphate (20–30% of parent excreted through urine), thiophosphate and phosphate esters. A subordinate biotransformation pathway is oxidation to the oxygen analogue, omethoate. There were no significant differences in the proportions of the various metabolites between the sexes (Kirkpatrick, 1995).

Toxicological data

The acute toxicity of dimethoate in mice and rats was studied by the oral route and the LD_{50} in mice was at 60 mg/kg bw (Sanderson & Edson, 1964; Ullman et al., 1985). In rats the LD_{50} was 245 mg/kg bw (Kynoch, 1986a; Shaffer, 1957). By the dermal route LD_{50} in rats was > 2000 g/kg bw (Kynoch, 1986b) and by inhalation in rats LC_{50} was 1.68 mg/L air (Jackson et al., 1986). Dimethoate is very slightly irritating to the skin of rabbits (Liggett & Parcell, 1985a) and mildly irritating to the eyes of rabbits (Liggett & Parcell, 1985b). Dimethoate is not a skin sensitizer in guinea pigs in the Magnusson and Kligman maximization test (Albrecht, 2008) or in the Buehler test (Madison, 1984).

In repeated-dose toxicity studies in rats and dogs, the predominant effect was inhibition of AChE.

In a four-week study in rats, dimethoate was administered in the diet at a concentration of 0, 5, 25 and 75 ppm (equivalent to 0, 0.5, 2.5 and 7.5 mg/kg bw per day) The NOAEL was 5 ppm (equivalent to 0.5 mg/kg bw per day) based on the decreased AChE activity in erythrocytes and brain at dose levels of 25 ppm (equivalent to 2.5 mg/kg bw per day) (Hellwig, 1983).

In a four-week dose range-finding study in rats dimethoate was administered in the diet at

concentrations of 0, 13, 38 and 157 ppm (equal to 0, 0.83, 2.48 and 10.4 mg/kg bw per day) for males and 0, 11, 35 and 135 ppm (equal to 0, 0.85, 2.68 and 11 mg/kg bw per day) for females. The NOAEL was 13 ppm (equal to 0.85 mg/kg bw per day) based on a decrease in erythrocyte AChE activity at 35 ppm (equal to 2.68 mg/kg bw per day) in females on day 29 (Kaspers, 2005).

The Meeting identified an overall NOAEL for rats as 0.85 mg/kg bw per day, and an overall LOAEL as 2.5 mg/kg bw per day.

In a 28-day dietary study in dogs, dimethoate was administered in the diet at dose levels of 0, 2, 10, 50, 250 and 1250 ppm (equal to 0, 0.09, 0.43, 2.20, 11.1 and 49.8 mg/kg bw per day) The NOAEL was 10 ppm (equal to 0.43 mg/kg bw per day) based on brain and erythrocyte AChE inhibition at a LOAEL of 50 ppm (equal to 2.20 mg/kg bw per day) (Buford et al., 1989).

In a 98-day dog dietary study, dimethoate was administered at dose levels of 0, 4, 6 and 9 ppm (equivalent to 0, 0.1, 0.15 and 0.225 mg/kg bw per day) The NOAEL was 9 ppm (equivalent to 0.225 mg/kg bw per day), the highest dose tested (Hutchison & Shaffer, 1968).

In a one-year dietary study, dogs were given dimethoate at a concentration of 0, 5, 20 or 125 ppm (equal to 0, 0.18, 0.70, 4.81 mg/kg bw per day for males; 0, 0.19, 0.76, 4.31 mg/kg bw per day for females). The NOAEL was 5 ppm (equal to 0.18 mg/kg bw per day) based on reduction in erythrocyte AChE activity in males at 20 ppm (equal to 0.70 mg/kg bw per day) (Burford et al., 1991).

The overall NOAEL for dogs was 0.43 mg/kg bw per day, based on an overall LOAEL of 0.70 mg/kg bw per day for erythrocyte AChE activity inhibition.

In a 78-week study of carcinogenicity in mice, dimethoate was administered at dietary concentrations of 0, 25, 100 or 200 ppm (equal to dosages of 0, 3.6, 13.7 and 31.1 mg/kg bw per day in males, and 0, 5.2, 18.2 and 35.6 mg/kg bw per day in females). The LOAEL for toxicity was 25 ppm (equal to 3.6 mg/kg bw per day), the lowest dose tested, based on decreases in RBC AChE activity at this dose. The NOAEL for carcinogenicity in mice was 200 ppm (equal to 31.1 mg/kg bw per day), the highest dose tested (Hellwig, Deckardt & Mirea, 1986a).

In a two-year combined chronic toxicity feeding and carcinogenicity study in Wistar rats dimethoate was administered at concentrations of 0, 1, 5, 25 and 100 ppm (equal to 0, 0.04, 0.23, 1.2 and 4.8 mg/kg bw per day in males and 0, 0.06, 0.3, 1.5 and 6.3 mg/kg bw per day in females). The $BMDL_{20}$ for inhibition of brain AChE, calculated by the Meeting, was 0.31 mg/kg bw per day in males and 0.37 mg/kg bw per day in females. The Meeting noted that the haemangiomas/haemangiosarcomas in spleen and mesenteric lymph nodes occurred at higher incidence in treated animals without any dose response or precursor lesion (endothelial proliferation). Consequently, the Meeting concluded that these tumours were not related to treatment. The NOAEL for carcinogenicity was 100 ppm (equal to 4.8 mg/kg bw per day), the highest dose tested (Hellwig & Gembart, 1986; Hellwig, Deckardt & Mirea, 1986b).

The Meeting concluded that dimethoate is not carcinogenic in mice or rats.

Dimethoate has been tested in a range of in vitro and in vivo genotoxicity studies. Dimethoate did not cause chromosomal aberrations in vivo, but appeared weakly positive at high concentrations in some in vitro mutagenicity tests. There were no adequate in vivo tests for mutagenicity. However, the Meeting noted that the critical (sometimes only) effects of dimethoate were related to inhibition of AChE. Consequently, the rate of phosphorylation of AChE appears to be the predominant reaction of dimethoate, whereas mutations resulting from reactions with DNA can only be detected at much higher concentrations. Therefore, the Meeting concluded that DNA alkylation is unlikely to occur at doses of dimethoate that are not strongly inhibitory to erythrocyte/brain AChE activity.

Based on the weight of evidence, the Meeting concluded that dimethoate is unlikely to be genotoxic at doses that do not inhibit AChE activity.

As dimethoate is unlikely to be genotoxic at doses that do not inhibit AChE and in view of the absence of carcinogenicity in mice and rats, the Meeting concluded that dimethoate is unlikely to pose a carcinogenic risk to humans.

In a non-GLP, three-generation study of reproductive toxicity in which mice were fed diets containing dimethoate at concentrations of 0, 5, 15 or 50 ppm (equal to 0, 1.3, 4.1 and 13.6 mg/kg bw per

day for males, 0, 1.5, 4.6 and 15.3 mg/kg bw for females, respectively) the NOAEL for reproductive and offspring toxicity was 50 ppm (equal to 13.6 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 15 ppm (equal to 4.1 mg/kg bw per day) based on tremors in dams at 50 ppm (equal to 13.6 mg/kg bw per day). Acetylcholinesterase activity was not measured in this study (Ribelin & Levinskas, 1965).

In a two-generation reproductive study in rats, when dimethoate was administered through diet during premating at levels of 0, 1, 15 or 65 ppm (equal to 0, 0.08, 1.2 and 5.46 mg/kg bw per day for males and 0, 0.09, 1.30 and 6.04 mg/kg bw per day for females). The NOAEL for parental toxicity was 1 ppm (equal to 0.08 mg/kg bw per day) on the basis of inhibition of erythrocyte and brain AChE activity and a slightly reduced body weight gain in parental females at 15 ppm (equal to 1.3 mg/kg bw per day). The NOAEL for reproductive toxicity was 15 ppm (equal to 1.2 mg/kg bw per day). It was based on decreased fertility in the F1b, F2a and F2b matings; decreased body weight during lactation in both sexes and generations; and decreased litter size at birth among F1a and F2b litters at 65 ppm (equal to 5.46 mg/kg bw per day). These effects on reproduction at the high dose level (65 ppm), decreased fertility in the F1b; F2a and F2b matings; decreased body weight during lactation in both sexes and generations; and decreased litter size at birth among F1a and F2b litters, are possibly a result of marked inhibition of AChE. The NOAEL for offspring toxicity was 1 ppm (equal to 0.08 mg/kg bw per day) based on reduced brain and erythrocyte AChE activity in the F1a generation at a LOAEL of 15 ppm (equal to 1.2 mg/kg bw per day) (Brooker et al., 1992).

In subsequent two-generation reproductive toxicity study in rats, dimethoate was administered in diets adjusted to provide dose levels of 0, 0.2, 1.0 and 6.5 mg/kg bw per day. The NOAEL for parental toxicity and offspring toxicity was 1.0 mg/kg bw per day based on statistically significant reduction in body weight gain shown by the F2B pups of high-dose F1 females, histopathological findings in prostate and epididymides of high dose F0 and F1 males, as well as statistically significant and toxicologically relevant reductions of erythrocyte and brain AChE activities in high-dose F0 and F1 parental generations of both sexes, effects that were apparent at 6.5 mg/kg bw per day. The NOAEL for reproductive toxicity was 6.5 mg/kg bw per day, the highest dose tested (Mellert et al., 2003).

The Meeting concluded that the more recent study has a higher number of animals and a more comprehensive evaluation of parameters, hence it was considered more reliable for identifying relevant toxicological effects.

In a developmental toxicity study in rats with gavage dosing at 0, 3, 6 and 18 mg/kg bw per day on days 6–15 of gestation, a NOAEL for maternal toxicity was 6 mg/kg bw per day based on body tremor, hypersensitivity, abnormal gait, reduced body weight gain and food consumption, at 18 mg/kg bw per day. The embryo/fetal NOAEL was 18 mg/kg bw per day, the highest dose tested. Acetylcholinesterase activity was not measured in this study (Edwards, Leeming & Clark, 1984b).

In a developmental toxicity study in rabbits using gavage dosing at 0, 10, 20 or 40 mg/kg bw per day on days 7–19 of gestation, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based upon effects on weight gain at higher dose levels in dams and an NOAEL for embryo and fetal toxicity which was 40 mg/kg bw per day, the highest dose tested. Acetylcholinesterase activity was not measured in this study (Edwards, Leeming & Clark, 1984d).

The Meeting concluded that dimethoate is not teratogenic.

An acute neurotoxicity gavage study in rats provided a single dimethoate dose of 0, 2, 20, or 200 mg/kg bw. The NOAEL for acute neurotoxicity in rats was 2 mg/kg bw on the basis of cholinergic signs at 20 mg/kg bw. Acetylcholinesterase activity was not measured in this study (Lamb, 1993b; JMPR, 2003).

In a study for acute neurotoxicity, dimethoate was given to rats at dietary concentrations adjusted to provide doses of 0, 1, 2, 3 or 15 mg/kg bw. The NOAEL was 2 mg/kg bw on the basis of inhibition of AChE activity in erythrocytes in males at 3 mg/kg bw (Schaefer, 1999; JMPR, 2003).

A 13-week dietary study for neurotoxicity fed rats a dose of 0, 1, 50 or 125 ppm (equal to intakes of 0, 0.06, 3.22 and 8.13 mg/kg bw per day for males; 0, 0.08, 3.78 and 9.88 mg/kg bw per day for females). This continued for 91, 92, 93 or 94 days. The NOAEL for systemic toxicity and neurotoxicity was 1 ppm (equal to 0.06 mg/kg bw per day) on the basis of inhibition of erythrocyte AChE activity at 50 ppm (equal to 3.22 mg/kg bw per day) (Lamb, 1994; JMPR, 2003).

In a dose range-finding gavage study for developmental neurotoxicity in rats, dose levels of 0, 0.2, 3 and 6.0 mg/kg bw per day were given in water to dams between GD days six and 20 or during GD 6 and PND 10, and to offspring during PND 11–21. The NOAEL was 0.2 mg/kg bw per day for dams and their offspring based on statistically significantly reduced maternal body weight gain from GD 6 until GD 20, and inhibition of erythrocyte and brain AChE in the dams and offspring at 3 mg/kg bw per day (Myers, 2001a).

In the main developmental neurotoxicity oral gavage study, rats were given a dose in water of 0, 0.1, 0.5 or 3 mg/kg bw per day during GD 10–PND10 (dams), and during PND 11–21 (offspring). The NOAEL in the offspring was 0.5 mg/kg bw per day based on poor general condition, developmental delay in some functional parameters and increased pup mortality at a dose of 3 mg/kg bw per day. A dose of up to 3 mg/kg bw per day, the highest dose tested, was not associated with any selective developmental neurotoxicity. Acetylcholinesterase activity was not measured in this study (Myers, 2001b; JMPR, 2003).

A study supplemental to a developmental neurotoxicity study was conducted to assess the influence on offspring survival of maternal exposure to dimethoate during gestation and the postnatal period. Groups of mated female rats were allocated one of three treatments: a control group, a 3 mg/kg bw per day group, or a 6 mg/kg bw per day group; offspring were then cross-fostered. Excess mortality, behavioural and clinical effects were observed in pups reared by treated dams, irrespective of pups' in utero exposure (Myers, 2004).

In a special study designed to assess effects on AChE activity, pregnant rats, preweaning rats and young adult rats received dimethoate by gavage at 0, 0.1, 0.5 or 3 mg/kg bw per day either once or for 11 days (preweaning and young adult), or from GD6 to GD20 (dams), or from GD6 to PND10 (dams) followed by pups treatment until PND 21. The NOAEL for dimethoate given as a single dose was 0.5 mg/kg bw per day on the basis of inhibition of erythrocyte AChE activity in preweaning females (26%) and in young adult females (27%) at 3 mg/kg bw per day. The NOAEL for dimethoate given as repeated doses was 0.1 mg/kg bw per day on the basis of inhibition (23%) of erythrocyte AChE activity in female pups on PND 21 at 0.5 mg/kg bw per day Myers, 2001c).

The Meeting identified an overall NOAEL of 2 mg/kg bw for acute treatment based on inhibition of erythrocyte AChE activity in an acute neurotoxicity study, and a special study in preweaning females and in young adult females with an overall LOAEL of 3 mg/kg bw per day.

In a GLP-compliant study it was concluded that administration of dimethoate, either orally or subcutaneously, at a dose level of 55 mg/kg bw (the oral LD_{50} value) did not produce any clinical signs of delayed neurotoxicity in domestic hens.

dimethoate is neurotoxic via inhibition of AChE but is not neuropathic.

No evidence of dimethoate-mediated estrogenic, androgenic or steroidogenic activity was observed in a battery of in vivo and in vitro tests.

No immunotoxicity studies were available, but there was no indication of immunotoxic effects in the short- or long-term toxicity studies.

Data on metabolites

Omethoate

The Meeting noted the conclusion of the 1996 JMPR:

"Omethoate has been extensively investigated for mutagenicity in vitro and in vivo. The Meeting concluded that it has clear mutagenic potential but that the weight of the evidence observed in vivo was negative; however, the positive result obtained in the mouse spot test could not be completely disregarded."

At that Meeting the ADI for omethoate was withdrawn. Although a number of new studies focused on the antiacetylcholinesterase activity of omethoate, no new genotoxicity studies were provided to the present Meeting. Consequently, the Meeting was unable to complete the assessment of omethoate with respect to its mutagenic potential.

Apart from omethoate, a number of plant metabolites have been identified, including dimethoate carboxylic acid, *O*desmethyl dimethoate carboxylic acid, *O*desmethyl dimethoate, *N*desmethyl dimethoate, *O*desmethyl omethoate, *O*Desmethyl-*N*-desmethyl omethoate, *O*desmethyl omethoate carboxylic acid, hydroxy-omethoate, isodimethoate, *O*desmethyl isodimethoate and hydroxydimethoate (and its glucose conjugate). These plant metabolites occur at different levels. Dimethoate carboxylic acid is a major rat metabolite, therefore, the Meeting concluded that the toxicity of this metabolite would be covered by that of dimethoate. As for the remaining metabolites, although the desmethylated metabolites are less potent inhibitors of AChE, the Meeting noted that they are also likely to retain the moiety for genotoxic activity. Therefore, in the absence of genotoxicity studies, no conclusion can be drawn as to the genotoxic potential of these metabolites.

Human data

The medical surveillance data of manufacturing personnel did not reveal any indications of negative health effects caused by the exposure to dimethoate during its manufacturing or formulation (Stephansen, 2016).

The Meeting also considered a number of studies in human volunteers, which indicated that single or repeated oral doses of dimethoate of up to 0.2 mg/kg bw did not induce clinical effects or inhibit AChE activity in the blood. Since these studies were not conducted according to current standards (no details on study design were given, for example age and sex of individual volunteers, nor were raw data provided) they could not form the basis for the ADI and ARfD.

A number of reported cases, both accidental and voluntary, of acute poisoning by dimethoate or omethoate are available in the literature. The effects observed appeared to be the cholinergic syndrome and no long-term consequences, including delayed polyneuropathy, have been described.

The Meeting concluded that the existing database on dimethoate was adequate to characterize the potential hazard to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting withdrew the previous ADI and established an ADI for dimethoate of 0–0.001 mg/kg bw on the basis of the NOAEL of 0.1 mg/kg bw per day for inhibition of erythrocyte AChE activity in female pups on PND 21 at 0.5 mg/kg bw in the developmental special study designed to assess effects of dimethoate on AChE activity in pregnant rats, preweaning rats and young adult rats. A safety factor of 100 was applied.

The Meeting reaffirmed the ARfD for dimethoate of 0.02 mg/kg bw on the basis of an overall NOAEL of 2 mg/kg bw per day based on inhibition of erythrocyte AChE activity in an acute neurotoxicity study and a special study in preweaning females and in young adult females with an overall LOAEL of 3 mg/kg bw per day. A safety factor of 100 was applied.

The Meeting noted that the data from the human volunteer studies are consistent with the proposed ADI and ARfD where there was apparent a NOAEL of about 0.2 mg/kg bw per day in studies in volunteers receiving single or repeated doses.

Levels relevant to risk assessment of dimethoate

Species	Study	Effect	NOAEL	LOAEL
Mouse	78-week study of toxicity and carcinogenicity[a]	Toxicity	-	25 ppm equal to 3.6 mg/kg bw per day[e]
		Carcinogenicity	200 ppm equal to 31.1 mg/kg bw per day[c]	-
	Three-generation reproductive toxicity [a, f]	Reproductive toxicity	50 ppm equal to 13.6 mg/kg bw per day[c]	-
		Parental toxicity	15 ppm equal to 4.1 mg/kg bw per day	50 ppm equal to 13.6 mg/kg bw per day
		Offspring toxicity	50 ppm equal to 14.4 mg/kg bw per day[c]	-
Rat	Four-week study on toxicity [a, b]	Toxicity	0.83 mg/kg bw per day	2.5 mg/kg bw per day
	Two-year study on toxicity and carcinogenicity[a]	Toxicity	$BMDL_{20}$: 0.31 mg/kg bw per day[g]	-
		Carcinogenicity	100 ppm equal to 4.8 mg/kg bw per day[c]	-
	Two-generation study of reproductive toxicity [a, b]	Reproductive toxicity	6.5 mg/kg bw per day[c]	-
		Parental toxicity	1.0 mg/kg bw per day	6.5 mg/kg bw per day
		Offspring toxicity	1.0 mg/kg bw per day	6.5 mg/kg bw per day
	Developmental Toxicity study [d, b]	Maternal toxicity	6 mg/kg bw per day	18 mg/kg bw per day
		Embryo and fetal toxicity	18 mg/kg bw per day[c]	-
	Acute neurotoxicity study [b, d]	Neurotoxicity	2 mg/kg bw per day	3 mg/kg bw per day
	91–94 day neurotoxicity study [a, f]	Neurotoxicity	0.06 mg/kg bw per day	3.22 mg/kg bw per day
	Study of acetylcholin-esterase activity after single and repeated doses of dimethoate	ChE activity after single dose in preweaning rats and young adults	0.5 mg/kg bw	3 mg/kg bw
		ChE activity after repeat dose in female pups on PND 21	0.1 mg/kg bw per day	0.5 mg/kg bw per day
	Developmental neurotoxicity[d]	Functional development of nervous systemic toxicity in offspring	0.5 mg/kg bw per day	3 mg/kg bw per day
		Developmental neurotoxicity	3 mg/kg bw per day[c]	-
Rabbit	Developmental toxicity study[d]	Maternal toxicity	10 mg/kg bw per day	20 mg/kg bw per day
		Embryo and fetal toxicity	40 mg/kg bw per day[c]	-
Dog	28-day, 98-day and oneyear studies of toxicity [a, b]	Toxicity	10 ppm equal to 0.43 mg/kg bw per day	20 ppm equal to 0.70 mg/kg bw per day

(continued on p340)

[a] Dietary administration
[b] Two or more studies combined
[c] Highest dose tested
[d] Gavage administration
[e] Lowest dose tested
[f] Cholinestrerase activity not measured
[g] $BMDL_{20}$ calculated with BMDS

Acceptable daily intake (ADI) for dimethoate

0–0.001 mg/kg bw

Acute reference dose (ARfD) for dimethoate

0.02 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from further epidemiological, occupational health and other such observational studies of human exposure; clarification on the genotoxic potential of omethoate and related metabolites.

Critical end-points for setting guidance values for exposure to dimethoate

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly and extensively absorbed (≥ 90%)
Dermal absorption	Approximately 40%
Distribution	Widely distributed, highest concentration found in kidney and liver
Potential for accumulation	None
Rate and extent of excretion	Excretion is rapid: 52–72% of the dose excreted in urine within the first six hours, and 80–90% by 24 hours
Metabolism in animals	Extensive
Toxicologically significant compounds in animals and plants	dimethoate, omethoate, dimethoate carboxylic acid, *O*desmethyl dimethoate carboxylic acid, *O*desmethyl dimethoate, *N*desmethyl dimethoate, *O*desmethyl omethoate, *O*Desmethyl-*N*-desmethyl omethoate, *O*desmethyl omethoate carboxylic acid, Hydroxy-omethoate, isodimethoate, *O*-desmethyl isodimethoate and hydroxydimethoate (and its glucose conjugate)
Acute toxicity	
Rat, LD_{50}, oral	245–414 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	1.68 mg/L
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Mildly irritating
Guinea pig, dermal sensitization	Not sensitizing (Buehler and maximization)
Short-term studies of toxicity	
Target/critical effect	Inhibition of acetylcholinesterase
Lowest relevant oral NOAEL	0.43 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	10 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	No data

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Inhibition of erythrocyte and brain acetylcholinesterase (rat, mouse)
Lowest relevant NOAEL	0.31 mg/kg bw per day (rat) ($BMDL_{20}$)
Carcinogenicity	Not carcinogenic in mice and rats[a]
Genotoxicity	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	1 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	1 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	1.2 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	No developmental effect (rat, rabbit)
Lowest relevant maternal NOAEL	6 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	18 mg/kg bw per day, highest dose tested (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL Lowest acetylcholinesterase activity NOAEL	0.06 mg/kg bw per day, (rat) 0.04 mg/kg bw per day
Developmental neurotoxicity NOAEL	0.5 mg/kg bw (rat)
Immunotoxicity	No specific studies available, but no indication of immunotoxic effects in the short- and long-term toxicity studies.
Human data	Studies on volunteers not adequate for risk assessment Poisoning case reports showing cholinergic toxicity

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.001 mg/kg bw	Special study in pups and young adults on acetylcholinesterase activity after repeated doses.	100
ARfD	0.02 mg/kg bw	Overall NOAEL of acute neurotoxicity study in rats and special study in pups and young adults, supported by studies in volunteers receiving single or repeated doses.	100

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Note: All references not published in peer-reviewed journals were submitted to WHO by Cheminova A/C, 7673 Harboør, Denmark

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METCONAZOLE

First draft prepared by
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Explanation

Metconazole is the ISO-approved common name for:
(1*RS*,5*RS*;1*RS*,5*SR*)-(chlorophenyl)methyl-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol, with the Chemical Abstract Service number 125116-23-6 (unstated stereochemistry). Metconazole is a fungicide and belongs to the chemical class of triazoles. As manufactured it consists of two diastereomers (*cis* 85% and *trans* 15%). Metconazole acts by inhibition of P450 sterol 14α-demethylase (CYP51).

Metconazole has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All studies were conducted in compliance with good laboratory practice (GLP) and other internationally recognized guidelines, unless otherwise specified. A literature search did not identify any toxicological information additional to that submitted for the current assessment.

Evaluation for acceptable intake

1. Biochemical aspects

Metconazole technical as originally manufactured was supplied as 95:5% *cis* isomer, but thereafter, the decision was taken to withhold the 85:15 *cis*:*trans* mixture from the market (designated KNF-S-474m, WL148271, AC 900768 or BAS 555F). A description of the metconazole enantiomers (actually two diastereoisomeric pairs) is shown below. Key toxicological studies were performed with either the *cis*/*trans* mixture or the *cis* isomer. For the two-generation study, only *cis*-metconazole was assayed. Technical metconazole has a total purity of at least 94%, and the total enantiomeric purity of the *cis* enantiomer is ≥ 80% and < 95%.

Metconazole enantiomers in isomer mix WL148271 (AC 900768)

Component 1:WL136184, CL354801. Conformation: *cis*
IUPAC chemical name:
(1RS,5SR)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol)-1-ylmethyl-cyclopentanol

Component 2:WL153996, CL354802. Conformation: *trans*
IUPAC chemical name:
(1*RS*,5*RS*)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol)-1-ylmethyl-cyclopentanol

1.1 Absorption, distribution and excretion

Study 1
The absorption and excretion of ^{14}C-metconazole were studied in bile-duct cannulated rats following oral administration. Bile-cannulated rats (Fischer 344), three/sex per dose, received [cyclopentyl-^{14}C]-metconazole, *cis* isomer WL136184 (radiochemical purity 98,2%; batch S.1190/2; specific activity 44.3 µCi/mg) at a dose level of 2 mg/kg body weight (bw). The radiolabelled metconazole was diluted with nonradiolabelled metconazole (batch 002/90) and then dissolved in polyethylene glycol 200 at a concentration of 1 mg/mL. Bile was immediately frozen (solid carbon dioxide) on collection during periods 0–6, 6–24 and 24–48 h. Urine was also collected during the periods 0–6, 6–24 and 24–48 h, and faeces were collected during the periods 0–24 h and 24–48 h. Cage washes were collected at termination. Survivors were sacrificed by cervical dislocation at 48 h, and gastrointestinal tracts (GITs) separated from carcasses.

During the first 48 hours folowing a single oral administration of [cyclopentyl-^{14}C]-metconazole (*cis* isomer) to bile-cannulated rats at a dose rate of 2 mg/kg bw, most of the applied dose was absorbed, 95.1% in males and 96.6% in females. Radioactivity was eliminated in bile and urine, with comparable amounts eliminated in bile (79–83%) for both males and females, whereas more radioactivity was eliminated by females in urine; 12% compared to males at 4%. Excretion in the faeces accounted for 0.2–0.3% of the administered radioactivity. In rats there were no apparent gender-related differences in the excretion of residues derived from [cyclopentyl-^{14}C]-metconazole.

Table 1. Excretion and retention of cis-metconazole in bile-cannulated rats following single low dosing

		Males			Females		
Substrate	**Time (h)**	**Mean** [§]	**±**	**SD**	**Mean** [§]	**±**	**SD**
Bile	0–6	50.2	±	20.7	68.5	±	6.6
	6–24	27.9	±	21.5	13.9	±	5.9
	24–48	0.6	±	0.5	0.9	±	0.3
	Total	78.7	±	13.0	83.3	±	1.4
Urine	0–6	1.4	±	0.5	5.3	±	1.7
	6–24	2.3	±	1.7	5.1	±	1.2
	24–48	0.6	±	0.5	1.7	±	0.7
	Total	4.3	±	1.9	12.1	±	0.8
Faeces	0–24	0.1	±	0.2	0.2	±	0.1
	24–48	0.1	±	0.1	0.1	±	0.1
	Total	0.2	±	-	0.3	±	0.1
GIT	48	8.5	±	11.7	0.2	±	0.1
Carcass	48	3.6	±	2.9	1.0	±	0.1
Cage wash	48	0.2	±	0.1	0.3	±	0.2
Total recovery		**95.5**	±	**1.7**	**97.2**	±	**1.6**
Absorbed fraction [#]		86.8			96.7		
Absorbed fraction [¶]		36.6			28.2		

SD Standard deviation

[§] Values expressed in % of administered radioactivity

[#] Including the biliary fraction 0–6 h

[¶] Excluding the biliary fraction 0–6 h; absorption taking into account biliary fraction, urine, carcass and cage wash

The overall recovery of radioactivity ranged from 96–97% of the total applied dose.

If it is assumed that the amount of radioactivity excreted via bile and urine represents the bioavailable amount of ^{14}C-metconazole, then the bioavailability is approximately 83–95%. (Hawkins, Elson & Kane, 1991).

Study 2

The absorption, distribution and elimination of [cyclopentyl-^{14}C]-metconazole were investigated in male and female Fischer 344 rats following oral administration at a dose level of 2 mg/kg bw of WL 148271, 80:20 *cis:trans* isomer; batch S. 1106/1). Five male and five female rats were used for this experiment. In a preliminary study, two rats/sex had been treated similarly, in order to measure radioactivity in the expired air over the first 24 h, then sacrificed at either 24 h (one rat/sex) or seven days after dosing (one rat/sex). In the main study, both urine and faeces was collected at 24 h intervals up to day 3 after dosing. Cage washes were collected at termination. Animals were sacrificed by exsanguination and whole blood, liver, spleen, kidneys, perirenal fat, adrenals, GIT, gonads, uterus, lung, heart, femoral muscle, femur bone, skin and brain were sampled and stored at –18°C (except blood at +4°C). Tissues were solubilized (skin) and casually homogenized.

Figure 1. Structure of cyclopentyl-labelled metconazole; cis and trans isomers

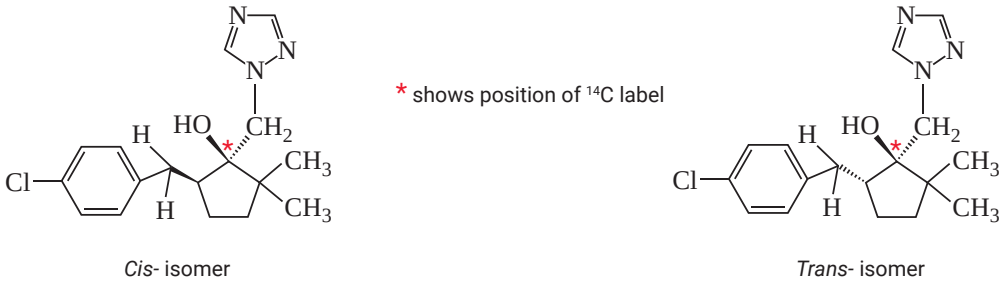


Table 2. Excretion of metconazole (cis/trans) in rats following single low dose (2 mg/kg bw) of ^{14}C-labelled metconazole (cyclopentyl label)

		Males			Females		
Substrate	**Time (h)**	**Mean** §	**±**	**SD**	**Mean** §	**±**	**SD**
Urine	24	10.7	±	0.7	17.1	±	3.5
	48	3	±	0.3	6.5	±	0.3
	72	1.1	±	0.3	2.3	±	0.2
	Total	14.8	±	0.8	25.9	±	3.7
Faeces	24	50.7	±	5.9	36.4	±	7.4
	48	21.7	±	5.7	22.1	±	9.3
	72	7.9	±	1.3	8.6	±	1.4
	Total	80.3	±	1.3	67.1	±	5.0
	Subtotal	95.1			93.0		
Cage wash	72	0.6	±	0.2	0.3	±	0.1
	Total recovery	**95.7**			**93.3**		

SD Standard deviation

§ Values expressed in % of administered radioactivity

Table 3. Levels of radioactivity in rat tissues harvested three days after administration of a single oral low dose (2 mg/kg) of ^{14}C-labelled metconazole

	Radioactive residues in tissues (mg/kg)			Radioactive residues in tissues (mg/kg)	
Matrix	**Males**	**Females**	**Matrix**	**Males**	**Females**
Adrenal	2.88	1.67	Liver	0.157	1.63
Blood	0.021	0.033	Lungs	0.022	0.021
Bone	ND	ND	Muscle	ND	ND
Brain	ND	ND	Ovaries	NA	0.017
Fat	ND	ND	Skin	0.015	0.011
GI Tract	0.75	0.559	Spleen	0.01	0.008
Heart	0.011	0.012	Testes	0.046	NA
Kidney	0.032	0.029	Uterus	NA	0.017

NA Not applicable

ND Not detected

In the preliminary study, only 0.05% of the radioactive dose had been expired in the air at 24 h after administration, whereas at that time 35–48% and 12–20% had been eliminated via the faeces and urine respectively. At day 3 after dosing about 90% of the radioactivity had been excreted. Examination of whole-body slides, processed for autoradiography, revealed that staining was concentrated in GIT, liver and adrenal glands 24 h after dosing. After seven days the staining in these organs was diminished but still present, especially in the adrenal cortex. The radioactivity was more pronounced in the male than in the female.

In the main study, on day 3 after dosing, 93–96% of the dose was excreted. Faecal elimination was the major excretion route, accounting for 67–80% of elimination. It was noted that elimination was rapid, as 54–61% of radioactivity was eliminated by day 1. It was also observed that renal excretion was more important in the female than the male. At termination on day 3, significant amounts of radioactivity were detected in the GIT (0.56–0.75 ppm), liver (0.16–1.63 ppm) and adrenals (1.67–2.88 ppm). Sex differences were apparent, as levels in the liver were greater in females than males, whereas, on the contrary, levels in the adrenals were higher in the males than in the females. Most other organs and tissues had values close to, or lower than, the levels in the blood. Radioactivity was not detectable in bone, brain, fats or muscle (Morrison & Richardson, 1990).

Study 3

The absorption, distribution and elimination of cyclopentyl-labelled *cis*-metconazole were investigated in female and male rats (Fischer 344) following multiple oral low doses of unlabelled and radiolabelled test substance (*cis*-isomer WL 136184; radiochemical purity 99.4% *cis* isomer of [cyclopentyl-^{14}C]-metconazole (WL 136184); lot/batch S1190/4). Six male and six female rats were administered 14 consecutive daily low doses (2 mg/kg bw) of unlabelled test substance. Of these animals, five male and five female rats were selected and given a single oral low dose of (2 mg/kg bw) of radiolabelled *cis*-metconazole (cyclopentyl label). Both urine and faeces were collected at 24 h intervals up to day 4 after dosing. Cage washes were collected at termination. Animals were sacrificed by exsanguination, and whole blood, liver, spleen, kidneys, perirenal fat, adrenals, GIT, gonads, uterus, lung, heart, femoral muscle, femur bone, skin and brain were sampled and stored.

Table 4. Excretion of cis-metconazole in rats following multiple low dosing

		Males			Females		
Substrate	**Time (h)**	**Mean** [§]	**±**	**SD**	**Mean** [§]	**±**	**SD**
Urine	24	10.2	±	0.3	18.5	±	3.6
	48	3.1	±	0.6	6.9	±	0.6
	72	1.0	±	0.1	3.0	±	0.5
	96	0.5	±	0.1	1.5	±	0.2
	Total	14.8	±	0.9	29.9	±	1.7
Faeces	24	32.4	±	5.8	19.5	±	10.3
	48	32.9	±	2.4	26.3	±	5.2
	72	11.6	±	2.1	14.6	±	4.0
	96	5.3	±	1.1	5.1	±	1.4
	Total	82.2	±	2.2	65.4	±	4.1
	Subtotal	97.0			95.4		
Cage wash	96	0.2	±	0.1	0.2	±	0.1
	Total recovery	**97.2**			**95.6**		

SD Standard deviation

[§] Values expressed in % of administered radioactivity

Table 5. Residues of cis-metconazole in rats following multiple low dosing

	Radioactive residues in tissues (mg/kg)	
Matrix	**Males**	**Females**
Adrenal	2.326	1.367
Blood	ND	ND
Bone	ND	ND
Brain	ND	ND
Fat	ND	ND
GI tract	0.562	0.491
Heart	ND	ND
Kidney	0.015	0.018
Liver	0.106	0.883
Lungs	0.008	0.007
Muscle	ND	ND
Ovaries	NA	ND
Skin	0.005	0.003
Spleen	0.004	0.005
Testes	0.054	NA
Uterus	NA	ND

NA Not applicable

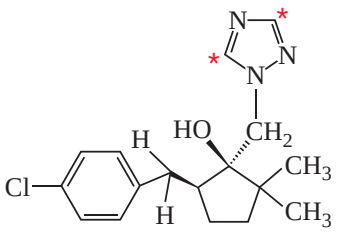
ND Not detected

During the first 96 hours after the last dose, most of the administered radioactivity was excreted in urine (15–30%) and faeces (65–82%). Female rats excreted more radioactivity in urine compared with male rats. The overall recovery of radioactivity in urine and faeces ranged from 96% to 97% of the total applied dose. Tissue residue was measured at 96 hours after the last dose. As in the single-dosing study, the highest concentrations of radioactivity were found in the adrenal followed by the GIT and/or liver, with liver values higher in females and adrenal values higher in males. Most other organs and tissues had values which approximated to, or were lower than, those detected in the blood. Radioactivity was not detectable in bone, brain, fat and muscle (Morrison & Richardson, 1992a).

Study 4

The elimination and metabolism of *cis*-metconazole (WL136184 labelled at the 3 and 5 positions of the triazole ring; lot/batch S1084/1) in six male Fischer 344 rats were investigated at a single oral high dose of 200 mg/kg bw. Urine and faeces were collected at 24 h intervals up to day 7 after dosing. Cage washes were collected at termination. Animals were sacrificed by carbon dioxide asphyxiation, but no necropsy was performed. Carcasses were were sampled and stored. At seven days after dosing the rats were sacrificed, and tissues collected for analysis.

Figure 2. WL136184; cis-metconazole labelled at the 3 and 5 positions of the triazole ring



Cis- isomer

Table 6. Excretion of cis-metconazole in male rats following a single high dosing

	Urine			Faeces			
Time (h)	**Mean** [§]	**±**	**SD**	**Mean** [§]	**±**	**SD**	**Total**
24	4.01	±	0.6	5.2	±	2.04	9.21
48	8.1	±	1.0	19.94	±	6.56	28.1
72	5.37	±	1.0	26.91	±	5.37	32.3
96	1.81	±	0.7	14.72	±	1.81	16.5
120	0.66	±	0.4	5.44	±	2.89	6.1
144	0.27	±	0.1	2.36	±	1.56	2.63
168	0.15	±	0.1	0.97	±	0.56	1.12
Total	**20.37**			**75.53**			**95.9**

SD Standard deviation

[§] Values expressed in % of administered radioactivity

Source: Richardson, 1991b

The elimination of metconazole-derived radioactivity was rapid, with approximately 70% of the applied dose excreted in three days and 96% in seven days. Most of the radioactivity was eliminated in faeces (75.5%) and urine (20.4%) (Richardson, 1991b).

Study 5

The absorption, distribution and elimination of [cyclopentyl-^{14}C]-metconazole (WL148271; *cis/trans* isomer ratio 79:21; lot/batch S1164/1) in male and female Fischer 344 rats were investigated at a dose level of 164 mg/kg bw (for structures see Fig.1). Five male and five female rats were used for this experiment. The urine and faeces were collected for five days. Five days after dosing, the rats were sacrificed and tissues collected for analysis.

Table 7. Excretion of metconazole (cis/trans) in rats following a single high dose

		Males			Females		
Substrate	**Time (h)**	**Mean** [§]	**±**	**SD**	**Mean** [§]	**±**	**SD**
Urine	24	2.488	±	0.492	7.585	±	0.8757
	48	6.005	±	0.795	12.29	±	1.785
	72	3.528	±	0.723	6.122	±	0.9916
	96	1.137	±	0.255	1.828	±	0.5409
	120	0.408	±	0.035	0.596	±	0.2963
	Total	13.57	±	1.775	28.42	±	1.65
Faeces	24	12.66	±	5.84	11.36	±	4.47
	48	34.46	±	6.112	24.49	±	6.842
	72	21.21	±	2.68	19.23	±	3.057
	96	9.596	±	1.626	8.286	±	4.199
	120	3.366	±	1.071	2.117	±	0.7059
	Total	81.28	±	1.656	65.48	±	2.021
Cage wash	120	0.44	±	0.08	1.35		0.52
Total recovery		**95.29**			**95.25**		

SD Standard deviation

[§] Values expressed in % of administered radioactivity

During the first 120 h after a single oral dose of 164 mg/kg bw of [cyclopentyl-^{14}C]-metconazole, most of the applied radioactivity was eliminated in urine (14–28%) and faeces (66–81%). There were no apparent gender-related differences in the excretion of metconazole-derived residues.

Table 8. Levels of radioactivity in rat tissues harvested five days after administration of a single oral high dose (164 mg/kg bw) of ^{14}C-labelled metconazole

	Radioactive residues in tissues (mg/kg)	
Matrix	**Males**	**Females**
Adrenal	4.27	2.63
Blood	1.02	1.11
Bone	0.18	0.19
Brain	ND	ND
Fat	0.83	0.87
GI tract	33.02	14.88
Heart	0.43	0.37
Kidney	1.38	1.13
Liver	4.99	3.43
Lungs	0.61	0.52
Muscle	0.17	0.15
Ovaries	NA	0.57
Skin	1.06	1.15
Spleen	0.4	0.35
Testes	0.27	NA
Uterus	n.a.	0.51

NA Not applicable

ND Not detected

Following the single oral dose of ^{14}C-metconazole at a dose level of 164 mg/kg bw, tissue radioactivity concentration was measured at 120 h after dosing. In general, tissue residue levels in males were slightly higher than those in females. The highest radioactivity concentrations were found in the GIT followed by liver and adrenals. Most other organs and tissues had residues close to, or lower than, the ones found in blood. Radioactivity was not detectable in the brain. The distribution of the radioactive residues in tissues is shown in Morrison & Richardson (1992b).

Study 6

Cyclopentyl-^{14}C radiolabelled KNF-474m mixed with unlabelled metconazole (*cis*:*trans* 83:16%; batch 9Z521; purity 98.88%) was administered by gavage to male and female Fisher F344/DuCrj strain rats at single doses of 2 or 200 mg/kg bw or at 14-day repeated doses of 2 mg/kg. There followed an investigation of the following pharmacokinetic parameters: time to reach maximum concentration (T_{max}), maximum concentration (C_{max}), half-life ($t_{½}$), area under the concentration–time curve from 0 to infinity ($AUC_{0\rightarrow\infty}$), and repetitive accumulation ratio (RAC_{24}). Also investigated were the distribution and concentration of labelled metconazole in major organs and tissues. Repetitive accumulation ratio was defined as the parameter showing the ratio of concentration at steady-state to the concentration in blood 24 hours after single dosing. Radioactivity was measured in appropriately processed tissues and organs by liquid scintillation counting (LSC).

Low dose level (2 mg/kg bw): ^{14}C concentration in plasma reached 0.251 mg equiv./L for males and 0.187 mg equiv./L for females within 15 minutes of administration and then decreased following monophasic first-order kinetics. Half-life times were approximately 20 h for males and 34 h for females, and $t_{½}$ was significantly longer in females than males. The ^{14}C concentrations in plasma decreased to 0.013 mg equiv./L for males and 0.038 mg equiv./L for females at 72 h after dosing. $AUC_{0\rightarrow\infty}$ values were ca 4.5 mg equiv. h/L for males and ca 7.2 mg equiv. h/L for females, suggesting that the systemic absorption in females was greater than in males (a finding also observed in mass balance studies).

High dose level (200 mg/kg bw): ^{14}C-concentration in plasma reached ca 17 mg equiv./L for both sexes at 4 h after administration and then decreased following monophasic first-order kinetics. ^{14}C concentrations in plasma decreased to 0.61 mg equiv./L for males and 1.48 mg equiv./L for females at 120 h after dosing. As was observed with the low dose, $t_{½}$ was greater in the female than the male, but the difference was not statistically significant (ca 25 h for males, ca 34 h for females). $AUC_{0\rightarrow\infty}$ values were 671.3 mg equiv. h/L for males and 787.2 mg equiv. h/L for females, and were nearly proportional to dose levels (at 100-fold dose differences, the values were 149 times greater for males and 109 times greater for females).

Results of pharmacokinetics: since $AUC_{0\rightarrow\infty}$ values were nearly proportional to dose levels, it was estimated that the absorption rate of the test substance was dose-independent, at least in the investigated dose range. However, the time to reach C_{max} (T_{max}) was dose-dependent: The maximum concentration was reached more slowly at the high dose level (4 h after dosing) than at the low dose level (within 0.25 h). This could be due to a saturation of the absorption process at the high dose level.

For any tested dose levels, the following was observed for female by comparison with male rats.

- The $t_{½}$ was longer for females.
- The ^{14}C concentration in plasma at final collection time point was higher for females.
- The $AUC_{0\rightarrow\infty}$ value was higher for females.

Table 9. Time course and total ^{14}C pharmacokinetics parameters for the plasma after oral administration of metconazole

	Low-dose (2 mg/kg bw)		High-dose (200 mg/kg bw)	
	Male	Female	Male	Female
Time after dosage (h)	**Concentration of ^{14}C in plasma; mean ± SD of 3 rats (mg equiv./L)**			
0.25	0.251 ± 0.119	0.187 ± 0.095	NA	NA
0.5	0.241 ± 0.063	0.171 ± 0.031	10.90 ± 4.26	12.12 ± 1.48
1	0.215 ± 0.033	0.170 ± 0.024	15.12 ± 3.62	14.32 ± 0.54
2	0.146 ± 0.009	0.144 ± 0.008	15.24 ± 2.52	15.98 ± 0.80
4	0.134 ± 0.016	0.143 ± 0.021	16.71 ± 4.76	16.62 ± 1.60
8	0.123 ± 0.002	0.118 ± 0.032	14.83 ± 2.83	14.18 ± 1.41
24	0.056 ± 0.006	0.072 ± 0.008	9.13 ± 2.98	8.32 ± 2.11
48	0.028 ± 0.012	0.058 ± 0.021	5.05 ± 0.53	6.57 ± 1.16
72	0.013 ± 0.002	0.038 ± 0.005	2.71 ± 1.11	3.61 ± 0.81
96	NA	NA	1.21 ± 1.45	2.22 ± 0.41
120	NA	NA	0.61[§]	1.48 ± 0.17
Pharmacokinetic parameters				
T_{max} (h)	0.25	0.25	4	4
C_{max} (mg equiv./L)	0.251 ± 0.119	0.187 ± 0.095	16.71 ± 4.76	16.62 ± 1.60[*]
β	0.035 ± 0.003	0.021 ± 0.002[*M]	0.029 ± 0.005	0.020 ± 0.002
$β_{½}$ (h)	20.0 ± 1.9	33.6 ± 3.1[*M]	24.6 ± 4.2	34.1 ± 3.9
AUC_{end} (mg equiv. h/L)	4.110 ± 0.452	5.359 ± 1.071	633.9 ± 43.2[*]	714.0 ± 8.9[*]
AUC_{inf} (mg equiv. h/L)	4.501 ± 0.558	7.225 ± 1.436	671.3 ± 70.1[*]	787.2 ± 19.9[*]
$C_{ss\,24h}$ (mg equiv./L)	0.188 ± 0.023	0.301 ± 0.060	27.97 ± 2.92[*]	32.80 ± 0.83[*]
$R_{ac\,24h}$	1.772 ± 0.109	2.560 ± 0.182[*]	2.037 ± 0.245	2.592 ± 0.231
γ	0.997 ± 0.001	0.986 ± 0.006	0.993 ± 0.010	0.993 ± 0.007

NA Not analysed [§] Mean of two rats

[*M] Significantly different from the corresponding male rats ($p < 0.01$)

[*] Significantly different from the corresponding low-dose group ($p < 0.01$)

(*Footnotes continued on following page*)

T_{max} The time at which the C_{max} is observed

C_{max} Maximum (or peak) plasma concentration

β Elimination rate constant of β phase

$\beta_{½}$ Biological half-life time of β phase

AUC_{end} Area under the concentration–time curve (calculated by a trapezoidal rule)

AUC_{inf} Area under the concentration–time curve (calculated by a trapezoidal rule, including extrapolation to infinity)

$C_{ss\,24h}$ Mean concentration of steady-state (interval 24 h)

$C_{ac\,24h}$ Repetitive accumulation ratio (minimum concentration of steady-state ÷ concentration 24 h after a single dose)

γ Correlation coefficient of β phase

Tissue distribution: the radioactivity in organs/tissues and residual carcass were determined:

- at 0.5, 24 and 72 h after single administration of 2 mg/kg bw,
- at 4, 24, 72 and 120 h after single administration of 200 mg/kg bw,
- at 0.5 and 72 h after final dosage after a 14-day repeated dosage of 2 mg/kg bw.

From the distribution study on major organs/tissues, the highest ^{14}C level after oral dosing was in the GIT and contents for all dose levels, frequencies, time intervals and both sexes. The ^{14}C activity in the adrenal gland and liver were constantly greater than in other organs/tissues for all dose levels, frequencies, time intervals, and in both sexes. However, since the concentrations from the 14-day repeated administrations were not so different to the concentrations from a single administration, the accumulation was estimated to be low. High levels of ^{14}C activity were found in adipose tissue as the characteristic phenomenon at 24 h after dosing; these then decreased to the same level as that in the plasma.

In the repeated administration group, radioactive residues recovered in erythrocytes were seven-fold higher than from a single administration, indicating the possibility of accumulation in erythrocytes. However, there was no significant difference in the levels of radioactive residues within erythrocytes and plasma. so it was estimated that the rate of accumulation was low (Yamamoto, 2002).

Table 10. Ratio of tissue:plasma rate in rats after oral administration of 2 mg/kg bw of ^{14}C-labelled KNF-474m **(mean ratio ± SD of three rats)**

	Time after dosage (h)					
	Male			**Female**		
Tissue	**0.5**	**24**	**72**	**0.5**	**24**	**72**
Whole blood	0.8 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Erythrocyte	0.5 ± 0.0	0.5 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Plasma	(1)	(1)	(1)	(1)	(1)	(1)
Adipose tissue	0.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	1.6 ± 0.3	0.3 ± 0.0	0.2 ± 0.0
Adrenal gland	**18.2 ± 2.4**	**39.0 ± 6.4**	**85.4 ± 7.9**	**17.9 ± 3.4**	**18.3 ± 1.0**	**25.3 ± 1.8**
Bone	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Brain	0.8 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	1.8 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Heart muscle	1.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	2.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0
Kidney	3.8 ± 0.6	2.4 ± 0.1	2.0 ± 0.2	4.1 ± 0.3	1.7 ± 0.1	0.8 ± 0.1
Liver	**46.3 ± 5.6**	**8.2 ± 1.0**	**6.3 ± 0.4**	**27.6 ± 1.8**	**36.5 ± 1.9**	**28.7 ± 3.0**
Lung	2.0 ± 0.3	1.9 ± 0.1	1.4 ± 0.2	2.7 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
Pituitary	1.2 ± 0.1	0.7 ± 0.1	< 0.9	1.7 ± 0.0	0.4 ± 0.0	< 0.4
Skeletal muscle	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.8 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Skin and hair	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.9 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
Spleen	0.9 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0

	Time after dosage (h)					
	Male			Female		
Tissue	**0.5**	**24**	**72**	**0.5**	**24**	**72**
Thymus	0.7 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	1.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Thyroid	1.2 ± 0.2	0.6 ± 0.1	< 0.6	1.6 ± 0.0	0.3 ± 0.0	< 0.5
Prostate	1.0 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	NA	NA	NA
Seminal vesicle	0.5 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	NA	NA	NA
Testis	0.5 ± 0.1	1.1 ± 0.1	1.9 ± 0.1	NA	NA	NA
Ovary	NA	NA	NA	1.7 ± 0.1	0.6 ± 0.0	0.3 ± 0.1
Uterus	NA	NA	NA	0.9 ± 0.0	0.5 ± 0.1	0.3 ± 0.2

NA Not applicable

Bold indicates values > 5.0

Table 11. Ratio of tissue:plasma rate in rats after oral administration of 200 mg/kg bw of ^{14}C-labelled KNF-474m; **mean ratio ± SD of 3 rats**

Tissue	**Time after dosage (h)**							
	Male				**Female**			
	4	**24**	**72**	**120**	**4**	**24**	**72**	**120**
Whole blood	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	13 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	1.0 ± 0.0
Erythrocyte	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	1.6 ± 0.2	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.1
Plasma	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Adipose tissue	**19.8 ± 1.0**	**6.8 ± 3.2**	1.0 ± 0.0	2.3 ± 0.7	**18.7 ± 0.4**	**12.1 ± 1.2**	1.1 ± 0.3	0.9 ± 0.4
Adrenal gland	**7.3 ± 0.3**	4.3 ± 0.1	1.8 ± 0.3	3.7 ± 0.7	**7.6 ± 0.2**	4.4 ± 0.7	1.4 ± 0.3	1.3 ± 0.1
Bone	0.9 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	1.1 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Brain	3.9 ± 0.1	1.3 ± 0.4	0.1 ± 0.0	0.1 ± 0.0	4.0 ± 0.1	1.8 ± 0.4	0.1 ± 0.0	0.1 ± 0.0
Heart muscle	3.5 ± 0.1	1.7 ± 0.3	0.4 ± 0.1	0.5 ± 0.0	3.3 ± 0.1	1.7 ± 0.3	0.4 ± 0.0	0.3 ± 0.0
Kidney	4.4 ± 0.0	2.8 ± 0.1	1.7 ± 0.2	2.0 ± 0.1	4.1 ± 0.1	3.0 ± 0.4	1.6 ± 0.1	1.1 ± 0.1
Liver	**8.2 ± 1.5**	**10.8 ± 2.5**	**6.5 ± 1.5**	**5.7 ± 0.5**	**8.9 ± 0.2**	**9.0 ± 1.1**	4.7 ± 0.6	3.0 ± 0.4
Lung	3.8 ± 0.1	2.0 ± 0.2	0.6 ± 0.1	0.7 ± 0.0	3.4 ± 0.1	2.0 ± 0.3	0.6 ± 0.0	0.4 ± 0.0
Pituitary	3.1 ± 0.1	1.4 ± 0.3	0.4	< 2.0	3.2 ± 0.1	1.6 ± 0.4	0.3	< 0.8
Skeletal muscle	1.7 ± 0.1	0.8 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	1.5 ± 0.0	0.8 ± 0.2	0.2 ± 0.0	0.1 ± 0.0
Skin and hair	2.1 ± 0.4	1.0 ± 0.1	0.5 ± 0.2	1.3 ± 0.3	2.5 ± 0.2	1.5 ± 0.3	0.4 ± 0.0	0.5 ± 0.1
Spleen	2.2 ± 0.1	1.1 ± 0.2	0.3 ± 0.0	0.5 ± 0.0	2.1 ± 0.1	1.1 ± 0.2	0.3 ± 0.0	0.3 ± 0.0
Thymus	1.9 ± 0.1	0.9 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	1.8 ± 0.0	1.0 ± 0.2	0.2 ± 0.0	0.1 ± 0.0
Thyroid	3.1 ± 0.1	1.6 ± 0.3	0.6 ± 0.1	1.2	2.8 ± 0.1	1.6 ± 0.3	0.5 ± 0.1	< 3.0
Prostate	4.2 ± 0.1	1.8 ± 0.3	0.7 ± 0.2	0.5 ± 0.2	NA	NA	NA	NA
Seminal vesicle	1.6 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	NA	NA	NA	NA
Testis	2.2 ± 0.1	1.0 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	NA	NA	NA	NA
Ovary	NA	NA	NA	NA	3.5 ± 0.2	1.9 ± 0.4	0.5 ± 0.0	0.4 ± 0.1
Uterus	NA	NA	NA	NA	1.3 ± 0.4	1.1 ± 0.2	0.4 ± 0.0	0.3 ± 0.0

NA Not applicable

Bold indicates values > 5.0

Table 12. Ratio of tissue:plasma rate in rats after 14-day repeated oral administrationof 2 mg/kg of ^{14}C-labelled KNF-474m; **mean ratio ± SD of 3 rats**

Tissue	Time after final dosage (h)			
	Male		Female	
	0.5	72	0.5	72
Whole blood	0.9 ± 0.0	2.3 ± 0.3	0.8 ± 0.0	0.9 ± 0.0
Erythrocyte	0.9 ± 0.1	3.6 ± 0.3	0.5 ± 0.1	0.8 ± 0.1
Plasma	***1.0 ± 0.0***	***1.0 ± 0.0***	***1.0 ± 0.0***	***1.0 ± 0.0***
Adipose tissue	0.5 ± 0.0	< 0.6	0.7 ± 0.1	0.1
Adrenal gland	**17.1 ± 1.6**	**49.9 ± 11.2**	**9.3 ± 0.3**	**9.3 ± 2.6**
Bone	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
Brain	0.5 ± 0.0	0.2 ± 0.0	0.7 ± 0.1	0.1 ± 0.0
Heart muscle	1.0 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	0.2 ± 0.0
Kidney	3.3 ± 0.2	3.1 ± 0.3	2.0 ± 0.2	0.8 ± 0.1
Liver	**22.8 ± 4.4**	**9.1 ± 1.5**	**19.5 ± 4.1**	**14.5 ± 8.6**
Lung	1.9 ± 0.0	1.4 ± 0.2	1.3 ± 0.1	0.4 ± 0.1
Pituitary	0.9 ± 0.1	< 4.4	0.7 ± 0.1	< 1.3
Skeletal muscle	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
Skin and hair	0.7 ± 0.1	0.9 ± 0.3	0.5 ± 0.1	0.2 ± 0.0
Spleen	0.8 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.2 ± 0.1
Thymus	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
Thyroid	1.0 ± 0.1	< 2.7	0.8 ± 0.0	< 1.5
Prostate	0.8 ± 0.0	0.3	NA	NA
Seminal vesicle	0.4 ± 0.0	0.3 ± 0.1	NA	NA
Testis	0.7 ± 0.1	1.6 ± 0.3	NA	NA
Ovary	NA	NA	0.9 ± 0.1	0.3
Uterus	NA	NA	0.6 ± 0.1	0.3 ± 0.0

NA Not applicable

Bold indicates values > 5.0

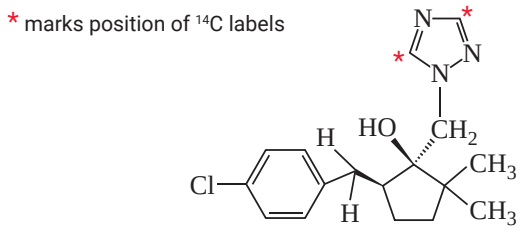
1.2 Biotransformation

Study 1

The metabolism of *cis*-metconazole (WL136184; labelled at the 3 and 5 positions of the triazole ring; lot/batch S1084/1; see Fig. 3) in six male Fischer 344 rats was investigated at a single oral high dose of 200 mg/kg bw. Both urine and faeces were collected at 24 h intervals up to day 7 after dosing. Cage washes were collected at termination. Carcasses were sampled and stored. Seven days after dosing, the rats were sacrificed, and tissues collected for analysis. Animals were sacrificed by carbon dioxide asphyxiation, but no necropsy was performed.

Metabolic profiles in urine and faeces were investigated. Urine samples were analysed directly, while faeces were extracted with acetonitrile. Metabolites were identified by co-chromatography against reference standards using high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS) or thin layer chromatorgraph (TLC) and/or nuclear magnetic resonance (NMR) analysis of the isolated fractions.

Figure 3. cis-Metconazole radiolabelled at the 3 and 5 carbons of the triazole ring

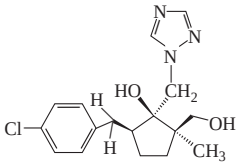
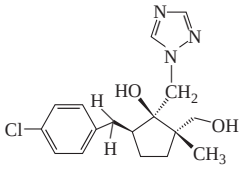
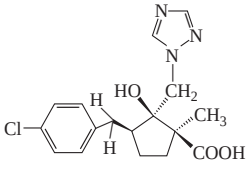
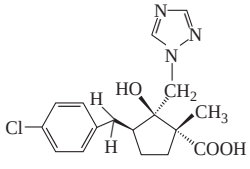
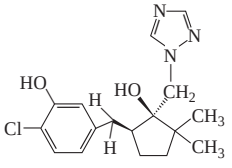


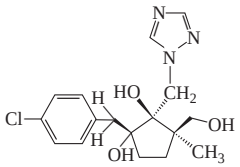
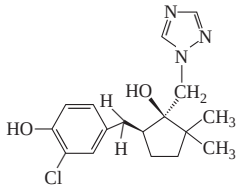
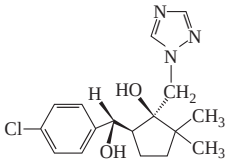
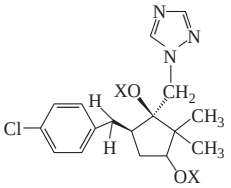
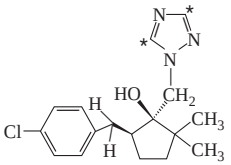
Little or no parent compound was retrieved in the excreta. It was proposed that the main rat metabolites of metconazole were:

- monohydroxy metabolites (such as M1 and M21), resulting from oxidation of the benzylic methylene groups, or the methyl, methylene or methine groups of the cyclopentane ring,
- hydroxyphenyl metabolites (M15 and M19),
- carboxy metabolites (M12 and M13),
- polyhydroxy metabolites (M18),
- mixed function metabolites,
- various sulfate conjugates of the above mentioned metabolites (M22).

In addition, the urinary metabolite 1,2,4-triazole (present at 5%), resulting from *N*-dealkylation, was also observed. Of note also was the presence of a wheat metabolite triazolylacetic acid, in trace amounts (0.03%), while another metabolite, triazolylalanine, was not detected in the rat.

Table 13. Summary of metabolites in excreta (expressed as percentage of total applied dose) following administration of a single oral high dose (200 mg/kg) of [triazole-^{14}C]-metconazole to male rats

Metabolite codes	Chemical structure	Faeces	Urine
M1 CL 359451		14	ND
M2 CL 359452		5	ND
M12 CL 359138		10	5
M13 CL 359139		4	1
M15 CL 359453		1	ND

Metabolite codes	Chemical structure	Faeces	Urine
M18		1	ND
M19 CL 395838		6	ND
M20		ND	5.0
M21 CL 197130		2	ND
M22	 Where X = H or SO_3^-	< 1	ND
Metconazole (*cis*) CL 354801		ND	ND
Others		< 1% each	< 1% each

ND Not detected

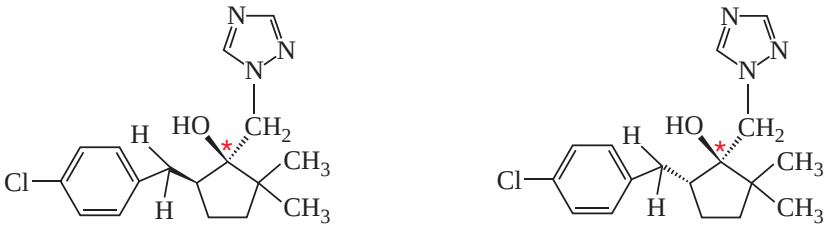
Numerous metabolites were found in faeces. The predominant metconazole-derived residues were identified as monohydroxylated metabolites (M1, M2, M15, M19) and carboxy metabolites (M12, M13). Other minor metabolites included dihydroxy-, hydroxy carboxy metabolites and conjugates (including sulfate conjugates).

In urine, the predominant metabolites were carboxy metabolites (M12, M13) and 1,2,4-triazole derivatives (M 20). Minor metabolites included carboxy , dihydroxy carboxy , polyhydroxycarboxylic metabolites (Richardson, 1991b).

Study 2

The metabolic profiles in excreta (urine and faeces) and liver were investigated. Five rats/sex per dose (Fischer 344) received [cyclopentyl-^{14}C]-metconazole WL148271 (*cis*:*trans* ratio 79:21; lot/batch S1106/1; see Fig. 4). Both urine and faeces were collected at 24 h intervals up to day 3 after dosing. Cage washes were collected at termination. Animals were sacrificed by exsanguination, and various tissues retained in order to assess radioactive residue. Urine samples were analysed directly, while faeces and liver samples were extracted with acetonitrile/water or acetonitrile before HPLC and/or TLC analyses. Relevant metabolites were identified by co-chromatography with reference standards and/or MS analysis of isolated fractions.

Figure 4. Structure of cyclopentyl-labelled metconazole; cis and trans isomers



The predominant metabolites found in faeces and urine were monohydroxylated metabolites (M1 and M19) and carboxy metabolites (M12). Levels of M12 were higher in female rat urine (8%) than in the male counterpart (1%). Other minor metabolites included monohydroxylated metabolites (M2, M15 and M21), carboxy metabolite (M13), other hydroxylated/carboxylated metabolites and sulfate conjugates.

Urine samples showed basically the same chromatographic profile in males as females, except that females excreted more M12. No explanation for the sex difference leading to the presence of this metabolite was given, but since it was a major metabolite, the finding was in line with the observed sex difference in urinary excretion in the mass balance study.

Table 14. Summary of metabolites in excreta (expressed as % of total applied dose) following administration of a single oral low dose (2 mg/kg) of [cyclopentyl-^{14}C]-metconazole to male rats

Metabolite code	Faeces	Urine
M1 CL 359451	12 (male) 13 (female)	ND
M2 CL 359452	4 (male) 2 (female)	ND
M12 CL 359138	10 (male) 14 (female)	1 (male) 8 (female)
M13 CL 359139	3 (male)	ND
M15 CL 359453	1 (male) 1 (female)	ND
M19 CL 395838	3 (male) 9 (female)	ND
M21 CL 197130	1 (male) < 1 (female)	ND
Metconazole, *cis*-isomer CL 354801	ND	ND

ND Not detected

In the liver, radioactivity was polar and consisted of one major peak; it was putatively associated with a conjugated metabolite. The compound could not be identified by co-chromatography with reference standards, however its retention time during reverse-phase HPLC was similar to that of M1. (Richardson, 1991a)

Five rats/sex per dose (Fischer 344) received [cyclopentyl-^{14}C]-metconazole WL148271 (*cis*:*trans* ratio 79:21; lot/batch:S1164/1) at a dose level of 164 mg/kg bw. The test material was administered as a suspension in dimethyl sulphoxide (DMSO). The dosing volume was 2 mL/kg bw. Two rats per sex was dosed with DMSO only, as a control.

Both urine and faeces was collected at 24 h intervals up to day 5 after dosing. Cage washes were collected at termination. Animals were sacrificed by exsanguination and whole blood, liver, spleen, kidneys, perirenal fat, adrenals, GIT, gonads, uterus, lung, heart, femoral muscle, femur bone, skin and brain were sampled and stored. The metabolic profiles in excreta (urine and faeces) and tissues (liver and adrenal) were investigated. Urine samples were analysed directly, while faeces, liver and adrenal samples were extracted with acetonitrile/water or acetonitrile before HPLC and/or TLC analysis. Metabolites were identified by co-chromatography with reference standards and/or MS analysis of isolated fractions.

Table 15. Summary of metabolites in excreta, expressed as percentage of total applied dose, following a single high dose (164 mg/kg) administration of [cyclopentyl-^{14}C]-metconazole to rats

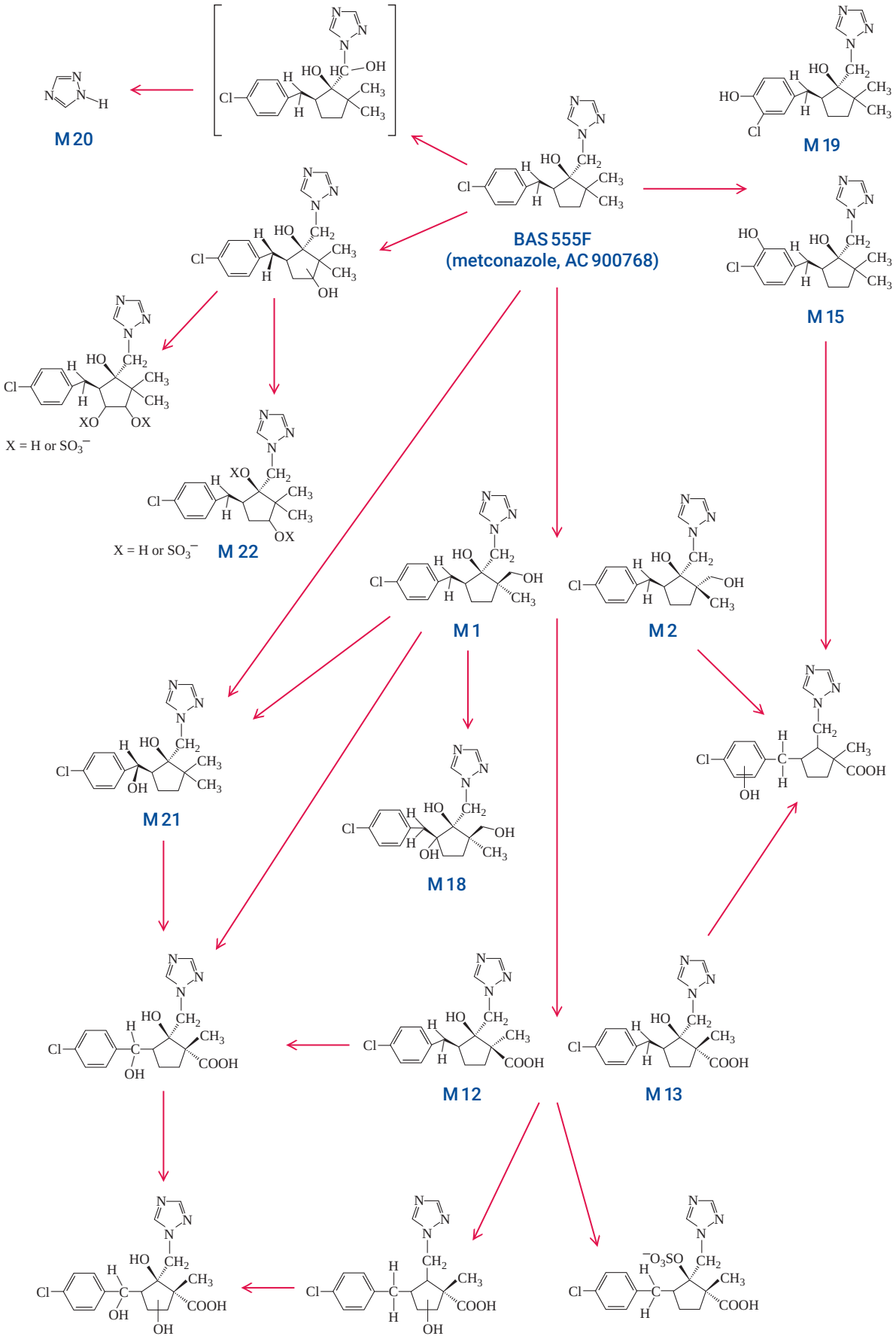
Metabolite code	Faeces	Urine
M1 CL 359451	21 (male) 15 (female)	(male)§ (female)§
M2 CL 359452	3 (male) 3 (female)	(male)§ (female)§
M12 CL 359138	11 (male) 6 (female)	2 (male) 7 (female)
M13 CL 359139	1 (male) ND (female)	ND
M15 CL 359453	3 (male) 2 (female)	(male)§ (female)§
M18	ND (male) 1 (female)	ND
M19 CL 395838	8 (male) 8 (female)	(male)§ (female)§
M21 CL 197130	6 (male) 1 (female)	ND
Metconazole BAS 555 F; AC 900768	2 (male) 2 (female)	ND
Mixture of metabolites	6 (male) 13 (female)	2 (male) 6 (female)

ND Not detected.

§ The conjugated metabolite was tentatively quantified as 5% (male) and 14% (female) of total dose

The rat metabolites, isolated in faeces and urine were very similar to those which were detected in both the low-dose and the high-dose administration and provide additional evidence for a similar pathway as has been described before. The major metabolites were again identified as hydroxy and carboxy derivatives. A higher proportion of urine metabolite M12 in the females was again observed. The major component, present in the adrenals on day 5 remained unidentified, although one peak was tentatively characterized as metabolite M1, and parent compound was observed at a low level. (Morrison & Richardson, 1992b).

Figure 5. Proposed metabolic pathway of metconazole in rats.



1.3 Comparative in-vitro metabolism studies

The objective of this study was the qualitative comparison of the metabolite patterns of ^{14}C-metconazole (BAS 555 F; Reg. No. 4056343) formed after incubation with human, rat and rabbit liver microsomes. A ^{14}C-labelled test item [3,(5)-triazole-^{14}C]-BAS 555 F; lot/batch 811-1201) was used for the investigation. For the comparison with human liver microsomes, the species rat and rabbit were chosen because they were used in toxicological studies with BAS 555 F. To address this question, the radiolabelled test item was incubated with liver microsomes from human, rat and rabbit (all mixed genders) at a final concentration of 10 µM. After incubation for 0, 10, 30, 60 or 180 minutes the reaction was terminated by addition of ice-cold acetonitrile containing 0.05% trifluoroacetic acid, and after centrifugation the resulting supernatant was analysed by HPLC. Selected samples were additionally investigated by HPLC-MS. All supernatants contained ≥ 90% of the applied radioactivity (AR), therefore pellet extraction was not required. Negative and positive controls were run in parallel to demonstrate the absence of nonmetabolic degradation and the metabolic activity of the microsomes (Phase I reactions), respectively. The control experiments yielded the expected results.

The test item was stable over the test period. Stability controls without microsomes and heat-inactivated microsomes showed nearly identical HPLC profiles that contained only peaks corresponding to the unchanged active substance, BAS 555 F.

The radio-HPLC analyses of human, rat and rabbit microsomal samples were compared in order to determine whether or not a unique human metabolite occurred. Selected human, rat and rabbit supernatant samples were also analysed by HPLC-MS to assign m/z values to prominent peaks which represented more than 5% AR in the human sample.

The triplicates of each negative control showed nearly identical HPLC profiles that contained only peaks corresponding to the unchanged active substance BAS 555 F. No significant metabolism or degradation of BAS 555 F occurred without the influence of liver microsomes.

The positive controls with testosterone showed that the metabolic activity of human liver microsomes with respect to 6β-hydroxylation reaction was sufficiently high. Testosterone 6β-hydroxylation rates observed in human liver microsomes were higher than the rate reported by the supplier for the corresponding lot of microsomes. The positive controls with 7-ethoxycoumarin showed that the metabolic activity of rat and rabbit liver microsomes with respect to 7-ethoxycoumarin *O*-dealkylation reaction was sufficiently high. Rates of 7-ethoxycoumarin *O*-dealkylation observed in rat and rabbit liver microsomes were higher than the mean of the rates given by the supplier for each lot of male and female liver microsomes.

Human microsomes

Radio-HPLC and HPLC-MS analysis of human microsome samples incubated with BAS 555 F allowed the assignment of six relevant peaks each accounting for more than 5% AR. Two of these peaks (P18 and P19) corresponded to the active substance BAS 555 F (*cis* and *trans* isomers). The peak at 22.7 minutes (P12; 6.1% AR) was detected after 60 minutes incubation. After 180 minutes incubation, the mean % AR for P12 increased to 11.3%. Peaks eluting at 19.8 minutes (P9), 21.8 minutes (P11) and 23.4 minutes (P13) with 8.0%, 7.0% and 6.2% AR, respectively, were detected in samples after 180 minutes incubation.

Rat microsomes

In rat microsome samples the relevant peaks, P9, P11, P12 and P13, from the incubation with human liver microsomes were detected in addition to the active substance BAS 555 F. Metabolite P12 accounted for 6.0% AR after 60 minutes incubation, and increased to 10.3% after 180 minutes. Metabolites P9, P11 and P13 accounted for 8.0%, 8.3% and 12.5% AR respectively after 180 minutes incubation. Amounts of P9, P11 and P12 were comparable in rat and human microsome samples. The amount of P13 in rat microsomes was twice as high as taht observed in human microsome samples after 180 minutes incubation.

Rabbit microsomes

In rabbit microsome samples the relevant peaks (P9, P11, P12 and P13) from the incubation with human liver microsomes were also detected in addition to the active substance BAS 555 F. Metabolite P12 accounted for 10.1% AR after 60 minutes incubation but did not increase significantly with incubation time, reaching 10.3% after 180 minutes incubation. Metabolites P9, P11 and P13 accounted for 17.1%, 15.1% and 13.6% AR respectively after 180 minutes incubation. Amounts of P12 observed in rabbit microsome samples after 180 minutes incubation were in the same range as for human microsomes. However, P9, P11 and P13 amounts observed in rabbit were more than twice the values observed in human microsome samples after 180 minutes incubation.

Table 16. Comparison of the relevant metabolites of BAS 555 F after incubation with human, rat and rabbit microsomes

Incubation time (minutes)	Species	Range of recovery in sample supernatants (% AR)	Relevant peak (% AR)					
			Peak 9	Peak 11	Peak 12	Peak 13	Peak 18	Peak 19
0	Human	99.3–105.1	ND	ND	ND	ND	16.7	82.4
	Rat	100.1–104.4	ND	0.1	ND	ND	16.9	81.9
	Rabbit	101.9–103.0	ND	ND	ND	ND	17.3	81.5
10	Human	100.2–103.5	0.7	0.9	1.4	0.6	16.6	78.1
	Rat	100.2–103.7	0.1	ND	1.3	4.3	16	69.9
	Rabbit	102.7–103.7	4.9	3.7	4.3	3	14.1	65.6
30	Human	101.5–104.7	2	2.4	3.6	1.4	16.1	72.3
	Rat	102.5–103.1	1.5	2.6	3.3	7	15.2	55
	Rabbit	103.4–104.1	9	7	7.9	5.6	11.4	50.8
60	Human	102.0–103.7	3.5	4.2	6.1	2.7	15.4	64.6
	Rat	102.7–105.7	3.2	6.7	6	8.5	13.3	40.2
	Rabbit	103.5–104.7	12.1	9.9	10.1	9	8.9	36.9
180	Human	99.1–105.7	8	7	11.3	6.2	13.1	45.2
	Rat	102.8–104.4	8	8.3	10.3	12.5	9	15.4
	Rabbit	101.5–104.3	17.1	15.1	10.3	13.6	4.3	15.6

% AR Percentage of applied radioactivity

ND Not detected

After the incubation with human liver microsomes, six ^{14}C peaks were detected that each represented more than 5% applied radioactivity in a human sample (supernatant) on at least one time point. Two of these signals represented the unchanged active substance BAS 555 F (m/z value 320.15), one corresponding to the *cis* isomer (peak at 28.9 minutes) and the other to the *trans* isomer (peak at 28.2 minutes). The other peaks (P9, P11, P12 and P13) corresponded to metabolites of BAS 555 F (peaks at 19.8, 21.8, 22.7 and 23.4 minutes) with an m/z value of 336.15.

Those metabolites and the two isomers of the active substance were also detected in rat and rabbit microsome samples. The comparison was based on retention time and m/z values.

It can be concluded, that no human-specific metabolites were found and that metabolic degradation in the tested species was similar (Thibaut, 2016).

2 Toxicological studies

2.1 Acute toxicity

Metconazole (BAS 555 F) has been tested in various species and via different routes of administration. Studies were carried out with WL 136184 (*cis:trans* 95:0.1 isomer mixture, effectively *cis* isomer) and/or BAS 555 F (WL 148271; *cis:trans* 80:15 isomer mixture, technical grade). Brief details of the respective studies, summarized in Table 17, are presented under the relevant subsections.

Table 17. Summary of acute toxicity studies with metconazole

Route Species Sex	Purity (%), *cis*/*trans* ratio, Batch number	Dose range (mg/kg bw) and vehicle	Result	Reference
		Metconazole *cis*/*trans* mixture		
Oral Rat, Fischer 344, M/F	95.3%, *cis*:*trans* 79.8:15.5 Batch: 89-01	255, 357, 500, 700, 980 in corn oil	LD_{50} (m) = 727 mg/kg bw LD_{50} (f) = 595 mg/kg bw LD_{50} (f+m) = 660 mg/kg bw	Gardner, 1990a
Oral Rat, Wistar F	98%, *cis*:*trans* 83.7:14.4 Batch: 42704	500, 2000 in olive oil	500 mg/kg bw < LD_{50} < 2000 mg/kg bw	Gamer & Leibold, 2005a
Oral Mouse, CD-1, M/F	95.3%, *cis*:*trans* 79.8:15.5 Batch 89-01	391, 625, 1000, 1600 in corn oil	LD_{50} (m) = 718 mg/kg bw LD_{50} (f) = 410 mg/kg bw LD_{50} (m+f) = 566 mg/kg bw	Gardner, 1990a
Dermal Rat, Fischer 344, M/F	95.3%, *cis*:*trans* 79.8:15.5 Batch: 89-01	2000 moistened with water	LD_{50} (m, f) > 2000 mg/kg bw	Gardner, 1990a
Dermal Rat, Wistar M/F	98%, *cis*:*trans* 83.7:14.4 Batch: 42704	2000 in CMC (0.5%)	LD_{50} > 2000 mg/kg bw	Gamer & Leibold, 2005b
Dermal Rabbit, NZW M/F	95.3%, *cis*:*trans* 79.8:15.5 Batch: 89-01	2000 moistened with water	LD_{50} (m, f) > 2000 mg/kg bw	Gardner, 1990a
Inhalation (head-only) Rat, Sprague Dawley M/F	95.3%, *cis*:*trans* 79.8:15.5 Batch: 89-01	5.588 mg/L air (4 h)	LC_{50} (m, f) > 5.588 mg/L air	Collins, 1990
Inhalation (head/nose-only) Rat, Wistar M/F	98%, *cis*:*trans* 83.7:14.4 Batch: 42704	5.2 mg/L air	LC_{50} > 5.2 mg/L air	Gamer & Leibold, 2005c

Route Species Sex	Purity (%), *cis*/*trans* ratio, Batch number	Dose range (mg/kg bw) and vehicle	Result	Reference
		Metconazole *cis* isomer		
Oral Rat, Fischer 344 M/F	95.29%, *cis*:*trans* 95.2:0.1 Batch: 12	850, 1190, 1666, 2332 in corn oil	LD_{50} (M) = 1627 mg/kg bw LD_{50} (F) = 1312 mg/kg bw LD_{50} (M/F) = 1459 mg/kg bw	Gardner, 1991
Dermal Rat, Fischer 344 M/F	95.29%, *cis*:*trans* 95.2:0.1 Batch: 12	2000 moistened with water	LD_{50} (M/F) > 2000 mg/kg bw	Gardner, 1991

LD_{50} Median lethal dose LC_{50} Median lethal concentration

Metconazole *cis*/*trans* is of moderate toxicity by the oral route of application as evident from its LD_{50} in rats of > 500 mg/kg bw but < 2000 mg/kg bw. The metconazole *cis* isomer (LD_{50} = 1312 mg/kg bw in rats) is also of moderate acute toxicity, however with a lower toxicity potential than the isomer-mix. Furthermore, female rats were slightly more susceptible than the males. Both compounds induced rather consistent clinical signs, which occurred rapidly within four hours of dosing. Non-specific signs were observed, such as unkempt appearance, hunched posture and diarrhoea, as well as abasia, ataxia, chromodacryorrhea, salivation and lacrimation. The animals recovered at the end of the observation period. Metconazole *cis*/*trans* is of low dermal and inhalation toxicity and is neither a skin nor eye irritant in rabbits. Slight eye irritation in the rabbit resolved within eight days of application. Furthermore, metconazole showed no skin sensitizing properties in Guinea pigs by the maximization and Buehler tests.

(a) Lethal dose

Oral

Mouse

Groups of five male and five female Albino CD-1 mice received metconazole (WL148271; purity 95.3%; *cis*/*trans* 79.8:15.5; batch 89-01) by gavage at dose levels of 391, 625, 1000 and 1600 mg/kg bw. The test material was administered as a suspension in corn oil. In a preliminary range-finding study, one mouse per sex had been dosed at 2000 and 4000 mg/kg bw. Common signs of reaction to treatment were abasia/ataxia, hunched posture, pallor of the skin and eyes and stereotype behaviour (circling movements). Prostration, coma, hypothermia or cyanosis were observed among mice at all dose levels, usually as a prelude to death. Mortality was observed between days 2 and 5 after treatment as follows: at dose level 391 mg/kg bw, one female; at 625 mg/kg bw, 2/5 males and 5/5 females; at 1000 mg/kg bw, 4/5 males and 5/5 females; at 1600 mg/kg bw, 5/5 males and 5/5 females. Among decedents, darkening or exaggerated lobular pattern of the liver, discolouration of the renal medulla, lung congestion and inflammation and/or abnormal contents of the stomach were observed. Among surviving mice sacrificed on day 14, enlarged or pale livers, distension of the caecum and, in a single animal, hyperaemia of the urinary bladder were observed. The acute oral median lethal dose (LD_{50}) was calculated to be 718 mg/kg bw and 410 mg/kg bw in males and female, respectively. The combined LD_{50} was 566 mg/kg bw for both sexes (Gardner, 1990a).

Rat

The test substance, metconazole (WL148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01) was evaluated for its acute oral toxicity potential in five male and five female Fischer 344 rats when administered as a gavage dose at levels of 255, 357, 500, 700, or 980 mg/kg bw. Mortality was observed between 2 and 7 days following treatment at dose levels of 500 mg/kg bw (1/5 males and 3/5 females), 700 mg/kg bw (1/5 males and 2/5 females), and 980 mg/kg bw (5/5 males and 5/5 females). Clinical

signs were first observed in all groups between 30 minutes and 2 hours following treatment and included unkempt appearance, lacrimation, diarrhoea and hunched posture. Additionally, ataxia, abasia, salivation, and chromodacryorrhea were observed at dose levels of 357 mg/kg bw and higher. Recovery of surviving rats was complete by 11 days after dosing. Body weight gains from days 1 to 7 were lower in the surviving rats that had received 500 and 700 mg/kg bw, compared to the animals that had received 255 mg/kg bw. However, all survivors gained weight during the 14 day observation period. Necropsy findings in decedents showed abnormal livers, discolouration of the renal medulla, lung congestion, stomach inflammation, and abnormal contents of liquid/gas in the stomach and intestines. At terminal necropsy macroscopic abnormalities in surviving animals considered to be possible effects of treatment included discolouration of the liver, enlargement of the liver and distention of the caecum and/or colon. The acute oral LD_{50} was calculated to be 727 mg/kg bw and 595 mg/kg bw in males and female respectively. The combined LD_{50} was 660 mg/kg bw for both sexes (Gardner, 1990a).

The acute oral toxicity potential of the test substance metconazole *cis* isomer (WL136184; purity 95.29%; *cis*:*trans* 95.2:0.09; batch 12) was investigated in five male and five female Fischer 344 rats when administered by gavage at dose levels of 850, 1190, 1666 and 2332 mg/kg bw. Mortality occurred in 1 male and 3 females of the 1190 mg/kg bw dose group on day 4. Three males (days 3 and 6) and three females (days 2 and 3) died from the 1666 mg/kg bw dose group, and four males (days 3, 5, 6) and five females (days 3, 4, 5) died in the 2332 mg/kg bw dose group. Although, body weight gain was reduced during the first week of the observation period at 1190 mg/kg bw and above, animals gained weight by the end of the 14 day observation period. Clinical symptoms recorded were unkempt appearance, staining (yellow) of the anogenital fur and a hunched posture, in addition to lethargy, salivation, lachrymation, periorbital encrustation and diarrhoea. Among rats dosed with 1190 mg/kg bw and above, the principal clinical signs were piloerection, abasia/ataxia or prostration. Tachypnoea and coma were apparent among rats that failed to survive treatment at the high-dose level. Recovery of rats surviving treatment, as judged by external appearance and behaviour, was generally advanced by day 7 but was incomplete until day 15. The principal necropsy findings among decedents were exaggerated hepatic lobular pattern, areas of pallor/darkening of the liver, darkening of the thymus, spleen and kidneys, abnormal fluid contents of the GIT and in four rats, inflammation of the stomach. Necropsy findings among the rats sacrificed on day 15 were limited to the presence of white/yellow areas on the liver of a single male rat treated at the high-dose level. The oral LD_{50} for male and female rats was 1627 mg/kg bw and 1312 mg/kg bw, respectively. The combined LD_{50} was 1459 mg/kg bw for both sexes (Gardner, 1991).

Single doses of 500 and 2000 mg/kg bw of metconazole preparations in olive oil (BAS 555 F; purity 98.0%; *cis*:*trans* 83.7:14.4; batch 42704) were administered to three treatment groups, each of three fasted female Wistar rats (2000 mg/kg bw to three females; 500 mg/kg bw to six females) by gavage in a sequential manner. Animals were observed for 14 days. Two animals of the high-dose group died on days 3 and 4. No mortality occurred in the 500 mg/kg bw groups. Clinical signs in the high-dose group included impaired and poor general state, dyspnea, abdominal position, staggering, piloerection, smeared fur, lacrimation and red clammy snout and eyelid. No clinical signs or findings were apparent in animals treated with 500 mg/kg bw. The mean body weights of the treatment groups increased throughout the study period. No macroscopic pathologic abnormalities were noted in the animals that died or that were examined at the end of the observation period. Under the conditions of this study the LD_{50} of metconazole (BAS 555 F) after oral administration was found to be greater than 500 mg/kg bw and less than 2000 mg/kg bw in rats (Gamer & Leibold, 2005a).

Dermal administration

In a dermal LD_{50} study, groups of 5 male and 5 female Fischer 344 rats received a single dermal application of metconazole (WL148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01) at a dose level of 2000 mg/kg bw to the clipped dorsal skin as a dry powder moistened with deionized water for 24 h under semi-occlusive conditions. Thereafter, test material was removed with warm dilute detergent solution and animals were observed for a period of 14 days. No mortalities or clinical signs of toxicity were observed during the study period. No dermal irritation was observed in any animal at the application sites. Body weight was not affected by the treatment. No relevant findings were recorded at necropsy. Under the conditions of this study the dermal LD_{50} for male and female rats was > 2000 mg/kg bw (Gardner, 1990a).

In a dermal LD_{50} study, groups of five male and five female Fischer 344 rats received a single dermal application of *cis*-metconazole, (WL136184; purity 95.29%; *cis*:*trans* 95.2:0.09; batch 12) at a dose level of 2000 mg/kg bw, to the clipped dorsal skin as a dry powder moistened with deionized water for 24 h under semi-occlusive conditions. Thereafter test material was removed with warm dilute detergent solution and animals were observed for a period of 14 days. No mortalities or clinical signs of toxicity were observed during the study period. Erythema was observed in 1/5 males and 2/5 females on day 2 but had recovered by day 3. Body weight was not affected by the treatment. No relevant findings were recorded at necropsy. Under the conditions of this study, the dermal LD_{50} for male and female rats was > 2000 mg/kg bw (Gardner, 1991).

Groups of five male and five female New Zealand White rabbits received a single dermal application of metconazole (WL148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01) at a dose level of 2000 mg/kg bw to the clipped dorsal skin as a dry powder moistened with deionized water for 24 h under semi-occlusive conditions. Thereafter, test material was removed with warm dilute detergent solution, and animals were observed for a period of 14 days. No mortalities or clinical signs of toxicity were observed during the study period. Sites of application showed no inflammatory reactions, but desquamation affected twp male rabbits during week 2 of the study. Body weight was not affected by the treatment. No relevant findings were recorded at necropsy. Under the conditions of this study, the dermal LD_{50} for male and female rabbits was > 2000 mg/kg bw (Gardner, 1990a).

In an acute dermal toxicity study groups of five male and five female Wistar rats were exposed to 2000 mg/kg bw of metconazole (BAS 555 F; purity 98.0%, *cis*:*trans* 83.7:14.4; batch 42704) preparation in 0.5% carboxymethyl cellulose (CMC) solution in doubly distilled water. The preparation was applied to the clipped skin under semi-occlusive conditions for 24 h. The animals were observed for 14 days after administration. No systemic clinical observations or skin effects were noted in the animals. The mean body weights of the animals increased throughout the study period. No macroscopic pathologic abnormalities were noted in the animals at the end of the study. No mortality occurred in any dose group. Accordingly, the dermal LD_{50} was greater than 2000 mg/kg bw (Gamer & Leibold, 2005b).

Inhalation

Groups of 5 male and 5 female Sprague Dawley rats (Crl:CD (SD) BR) were exposed to dust of metconazole (WL148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01) in a head-only inhalation system, at a nominal concentration of 18.702 mg/L equivalent to the analytical concentration of 5.588 mg/L (by gravimetry) for four hours. A concurrent negative control group of five animals per sex was treated with filtered air. The mass median aerodynamic diameter (MMAD) of the particles in the atmosphere was 3.73 µm ± 1.40 µm. The relative humidity of the chamber was between 24 and 58%. No mortalities occurred during the study period. Clinical signs of toxicity consisted of piloerection and hunched posture on the day of exposure. Other clinical signs during days 0–12 included transient lethargy, sores on both front paws and unkempt fur. Treated animals showed a small reduction in body weight gain, which persisted in week 2 of the study in males. Necropsy findings revealed decreased lung weight of uncertain toxicological importance in the treated males but not the females. No treatment-related macroscopic abnormalities were observed. Under the conditions of this study the inhalation LC_{50} for male and female rats was > 5.588 mg/L air (Collins, 1990).

In an acute inhalation toxicity study, groups of five male and five female Wistar rats were exposed to dust of *cis*/*trans*-metconazole (BAS 555 F; purity 98.0%; *cis*:*trans* 83.7:14.4; batch 42704) at a concentration of 5.2 mg/L for four hours. The animals were observed for 14 days after exposure. No mortality occurred at the limit concentration of 5.2 mg/L. Accordingly, the acute inhalation LC_{50} for *cis*/*trans*-metconazole for male and female rats after dust inhalation exposure was > 5.2 mg/L. Clinical signs of toxicity in animals exposed to 5.2 mg/L comprised visually accelerated respiration, respiratory sounds, crust formation at the nose, apathy, eyelid closure, squatting posture, piloerection, smeared fur, exsiccosis and reduced general state. Findings were observed from hour 0 of exposure until study day 14. The mean body weights of the male animals decreased during the first postexposure observation week but increased during the second week. The mean body weights of the female animals did not

increase adequately during the first postexposure observation week, but increased during the second week No gross pathological abnormalities were noted in the animals necropsied at termination of the postexposure observation period, except in one male animal where diffuse red discolouration of all lung lobes and oedema were observed. Cascade impactor measurements resulted in particle size distributions with a MMAD of 4.0–4.7 μm and a geometric standard deviation of 3.0 – 3.1 μm. Under the conditions of this study, the four-hour inhalation LC_{50} for male and female rats was estimated to be > 5.2 mg/L of *cis*/*trans*-metconazole (Gamer & Leibold, 2005c).

(b) Dermal and ocular irritation/sensitization

Table 18 summarizes studies carried out on dermal and ocular irration and sensitization by metconazole.

Table 18. Summary of skin irritation, eye irritation and skin sensitization with metconazole

Route **Species** **Sex**	**Purity (%),** ***cis*/*trans* ratio,** **batch number**	**Dose range and vehicle**	**Result**	**Reference**
		Metconazole *cis*/*trans* mixture		
Skin irritation Rabbit, NZW M/F	95.3%, *cis*:*trans* 79.8:15.5, batch 89-01	0.5 g moistened with water	Not irritating	Gardner 1990b
Skin irritation Rabbit, NZW M/F	98%, *cis*:*trans* 83.7:14.4, batch 42704	0.5 g	Not irritating	Remmele & Leibold, 2005a
Eye irritation Rabbit, NZW M/F	95.3%, *cis*:*trans* 79.8:15.5, batch 89-01	65 mg (0.1 mL bulk volume)	Not irritating	Gardner, 1990b
Eye irritation Rabbit, NZW M/F	98%, *cis*:*trans* 83.7:14.4, batch 42704	32 mg (0.1 mL bulk volume)	Not irritating	Remmele & Leibold, 2005b
Skin sensitization (mod. Buehler test) Guinea pig, Dunkin-Hartley M/F	95.3%, *cis*:*trans* 79.8:15.5, batch 89-01	Induction/challenge: 60% as Vaseline paste	Not sensitizing	Gardner, 1990b
***Skin sensitization*,** (Maximization test) Guinea pig, albino Hartley, M	97.4%, *cis*:*trans* 83.7:13.7, batch AC 9339-114	Intradermal (5% and 10%; 0.1 mL): in mixture Freund's adjuvant /mineral oil Epidermal/challenge: 25% in petrolatum	Not sensitizing	Glaza, 1995
Skin sensitization (Buehler test) Guinea pig, Dunkin-Hartley, F	98.6%, *cis*:*trans* 83.5:15.1, batch 39513	Induction/challenge: 0.5 mL, 50% in 1% CMC	Not sensitizing	Gamer & Leibold, 2005d

Route Species Sex	Purity (%), *cis*/*trans* ratio, batch number	Dose range and vehicle	Result	Reference
Skin sensitization (Buehler test) Guinea pig, Dunkin-Hartley, F	99.1%, *cis*:*trans* 85:14.1, batch 43707	Induction/challenge: 0.5 mL, 50% in 1% CMC	Not sensitizing	Gamer & Leibold, 2005e
		Metconazole *cis* isomer		
Skin irritation Rabbit, NZW, M/F	95.29%, *cis*:*trans* 95.2:0.1, batch 12	0.5 g moistened with water	Not irritating	Gardner, 1991
Eye irritation Rabbit, NZW, M/F	95.29%, *cis*:*trans* 95.2:0.1, batch 12	42 mg (0.1 mL bulk volume)	Not irritating	Gardner, 1991
Skin sensitization (Buehler test) Guinea pig, Dunkin-Hartley, M/F	95.29%, *cis*:*trans* 95.2:0.1, batch 12	Induction/challenge: 50% as vaseline paste	Not sensitizing	Gardner, 1991

2.2 Short-term studies of toxicity

(a) Oral administration

The short-term toxicity of metconazole for both the isomer mix and the *cis* isomer was evaluated in subacute (rat, dog) and subchronic studies (mouse, rat, dog).

Mouse

Groups of 12 male and 12 female Crl:CD1 (ICR)BR mice were fed with diets for 90 days containing 30, 300, and 3000 (days 1–7) reducing to 2000 (days 7–90) ppm of metconazole (WL 148271; purity 95.3%; *cis*:*trans* ratio 83.7:16.3; batch 89-01). The dietary concentration of 3000 ppm was decreased to 2000 ppm for weeks 2–13 due to decreased food consumption and an actual loss of body weight (16% loss for males, 13% for females) in this group during week 1. Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. Achieved doses for males were 4.6, 50.5, and 341.1 mg/kg bw per day and for females 6.4, 60.7, and 438 mg/kg bw per day. The correctness of the concentrations was demonstrated.

One female dosed at 3000 ppm was in a poor condition at the end of week 1 but was replaced with an animal of the same weight from week 2 (to be dosed 2000 ppm). In the 30 ppm group, one male was humanely killed (week 11) because of sore, swollen shoulders/hind paws, hunched posture and thin appearance, and one male was found dead as a result of urinary tract lesions. Other fatalities included three males and one female from the control group and two males at 30 ppm, which died subsequent to blood sampling problems at termination and thus these mortalities were not considered treatment-related. No information on clinical signs, urinalysis or ophthalmology was provided.

Feed consumption was impaired in all animals treated at 2000 ppm and statistically significantly decreased between week 1 and week 6 for males (11%), and between weeks 1 and 13 for females (14%). As a consequence, body weight was decreased at this dose level during the whole treatment period, leading to significantly decreased mean terminal body weights for males (16%) and females (14%) and a significant drop in body weight gain for males (61%) and females (50%).

The statistically significant decreased values of hematocrit (Ht), mean cell volume (MCV) and cell hemoglobin (Hb) concentration at the top dose were indicative of a slight microcytic anemia. In addition, increased leukocyte counts, with increased neutrophil but decreased lymphocyte fractions were observed in the top dose females. Other statistically significant deviations from control groups at the lower dose levels were considered irrelevant in the absence of a dose–response relationship.

The observed increase of alanine transaminase (ALT) and aspartate transaminase (AST) activities at 300 ppm (males) and above (both sexes) and alkaline phosphatase (ALP) activity at the top dose was indicative of liver toxicity. The marginal increase of AST at 30 ppm was probably substance-related, but not considered adverse in the absence of other relevant effects at this dose. Bilirubin levels were decreased at 300 ppm (males) and above (both sexes). The decreased cholesterol levels at 300 ppm and above may be related to an alteration in lipid metabolism. Creatinine was increased in males at 300 ppm and above, possibly reflecting an effect on the kidney function, although this was not confirmed by either organ weight or histopathology. In addition, slightly decreased protein levels were detected in males at 300 ppm and above. Finally, electrolyte concentrations appeared to be disturbed at the top dose, as illustrated by increased Ca^{2+} and K^{+} (females) and inorganic phosphate (males), and decreased Cl^{-} (males). Changes at lower doses in the absence of such changes at the top dose were not considered relevant.

At 300 ppm and above, dose-related increases in absolute and relative liver weight (at 300 ppm relative liver weight by 24% in females; at 2000 ppm by 114% in males and 122% in females) and increased absolute and relative spleen weight was observed (relative spleen weight by 30% and 47% in females at 300 and 2000 ppm, respectively). In males at the top dose the spleen weight increase was marginal.

Gross pathological examinations revealed both liver and spleen enlargement in males and females at the top dose. In addition, spleen enlargement was detected in one female at 300 ppm. The macroscopic findings were confirmed histopathologically.

In the liver, hypertrophy and vacuolation was observed at 300 ppm and above, while slight to moderate multifocal leukocyte infiltration was present at the top dose. At 300 ppm liver hypertrophy and vacuolation was graded mainly slight to moderate, while the finding was moderately severe to severe at the top dose. The vacuolation (mainly fine at 300 ppm, more crude at the top dose) was considered to reflect a fatty degenerative event (oil red O staining). In addition the presence of single-cell necrosis and pigmented Kupffer cells confirmed the accentuation of degenerative changes at the top dose. In males on the top dose relative testes weight (but not absolute) was increased without any histopathological correlate. Additionally, increased relative weight of adrenals was noted but without macroscopic or microscopic correlates. Furthermore, some histopathological findings were recorded in thyroids, but they were also observed in concurrent control group males and no organ weight effect or macroscopic correlate were observed. In females at the top dose, reduced absolute ovary weights were recorded, but without microscopic or macroscopic correlates. Additionally in females, some macroscopic (enlargement) and microscopic (cortical vacuolation) findings were observed in the adrenals. However, the histopathological incidences were also observed in the concurrent control females and no effect on the organ weight was detected.

Table 19. Key findings of 90-day dietary toxicity study in mice

Dose (ppm)		**0**		**30**		**300**		**2000**	
(mg/kg bw per day)		**0**		**4.6**		**50.5**		**341.1**	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Feed and weight (% change relative to controls)									
Food consumption	weeks 1–6							**↓11%***	**↓17%*****
	weeks 1–13							↓9%	**↓14%***
Body weight	week 13							↓16%	↓14%
Body weight gain	weeks 0–13							**↓61%*****	**↓50%*****
Haematology (% change relative to controls)									
Haemoglobin , Hb								-	**↓6%***
Haematocrit, Ht								↓4%	**↓6%***
Mean corpuscular volume, MCV								**↓9%****	**↓9%****
Mean corpuscular Hb, MCH								**↓7%*****	**↓7%*****
White blood cell count, WBC						(↓)	-	-	**↑11%***
Neutrophils								-	**↑135%*****
Lymphocytes								-	**↓12%****

Dose (ppm) (mg/kg bw per day)		0 0		30 4.6		300 50.5		2000 341.1	
		M	F	M	F	M	F	M	F
Clinical chemistry (% change relative to controls)									
Total protein						-	**↓7%****	-	**↓9%*****
Bilirubin						**↓28%*****	-	**↓46%*****	**↓28%*****
Creatinin						**↑20%****	-	**↑26%****	-
Cholesterol						**↓35%*****	**↓50%*****	**↓65%*****	**↓60%*****
Ca^{2+}								-	**↑6%****
K^{+}								-	**↑21%****
Cl^{-}								**↑3%*****	-
Inorganic phosphate								**↑18%***	-
Aspartate transaminase, AST				**↑32%***	-	**↑61%*****	-	**↑172%*****	**↑146%*****
Alanine transaminase, ALT						**↑61%****	-	**↑455%*****	**↑508%*****
Alkaline phosphatase, ALP								**↑245%*****	**↑332%*****
Organ weights (% change relative to controls)									
Liver	absolute					↑12%	**↑27%***	**↑78%*****	**↑95%*****
	relative					**↑22%*****	**↑24%*****	**↑113%*****	**↑121%*****
Spleen	absolute					-	**↑32%****	↑4%	**↑30%****
	relative					-	**↑30%****	**↑24%***	**↑48%*****
Adrenals (L+R)	absolute						-	↑63%	↓11%
	relative						-	**↑91%****	↑9%
Testes	absolute							↑4%	-
	relative							**↑25%****	-
Ovaries (L+R)	absolute							-	**↓27%****
	relative							-	↓20%
Histopathology									
Number of animals examined		9	11	12	12	8	12	12	12
Liver	leukocyte foci slight	1	0					8	10
	moderate	0	0					0	1
	hepatocyte hypertrophy/ vacuolation §	0	0			8	12	12	12
Spleen	lymphoid hyperplasia §	0	0			1	0	9	8
Thyroid	cystic follicle	0	1					4	0
Adrenal	subcapsular cell focus	1	3	0	0	0	0	1	4 −33%
	cortico-medullary vacuolation	0	4	0	0	0	0	0	5 −42%
Uterus	cystic endometrial gland(s)		1		1		2		2

Statistically significant modification, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

§ Total incidence for score expanded finding

The NOAEL for this study was established at 30 ppm (equivalent to 4.6 mg/kg bw per day) based on increased liver and spleen weights with corresponding macroscopic and microscopic changes corroborated by clinical chemistry findings observed at 300 ppm (equivalent to 50.5 mg/kg bw per day) (Clay, 1991).

Rat

Study 1

Groups of seven male and seven female Fischer 334 rats were fed for 28 days on diets containing 30, 100, 1000, and 3000 ppm metconazole (BAS 555 F; purity 96.0%; *cis*/*trans* 83:17, nominal; batch 88-10). Achieved doses for males were 2.7, 9.1, 90.5 and 261.2 mg/kg bw per day and for females 3.1, 10.1, 97.0 and 287.4 mg/kg bw per day, respectively. Animals were individually housed. The stability and homogeneous distribution of the test substance in the diet were confirmed by analysis. The correctness of the concentrations was analytically confirmed.

No mortality was observed. Clinical signs of toxicity included food spillage at 3000 ppm indicating palatability problems.

Feed consumption was statistically significantly reduced during the entire four-week treatment period, for males in the 1000 ppm group, and for males and females in the 3000 ppm groups. Mean body weights at study termination were statistically significantly decreased compared to controls for males in the 1000 ppm group (15%), and for males and females in the 3000 ppm groups (53% and 23% respectively). Compared to controls, overall body weight gain during the four-week treatment period was decreased for males in the 1000 ppm group (27%) and for males and females in the 3000 ppm group (71% and 58% respectively). No effects on body weight, body weight gain, or food consumption were observed in other treatment groups.

Haematology findings included mild hypochromic microcytic anaemia as illustrated by statistically significant decreased corpuscular volume (3000 ppm) and decreased Hb concentration at 1000 ppm (males) and above (both sexes). Moderate thrombocytopenia was observed in both sexes at the top dose. Increased leukocyte (females) and monocyte counts (both sexes) were observed at the top dose, but in the absence of further observations in differential counts, the finding is probably without toxicological relevance.

The compound was hepatotoxic as illustrated by statistically significant elevations in aspartate and alanine amino*trans*ferase and alkaline phosphatase activities (both sexes) at the top dose. In addition, statistically significant increases in γ-glutamyl transpeptidase (GGTP) were apparent at 1000 ppm and above in animals of both sexes. The slightly increased bilirubin levels in the top-dose females were possibly subsequent to the observed anaemic events (increased Hb catabolism). Further changes at the top dose included statistically significant serum glucose decrease (both sexes), and increased albumin, blood urea nitrogen (BUN), creatinine, Ca^{2+}, and inorganic phosphorus levels (females). Protein level was increased in females, and cholesterol decreased at 1000 ppm (males) and above (both sexes).

The observed absolute weight decreases of the heart, kidneys (both sexes) and testes at ≥ 1000 ppm, and of brain (both sexes), ovaries and adrenals (females) at the top dose, were considered secondary to the body weight loss noted at termination. Further alterations included a statistically significant increase in relative liver weight at 1000 ppm and above in both sexes. The slight, but statistically significant increase in spleen weight at the top dose could be in line with the observed platelet changes. Furthermore, a treatment-related absolute weight reduction of the testes was observed in male animals of both sexes at 3000 ppm as well as in males at 1000 ppm. As a consequence of the severely reduced testes weight at the top dose, reduced testis size (5/7 males) was observed during gross pathology, and reduced spermatogenesis (see below) in all animals as a histopathological correlate. In females only, reduced absolute adrenal weight was observed without an effect on the relative weight. Furthermore, absolute weight of ovaries was decreased.

At 100 ppm and above, liver enlargement was observed in the males (3/7 animals). This finding, as well as pale appearance, was observed at 1000 ppm (6/7 males, 3/7 females) and above at 3000 ppm (7/7 males, 7/7 females) in animals of both sexes. At the top dose, indications of stomach irritation were observed, as well as testis wastage. The gross pathological findings in the liver were confirmed by either slight to moderate (1000 ppm; 6/7 males and 7/7 females) and moderate to very severe (3000 ppm; 5/7 males and females) fatty degeneration, and dose-dependent severe diffuse (males) or centrilobular (females) hypertrophy. The observed liver enlargement/pallor at 100 ppm was not corroborated by any

histological finding. Occasional liver focal necrosis was observed at the top dose. Further observations at the top dose included: slight to moderate reduction in spermatocyte presence, slight (6/7 males) to very slight (7/7 females) vacuolation of the adrenal cortex, and moderate hyperkeratosis in the fore-stomach which appeared grossly abnormal.

All organ findings correlated to severe body weight reductions at dose levels of 1000 ppm (−14% in males) and 3000 ppm (−34% in males and −19% in females) as a consequence of markedly reduced food consumption at dose levels of 1000 ppm (−12% in males) and 3000 ppm (32% in males and −18% in females). Furthermore stomach irritation, a sign of local toxicity, was observed at 3000 ppm in animals of both sexes, and this is associated with pain and distress. The observed adrenal vacuolation did not result in organ damage, necrosis, fibrosis or granuloma formation, organ dysfunction or cell death, even at longer exposure times (chronic exposure duration). Nevertheless, from the overall data, it cannot be excluded that the observed adrenal vacuolation, which was also observed in one of two 90-day rat studies with metconazole, is treatment-related, but the effect per se is not an adverse one. There is no evidence of a functional impairment of the adrenal glands.

Table 20. Key findings of 28-day dietary toxicity study in rat

Dose (ppm)		**0**		**30**		**100**		**1000**		**3000**	
mg/kg bw per day		**0**		**2.7**		**9.1**		**90.5**		**261**	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Food consumption								**↓12%****	-	**↓32%****	**↓18%****
Body weight								**↓14%****	-	**↓34%****	**↓19%****
Haematology (% change relative to controls)											
Mean corpuscular volume, MCV										**↓2 %****	**↓3 %****
Mean corpuscular Hb, MCH										**↓5%****	**↓4%****
Platelet count										**↓25%****	**↓17%****
Clinical chemistry (% change relative to controls)											
Glucose										**↓29%****	**↓14%****
Cholesterol								**↓23%****	↑16%	**↓24%****	**↓28%****
Triglycerides								↓25%	-	**↓46%****	-
Protein								-	**↑4 %***	-	**↑12%****
Albumin										-	↑13.0%
Bilirubin										-	**↑25%****
Blood urea nitrogen, BUN										-	**↑13%****
Creatinine										-	↑8%
Aspartate transaminase, AST								↑11%	↑13%	**↑130%****	**↑166%****
Alanine transaminase, ALT								↑14%	↑13%	**↑139%****	**↑141%****
γ-Glutamyl transpeptidase, GGTP[§]		0	0.1					0.4	0.6	**14.6****	**19.9****
Organ weight (% change relative to controls)											
Liver	absolute							↑4%	**↑22%****	↑8%	**↑67%****
	relative[#]							**↑27%****	**↑22%****	**↑65%****	**↑102%****
Spleen	relative[#]									**↑23%***	**↑33%****
Testes	absolute							**↓14%***	-	**↓45%****	-
	relative[#]							↑1%	-	↓8%	-
Ovaries	absolute									-	**↓50%****
	relative[#]									-	-
Adrenals	absolute							↓14%	↓13%	↓11%	**↓28%****
	relative[#]							↓9%	↑2%		↓13%

Dose (ppm) mg/kg bw per day			0 0		30 2.7		100 9.1		1000 90.5		3000 261	
			M	F	M	F	M	F	M	F	M	F
						Histopathology						
Adrenal	No. examined		7	7	7	7	7	7	7	7	7	7
	cortical vacuolation										**6****	**7*****
Stomach	No. examined	**0**	0	0	0	0	0	0	0	0	5	5
	forestomach hyperkeratosis										5	5
Liver	No. examined		7	7	7	7	7	7	7	7	7	7
	diffuse fatty vacuolation								**7*****	**7*****	**7*****	**7*****
	diffuse hypertrophy								**6****	0	**7*****	2
	centrilobular hypertrophy								1	3	0	**5***
	focal necrosis										1	1
Testes	No. examined		7		1		0		1		7	
	spermatogenic reduction										**7*****	-

§ Numerical value in IU

Actually not the proportion between organ weight and body weight, but covariate analysis with "terminal body weight as a covariate"

Statistically significant modification, Williams', Wilcoxon or Fisher's test: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

The NOAEL for this study was established at 100 ppm (equivalent to 9.1 mg/kg bw per day) based on reduced body weight and food consumption as well as increased liver weight with corresponding macroscopic and microscopic changes: liver pallor/enlargement and hepatocellular vacuolation/ hypertrophy corroborated by clinical chemistry findings (enhanced AST, ALT reduced cholesterol and triglycerides) observed at 1000 ppm (equivalent to 90.5 mg/kg bw per day) (Esdaile, 1990).

Study 2

Groups of seven male and seven female Fischer 334 rats were fed with diets for 28 days containing 30, 100, 300, 1000, and 10 000 ppm of metconazole (WL1361; purity 84 98%; *cis*:*trans* 97:1; batch: ST87-181). Achieved doses for males were 2.7, 9.2, 27.3, 89.3, and 720.6 mg/kg bw per day and for females 3.0, 9.5, 29.8, 100.7 and 784.3 mg/kg bw per day. Animals were housed individually. The stability and homogeneous distribution of the test substance in the diet were confirmed by analysis. The correctness of the concentrations was analytically confirmed.

One top-dose female was killed moribund on day 15 and had similar necropsy findings as the other animals at this dose level. The top-dose animals spilled their feed, leading to body weight loss during week 1, and reduced body weight gain until week 4. They were emaciated, unkempt, showed alopecia (males), and had discolored urine. A statistically significant decrease in feed intake was observed at 1000 ppm in males (10%), and above in animals of both sexes (56% in males, 48% in females) at all sampling times during the entire four-week treatment period. At 300 ppm, a single statistically significant drop of 7% was noted only during week 3 in the males.

Body weight and body weight gain were statistically significantly decreased at 1000 ppm and above on weeks 1 to 4 in animals of both sexes. Compared to controls, mean body weights at study termination were statistically significantly decreased for males and females at 1000 ppm (8% and 7%, respectively) and 10 000 ppm (61% and 52%, respectively). Overall body weight gain during the

four-week treatment period was decreased at 1000 ppm (by 16% for males, 15% for females) and at 10 000 ppm (26% and 28%, respectively). During weeks 1–3 at 100 ppm (females) and at 300 ppm (both sexes), the sporadic decreases observed were small (below 5%) and not dose-related.

At the top dose animals of both sexes showed signs of a microcytic hypochromic anaemia (as illustrated by statistically significantly decreased MCV, decreased Hb concentration and decreased Ht), associated with increased levels of red blood cell (RBC) precursor cells like reticulocytes and erythroblasts (normoblasts), indicating a regenerative process. The aetiology was likely a combination of haemolysis, haemorrhage or iron-deficiency due to decreased feed intake. The high incidence of platelets was reported as an instrumental artefact (interference between nucleated RBC precursors and leukocytes, and of cell debris with platelets), since no abnormally high platelet or leukocyte numbers were counted on smears. The increased neutrophil content was in line with both regenerative anaemia, and acute inflammation events (gastric inflammation).

Total protein and albumin levels were increased, and the albumin:globulin ratio decreased in the top-dose females, reflecting both an effect on liver and kidney physiology (see also increased plasma urea nitrogen). Liver toxicity was demonstrated by the huge statistically significant increase in both transaminases (such as AST and ALT), and ALP and GGTP activities in all top-dose animals. The elevated bilirubin level could be a result of both hepatotoxicity and anaemia.

Both absolute and relative (adjusted for terminal body weight) liver weight increases were observed at 300 ppm and above in females, and at the top dose in males. Testes weight was decreased at 1000 ppm (absolute) and above (absolute and relative). Statistically significant decreases of absolute (but not relative) adrenal weight were observed at 300 ppm (males) and at 1000 ppm (both sexes) but not at the top dose. Other decreases (absolute) or increases (relative) of organ weights were explained by the body weight decrease at termination.

At necropsy, top-dose animals showed enlarged and pale livers, dark kidneys, and forestomach irritation lesions. Liver pallor was also observed at 300 ppm and above in males and at 1000 ppm in animals of both sexes. Small testes were observed at 300 ppm and above.

Histological evidence of liver toxicity was observed in the top-dose animals, and occasionally at 1000 ppm, including both fibrotic or necrotic areas and single-cell necrosis, plus both microvesicular and macrovesicular fatty degeneration. In addition, marked parenchymal hypertrophy (indicating enzyme induction) and hyperplasia (increased mitoses) were observed. The liver weight increase and gross lesions observed at 300 ppm were not corroborated by histological findings. Therefore, the effects at 300 ppm could be considered as adaptive and not toxic changes. Renal toxicity at the top dose was evidenced by cortical tubular vacuolation, basophilic change, pigmentation, dilatation and protein casts. Tubular necrosis and acidophilic change was observed on one occasion in females. In the forestomach, papillomatous hyperplasia with associated inflammatory lesions were observed at the top dose. At this dose level, further effects were noted on male gonads (testicular and seminal vesicle degeneration) and on the adrenals (increased cortical vacuolation). While liver, adrenals glands, and kidney were obviously detected as target organs for systemic toxicity, and stomach for local toxicity, it is probable that the effects on gonads were subsequent to feed restriction and associated stress at the top dose. In testes, macroscopic findings ($\geq$ 300 ppm) were corroborated by histopathological findings (atrophy and aspermatogenesis) only at 10 000 ppm, correlating with organ weight reductions at 1000 ppm (absolute) and 10 000 ppm (absolute and relative). Any changes at the top dose on the organ weights adjusted for terminal body weight were associated with the severe body weight decrease at termination. Additionally, reduced absolute adrenal weights were recorded in males at $\geq$ 300 ppm without any changes on their relative weights. At 10 000 ppm adrenal weight reduction was only marginal, leading to a highly significant weight increase when adjusted for the terminal body weight. A histopathological correlate for the adrenal weight effects in males was found at the top dose, where adrenal cortical vacuolation was observed in all animals. In females at 10000 ppm reduction in ovary size and uterus thinning/atrophy were recorded in 1/7 animals. Additionally, marginal adrenal weight reduction led to a significant weight increase when adjusted for the terminal body weight. Adrenal weight effects at 10 000 ppm correspond with adrenal cortical vacuolation in all animals.

Table 21. Key findings of 28-day dietary toxicity study in rats

Dose (ppm) mg/kg bw per day		0 0		30 2.7		100 9.2		300 27		1000 89.3		10 000 720.6	
		M	F	M	F	M	F	M	F	M	F	M	F
Food consumption,	week 4									**↓10%****	-	**↓56%****	**↓48%****
Body weight,	week 4									**↓8%****	**↓5%***	**↓61%****	**↓52%****
Haematology (% change relative to controls)													
Red blood cells, RBC												**↓30%****	**↓31%****
Haemoglobin, Hb												**↓34%****	**↓36%****
Haematocrit, Ht												**↓33%****	**↓33%****
Mean corpuscular Hb, MCH												**↓6.3%****	↓5.0%
Mean corpuscular Hb concentration, MCHC												↓3%	**↓3.0%***
Reticulocytes												**↑214%****	**↑756%****
Clinical chemistry (% change relative to controls)													
Protein												-	**↑21%****
Albumin												-	**↑9%***
Albumin:globulin ratio, A:G												**↓11%****	**↓22%****
Bilirubin												**↑63%****	**↑108%****
Blood urea nitrogen, BUN												**↑59%****	**↑75%****
Aspartate transaminase, AST												**↑240%****	**↑433%***
Alanine transaminase, ALT												**↑487%****	**↑769%****
γ-Glutamyl transpeptidase, GGTP[§2]												**130.0****	**113.6****
Alkaline phosphatase, ALP												**↑136%****	**↑272%***
Organ weight (% change relative to controls)													
Liver	absolute							-	↑8%	-	**↑13%****	**↑11%***	**↑67%****
	relative							-	**↑9%****	**↑13%****	**↑20%****	**↑193%****	**↑229%****
Testes	absolute									**↓7%****	-	**↓89%****	-
	relative									-	-	**↓72%****	-
Histopathology													
Adrenals	No. examined	**7**	7	0	0	0	0	0	0	7	7	7	7
	vacuolation											**7****	**7****
Kidney	No. examined	**7**	7	0	0	0	0	0	0	2	0	7	7
	tubular cortical vacuolation											**7****	**7****
	tubular basophilia	1	2									**7****	5
	tubular pigment deposit											4	7
	tubular dilatation											4	4
	protein casts											2	**6****

Dose (ppm) mg/kg bw per day		0 0		30 2.7		100 9.2		300 27		1000 89.3		10 000 720.6	
		M	F	M	F	M	F	M	F	M	F	M	F
Forestomach	No. examined	7	7	0	0	0	0	0	0	1	0	7	7
	hyperplasia/ acanthosis/hyper-parakeratosis											**7****	**7****
	epithelial microabscesses											4	4
	submucosal inflammation											1	**5***
	erosion/ ulceration											3	0
Liver	No. examined												
	mineralisation/ fibrosis/necrosis											**6****	**6****
	single cell necrosis											**7****	4
	microvesicular vacuolation	1	2							**6***	0	**6***	**7***
	diffuse hypertrophy											**7****	**7****
	hyperplasia											**7****	**7****
	atrophy											**6****	-
Testes	No. examined	7		0		0		0		7		7	
	tubular atrophy/ aspermatogenesis											**7****	-

Statistically significant modification, Williams', Wilcoxon or Fisher's test: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

All organ findings in animals of both sexes correlated with the dramatic terminal body weight reduction as a consequence of impaired food consumption, particularly at the top dose.

The NOAEL for this study was established at 300 ppm (equivalent to 27.3 mg/kg bw per day) based on reduced body weight and food consumption as well as liver weight increase with corresponding macroscopic (pallor of the liver) and microscopic (hepatocellular vacuolation) changes observed at 1000 ppm (equivalent to 89.3 mg/kg bw per day) (Esdaile, 1991a).

Study 3

Groups of 10 males and 10 females Fischer rats were fed with diets for 90 days containing 30, 100, 300, 1000, and 3000 ppm metconazole (BAS 555 F; purity 94.5%; *cis*:*trans* 76.5:18; batch 88-10). An additional seven week recovery experiment was set up, where 10 rats/sex per dose received metconazole in the diet at dose levels of 0 or 3000 ppm. Animals were housed individually. Achieved doses for males were 1.9, 6.4, 19.2, 64.3, and 192.7 mg/kg bw per day and for females 2.1, 7.2, 22.1, 71.4, and 208 mg/kg bw per day. The stability and homogeneous distribution of the test substance in the diet were confirmed by analysis. The correctness of the concentrations was analytically confirmed.

No mortality was observed throughout the study period. Feed spillage and evident emaciation was observed in all top-dose animals. During ophthalmoscopic examination, increased lacrimation and eye orbit alopecia were observed in most top-dose animals of both sexes (intermediate dose levels not examined). Both body weight and feed consumption were reduced during the whole treatment period at 1000 ppm (male) and above (both sexes). The differences in body weight were attenuated during the recovery period but still noticeable in all top-dose animals at study termination.

Hematology parameters, at the 1000 ppm and above were consistent with a mild hypochromic microcytic anemia. At the top dose, platelets were decreased and showed increased variation in size, reflecting platelet anisocytosis. In the males this was accompanied by a marginal fibrinogen clottability deficiency. However, the activated partial thromboplastin time (APTT) was apparently decreased, and no clinical signs of coagulation deficiency were observed. The increased number of leukocytes (and lymphocytes) in the females was highly significant at the highest dose; statistically significant values at the low- and mid-dose levels did not show any dose-dependence. However, differential counts did not reveal any pertinent disturbance in the blood formula, and no atypical cells were observed. Following the seven-week recovery period, Hb concentration, Ht and erythrocyte mean diameter returned to near control values for the top-dose males and females, but the platelet count did so only for females.

In the top-dose males, a significant increase in ketone bodies was observed (+770%), which was related to inanition. In addition, urine showed a more intense colour, which persisted after recovery. Protein fractions were disturbed in the top-dose animals, with increased β-globulin fraction as the most consistent modification. The decreased glucose, cholesterol and triglyceride levels were in line with feed deprivation status. The statistically significant increase in transaminases (ALT and AST), ALP and GGTP levels were consistent with the effect of the substance on the liver.

The liver was detected as the target organ; both absolute and adjusted liver weights were increased at 1000 ppm and above. Histopathological evaluation revealed microvesicular, diffuse or periportal fatty vacuolation in both sexes, which was graded slight at 300 ppm (males) and at 1000 ppm (females), moderate at 1000 ppm (males) and at 3000 ppm (females) and severe at 3000 ppm in males. After recovery, about half of the animals still showed slight parenchymal vacuolation. Centrilobular hypertrophy was detected at 1000 ppm (males) and above (both sexes), and in one animal at 300 ppm. At the top dose the presence of Fe^{3+}-containing pigment within Kupffer cells was observed in both sexes but was more pronounced in the females. Persistence of hypertrophy in two males and Kupffer cell pigmentation in two females was demonstrated in the recovery group.

At the top dose, the increase in adjusted splenic weight was highly significant. The effects at other dose levels on spleen weight were not always dose-related or were not corroborated by histological findings. Other weight changes were considered secondary to terminal body weight changes. The macroscopic alterations in spleen were restricted to the top dose. Histopathological spleen evaluations revealed a decrease of both red and white pulp and aggregates of pigmented macrophages in the marginal zone of top-dose animals, indicating haemosiderosis. Absolute (but not relative) testis weight reductions were observed at 3000 ppm (−8%, significant at $p = 0.01$). After the recovery period, organ weight reduction was recorded at the top dose (−14%, significant at $p = 0.05$), but only one animal showed a correlating macroscopic finding (reduction in size and pale focus), and no relevant histopathological effects were noted. A dose-related significant reduction in absolute adrenal weight was observed in males at 300 ppm and above and in females at the top dose. In top-dose males and females the adrenal weight effect was corroborated by histopathological findings (adrenal cortical vacuolation; moderate in males and very slight in females). Significantly reduced absolute ovary weight was restricted to the top dose (−25%), was not confirmed by any macro- or microscopic findings, and was not observed at the end of the recovery period, except of a small incidence of reddening of the organ. Macroscopic uterus findings (thinning) were confirmed by histopathological evaluation (atrophy), however the severity grade was reduced markedly during recovery period.

All effects on the organs of the endocrine system were reversible within seven weeks with the exception of the testes absolute weight (top dose; −14%) and adrenal absolute weight (top dose; –20% in males and −11% in females) which persisted until the end of the recovery period. Nevertheless, the adrenal findings present at the end of the recovery period also correlated with markedly reduced body weight reductions induced by low food consumption. Macroscopic stomach alterations with correlating forestomach focal hyperplasia or hyperkeratosis were seen mainly in the male top-dose animals. On occasions, top-dose females exhibited submucosal inflammatory changes (polymorphonuclear leukocyte infiltration/oedema).

Results are summarized on the following page in Table 22.

Table 22. Key findings of 90-day dietary toxicity study in rat

Dose (ppm) mg/kg bw per day	0 0		30 1.9		100 6.4		300 19.2		1000 64.3		3000 192.7	
	M	F	M	F	M	F	M	F	M	F	M	F
Feed consumption, week 13									**↓12%****	**↓9%****	**↓33%****	**↓22%****
Body weight, week 13									**↓9%****	-	**↓35%****	**↓19%****
Haematology (% change relative to controls)												
Haemoglobin, Hb									-	**↓4%****	**↓8%****	**↓10%****
Haematocrit, Ht											**↓5%****	**↓6%****
Mean corpuscular volume								**↑1.3%****	-	**↑2%****	**↓7****	**↓10%****
Mean corpuscular Hb									-	**↓2%***	**↓9%****	**↓13%****
Mean corpuscular Hb concentration, MCHC									-	**↓2.7%****	**↓3%****	**↓4%****
Platelets											**↓27%***	**↓23%****
Prothrombin time, PT									**↓3%****	-	**↓3%****	-
APTT											**↓10%****	-
White blood cells								**↑12%***	-	**↑6%***	-	**↑24%****
Lymphocytes											-	**↑23%****
Clinical chemistry (% change relative to controls)												
β-globulin									**↑17.6%****	-	**↑27%****	**↑33%****
Albumin:globulin ratio, A:G												**↓17%****
Glucose											↓17%	**↓15%***
Cholesterol									**↓36%****	-	**↓48%****	-
Triglycerides									**↓69%****		**↓96%****	**↓70%****
Aspartate transaminase, AST											**↑77%****	**↑70%****
Alanine transaminase, ALT				↓18.3		↓12.5			**↑13.6%****		**↑136%****	**↑94%****
γ-Glutamyl transpeptidase, GGTP [§]	0	1.1							0.1	**2.9****	**11.0****	**17.5****
Alkaline phosphatase, ALP											**↑35%****	**↑42%****
Organ weight (% change relative to controls)												
Liver absolute									**↑18%****	**↑20%****	**↑11%****	**↑53%****
relative									**↑31%****	**↑20%****	**↑59%****	**↑81%****
Spleen absolute							-	**↑8%***	**↓8%***	**↑8%***	↓6%	**↑5%***
relative							-	**↑8%***	-	**↑8%***	**↑20%****	**↑24%****
Adrenals absolute							**↓6%***	-	**↓10%****	-	**↓23**** [a] **↓20%**** [b]	↓24* [a] **↓11%**** [b]
relative							-	-	-	-	**↓11%***	-
Testes absolute											**↓8**** [a] **↓14%*** [b]	-
Ovaries absolute											-	**↓25%****
relative											-	-

Dose (ppm) mg/kg bw per day		0 0		30 1.9		100 6.4		300 19.2		1000 64.3		3000 192.7	
		M	F	M	F	M	F	M	F	M	F	M	F
							Histopathology						
Adrenals	No. examined	20	20	0	1	1	0	0	0	10	10	10	10
	vacuolation total [#]	19	0	0	0	1	0	0	0	10	0	**10*****	**6*****
Liver	No. examined	20	20	10	10	10	10	10	10	10	10	10	10
	week 13; fatty vacuolation	0	0					4**	0	**10*****	**5****	**10*****	**10*****
	week 20; fatty vacuolation	0	0									4	6*
	centrilobular hypertrophy	0	0					1	0	**10*****	0	**10*****	**10*****
	pigment deposit [#]	0	0									**4****	**10*****
Spleen	No. examined	20	20	0	0	0	0	1	0	10	10	10	10
	decreased haematopoiesis	0	0									**10*****	**10*****
	pigment deposit	0	0									**9*****	**10*****
	white pulp depletion	0	0									**3*****	**9*****
Forestomach	No. examined	20	20	1	0	0	0	0	1	0	0	10	10
	junctional ridge hyperplasia	0	0									**3***	0
	hyperkeratosis	0	0									2	1
Uterus	atrophy	-	0									-	**9*****
Testes	focal mineralization									1	-	2	-
	tubular atrophy											1	

APTT Activated partial thromboplastin time

[§] Numerical value in IU

[#] total incidence for score expanded finding

[a] Mean group, [b] recovery group

Statistically significant modification, Williams' or Dunnett's or Fisher's test: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

The NOAEL for this study is established at 100 ppm (equivalent to 6.4 mg/kg bw per day) based on hepatocellular fatty vacuolation observed at 300 ppm (equivalent to 19.2 mg/kg bw per day) (Esdaile, 1991b).

Study 4

Groups of 10 males and 10 female Fischer rats were fed with diets for 90 days containing 50, 150, 450, 1350, and 4050 ppm metconazole (KNF-S-474c/WL136184; purity 98%; *cis*:*trans* 99:1; batch 88-05). An additional seven-week recovery experiment was set up in which 10 rats/sex per dose received *cis*-metconazole in the diet at dose levels of 0 or 4050 ppm. Achieved doses for males were 3.2, 9.7, 28.8, 88.6, 264.6 mg/kg bw per day and for females 3.7, 11.0, 33.0, 96.8, and 267.3 mg/kg bw per day.

The stability and homogeneous distribution of the test substance in the diet were confirmed by analysis. The correctness of the concentrations was analytically confirmed.

No mortality was observed throughout the study period. A dose-dependent increase of feed spillage incidence was observed from 450 ppm (female) and 1350 ppm (male) upwards. Body weight loss and thin appearance was visible at the top dose in both sexes. In the top-dose males crusting eyes were additionally observed. At the top dose a slightly increased incidence of chromodacryorrhea was observed, possibly connected to the poor general state of the animals at this dose.

Feed consumption was altered in all top-dose animals throughout the whole treatment, and at several sampling times at 1350 ppm, the effect being slightly more pronounced in the males. After cessation of the treatment the feed consumption rebounded from the first week returning to the control level. Body weight decrease was observed throughout treatment in both sexes at 1350 ppm and above. Overall body weight gain was reduced 13% for males and 14% for females at 1350 ppm, and 62% for males and 50% for females at 4050 ppm. Body weight decrease moderated but remained noticeable until recovery on week 20. No urinalysis was performed.

Evaluation of haematology parameters at termination of the study revealed statistically significantly decreased Hb concentration, Ht, erythrocyte mean diameter, MCV, mean corpuscular hemoglobin (MCH) and platelet count for males and females in the 4050 ppm group. In females at 1350 ppm, there were slight, but statistically significant decreases in Hb concentration, erythrocyte mean diameter and MCH. After recovery, MCV and MCH (both sexes) remained low, and red cell distribution width (RDW) in females and platelet distribution width (PDW) in males) remained higher than the control group value. However, RBC counts (both sexes), Ht and RBC diameter (males) were increased compared to control values, indicating animals at the top dose had partially recovered from their anaemic state.

Evaluation of clinical chemistry parameters revealed mostly enzymatic modifications (increased AST, ALT, GGTP, AP) at the top dose. Moderate increases were also noted at 1350 ppm in the males (transaminases) or in the females (GGTP). At the top dose changes in both ALP (males) and GGTP (females; increased by 150% compared to1256% at termination of treatment) were still detectable after the recovery period. Decreased levels of both cholesterol and triglycerides at 1350 ppm (males) and above (both sexes) may indicate an effect on lipid metabolism. Slight increases in total protein, albumin and bilirubin levels were demonstrated at the top dose. Altered protein level findings were further investigated by electrophoresis, leading to the observation of decreased α_1 and α_2 fractions at the top dose (males) and increased β-globulin fractions at 1350 ppm (males) and above (both sexes).

At the top dose, an increase in adjusted splenic weight was observed for both sexes, and a decrease of absolute (females and males) and adjusted (females) adrenal weight was observed. Additionally, at 1350 ppm and above, males and females revealed statistically significant increases in mean absolute and relative (to body weight) liver weights. For both liver and spleen, the effects were considered substance-related in the presence of histological abnormalities observed at study termination.

At necropsy most top-dose animals appeared small and showed reduced abdominal fat. Liver gross lesions (pallor, marked liver lobes, enlargement) were detected at 1350 ppm (males) and above (both sexes) with an isolated case in one male at 450 ppm. Forestomach or junctional ridge thickening was evident at the top dose, with some cases of ulcers at 1350 ppm (males). Spleens were characterized by a roughened surface in top-dose animals.

Histopathological liver changes included hepatocellular fatty degeneration as illustrated by the increased incidence of microvesicular vacuolation at 1350 ppm (males) and 4050 ppm (males and females), which tended to persist in the females after recovery. The finding was in line with observed modifications of lipid clinic chemical parameters at these dose levels. The centrilobular hypertrophy (observed at the highest dose level in males and females and at 1350 ppm in males) or pallor (in both sexes at 1350 ppm and above) was an indication of possible enzymatic induction. In the spleen, both red pulp effects (decreased haematopoietic activity) and white pulp effects (aggregates of pigment-loaded macrophages) were observed at the top dose. The inflammatory and hyperplastic changes at 1350 ppm (males) and above (both sexes) were indicative of the irritating effect of the test article on the (fore) stomach.

Absolute (but not relative) testis weights were reduced at the top dose. Prostate (8/10 animals) and seminal vesicles (7/10 animals) appeared small at the top dose. However, the small appearance of both prostate and seminal vesicles was not corroborated by any histological abnormality. At the top dose a decrease in absolute adrenal weights was observed, whereas when adjusted, adrenal weight was increased in males and decreased in females at this dose. At the lower dose levels absolute adrenal weight decreased in males only, but without a dose–response relationship, the magnitude was low, and concomitant histological lesions were not reported. The observed cortical vacuolation of the adrenals in the male recovery group at the top dose was unexplained in the absence of similar findings in the non-recovery group. Uterus thinning was apparent at 1350 ppm and above and the effect persisted in 2/10 females after recovery. At the top dose, uterus wall atrophy confirmed the thin appearance of this organ at necropsy.

Table 23. Key findings of 90-day dietary toxicity study in rat

Dose (ppm) **mg/kg bw per day**		**0** **0**		**50** **3.2**		**150** **9.7**		**450** **28.8**		**1350** **88.6**		**4050** **264.6**	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Food consumption,	week 13									**↓11%****	↓6%	**↓36%****	**↓18%****
Body weight,	week 13									**↓10%***	↓5.5%*	**↓38%****	**↓20%****
	week 20									NA	NA	**↓18%****	↓5%
Haematology (% change relative to controls)													
Red blood cells, RBC												**↑3%***	**↑7%****
Haemoglobin, Hb										-	**↓4%****	**↓6%***	**↓12%****
Haematocrit, Ht												**↓4%***	**↓8%****
Mean corpuscular volume												**↓7%****	**↓14%****
Mean corpuscular Hb, MCH										-	**↓3%****	**↓9%****	**↓17%****
Mean corpuscular Hb concentration, MCHC										-	**↓3%****	**↓3%****	**↓4%****
Platelets												**↓15%***	**↓18%***
White blood cells, WBC												-	**↑19%****
Lymphocytes												-	**↑23%****
Clinical chemistry (% change relative to controls)													
β-globulin (g/L)										**↑11%****	-	**↑35%****	**↑46%****
Protein (g/L)												**↑6%****	**↑9%****
Albumin (g/L)												**↑6%****	**↑7%****
Albumin:globulin ratio, A:G										-	**↓5%***	-	**↓6%****
Glucose												-	**↓21%***
Bilirubin												**↑18%****	**↑11%****
Cholesterol										**↓21%****	-	**↓48%****	-
Aspartate transaminase, AST										↑14%	-	**↑63%****	**↑75%****
Alanine transaminase, ALT										**↑42%****	-	**↑107%****	**↑73%****
γ-Glutamyl transpeptidase, GGTP[§]		0	1.6							-	**4.7****	**10.9****	**21.7****
Alkaline phosphatase, ALP												**↑17%****	**↑33%****
Organ weight (% change relative to controls)													
Liver	absolute							-	↑7%	**↑15%****	**↑21%****	**↑10%****	**↑68%****
	relative							-	**↑9%****	**↑30%****	**↑30%****	**↑66%****	**↑95%****
Spleen	absolute									-	-	-	-
	relative									-	**↑13%****	**↑20%****	**↑8%****

Dose (ppm) mg/kg bw per day		0 0		50 3.2		150 9.7		450 28.8		1350 88.6		4050 264.6	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Adrenals	absolute					**↓8%***	-	**↓10%****	-	**↓10%****	-	**↓19%****	**↓21%****
	relative					-	-	↓11%	-	-	-	-	**↓13%***
Testes	absolute											**↓11%***	-
	relative											-	-
Ovaries	absolute											**↓29%***	-
	relative											-	-
						Histopathology							
Adrenals	cortical vacuolation week 13	0	0									0	0
	cortical vacuolation week 20	1	0									**10*****	0
Liver	centrilobular vacuolation week 13	0	0							**9*****	**3***	0	**5****
	periportal vacuolation week 13	0	0							0	0	**10*****	**5****
	diffuse vacuolation week 20	0	0							NA	NA	0	**8*****
	centrilobular hypertrophy	0	0							2	0	**9*****	**10*****
	centrilobular pallor	0	0							3	0	0	0
Spleen	decreased haematopoiesis	0	2									**4****	**5***
	pigment deposit	0	0									**9*****	**5****
Forestomach	epithelial hyperplasia	0	0							2	0	**9*****	**5****
	hyperkeratosis	0	0							2	0	**10*****	**3***
	submucosal oedema	0	0							1	0	**6*****	**3***
	submucosal neutrophil infiltration	0	0							1	0	**9*****	1
	junctional ridge hyperplasia	0	0									0	3*
	ulceration/ erosion	0	0									**3***	0

Dose (ppm) mg/kg bw per day		0 0		50 3.2		150 9.7		450 28.8		1350 88.6		4050 264.6	
		M	F	M	F	M	F	M	F	M	F	M	F
Prostate	interstitial oedema (week 20)											**3***	
Uterus	atrophy	-	2							-	**3****	-	**9*****
Seminal vesicles	reduced secretion		0									2	
Testes	focal mineralization		2									2	

NA Not applicable

Statistically significant modification, Williams' or Dunnett's or Fisher's test: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

The NOAEL for this study was established at 450 ppm (equivalent to 28.8 mg/kg bw per day) based on reduced body weight and food consumption as well as increased liver and spleen weights with correlating hepatic macroscopic and microscopic changes corroborated by clinical chemistry findings observed at 1350 ppm (equivalent to 88.6 mg/kg bw per day) (Fokkema, 1992).

Dog

Study1

Groups of two males and two females Beagle dogs were fed for 28 days with diets containing 100 or 1000 ppm (days 1–29), and 10 000 (days 1–2)→8000 (days 3–4)→7000 ppm (days 5–29) metconazole (WL 148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01). Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. Achieved doses for males were 3.7, 37.8, and 170.5 mg/kg bw per day and for females 4.0, 41.2, and 178.4 mg/kg bw per day.

In a preliminary palatability study the test substance was administered at increasing doses of 200, 400, 800, 1600, 2400, 3200, 4800, 6400 and 9600 ppm (dose levels increased approximately every three days if no apparent treatment effects were seen). Critical decline of body weight occurred at 4800 ppm and above, feed consumption was markedly reduced at 9600 ppm, and the majority of the animals showed red areas on duodenum and colon. The stability and homogeneous distribution of the test substance in the diet were confirmed by analysis. The correctness of the concentrations was analytically confirmed (Pickersgill, 1991b).

No mortality was observed. Emesis was noted in one female 5 h after dosing at 10 000 ppm. Food consumption was reduced in males and females treated with the top dose, by 48% and 36%, respectively. In the top-dose males, body weight decreases of 15% (week 1), 20% (week 2), 23% (week 3) and 24% (week 4) were recorded. Body weight losses in the females at this dose-level were hardly interpretable (variation too high at the start of the study), but when comparing to week –1, body weight losses were noted at the top dose, whereas animals gained weight in the other dose groups. At termination, body weights in males treated with the top dose was decreased by 27% compared to the control animals. Urine of top-dose animals showed an increased specific gravity, a more intense color, and a lower pH when compared to controls. No relevant haematology findings were observed. Clinical chemistry revealed increased ALP activities at the top dose (14- to 23-fold on week 2, 16- to 17-fold on week 4). At 1000 ppm a trend toward higher values was also noted, but the increases were weak. In the top-dose females absolute and relative weight increases were noted in the spleen (by 26% and 22%, respectively), and in the liver (by 26% and 27%, respectively). In top-dose males, elevation of relative spleen weight (by 39%) and liver weight (by 10%) was noted. Finally, in the females, absolute and relative thyroid weight increases were observed at 1000 ppm and at the top dose. No relevant findings were observed in gross pathology.

Results are summarized on the following page in Table 24.

Table 24. Key findings of preliminary dietary toxicity study in dog

Dose (ppm)	0		100		1000		10 000[§]	
	M	F	M	F	M	F	M	F
Liver wt, absolute (g)	367.9	232.7	328.8	221.9	299.3	259.8	297	300.3
Liver wt, relative (%)	3.34	3.23	3.06	3.04	2.86	3.24	3.66	4.11
Spleen wt, absolute (g)	28.495	25.821	28.514	25.086	24.536	26.122	28.951	32.647
Spleen wt, relative (%)	0.2578	0.3669	0.2655	0.3373	0.2348	0.3233	0.359	0.4477
Thyroid wt, absolute (g) L/R	0.475/ 0.517	0.283/ 0.247	0.408/ 0.388	0.29/ 0.32	0.345/ 0.368	0.428/ 0.421	0.376/ 0.370	0.434 /0.442
Thyroid wt, relative (%) L/R	0.0043/ 0.0047	0.0040/ 0.0034	0.0038/ 0.0036	0.004/ 0.0043	0.0033/ 0.0035	0.0053/ 0.0051	0.0046 0.0045	0.0059 /0.0060

[§] Dose adjusted 10 000 ppm (days 1–2)→8000 ppm (days 3–4)→7000 ppm (days 5–29)

No histopathology was performed. The maximum tolerated dose (MTD) for this study was established at 7000 ppm (170–178 mg/kg bw per day) (Pickersgill, 1991a).

Study 2

Groups of five males and five females Beagle dogs were fed with diets for 90 days containing 60, 600, and 6000 ppm metconazole (WL 148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01). Achieved doses for males were 2.5, 24.4, and 225.2 mg/kg bw per day and for females 2.6, 24.3, and 206.6 mg/kg bw per day. The stability of the test substance in the diet and dietary preparation was verified. The homogeneity of the mixtures was verified prior to the study. The correctness of the concentrations was demonstrated.

No mortality was observed throughout the study period. Thin appearance and fur staining was observed from weeks 7–13 in 2/5 top-dose females as stated in the report. No raw data information was available concerning clinical signs. Feed consumption, calculated over the whole treatment period, was decreased at 600 ppm (14%, females) and above (14%, males, 31%, females). The decrease occurred mainly during weeks 1–4 of treatment and was more pronounced in the females than the males. Consequently, body weights of top-dose animals were depressed throughout the whole treatment period, leading to the diminished body weight gains noted at termination. At the top dose, decreases in body weight gain were up to −60% (male) and −98% (female), while at 600 ppm a trend toward lower values was noticeable (−29% in females) compared to controls.

Ophthalmology raw data were not available, but in the report it was stated that all top-dose animals showed lenticular degeneration (cataract). The observed opacities were considered test article-related.

At the top dose urine from 3/5 females contained bilirubin. There was also a tendency towards increased blood cells in the urine of this group.

At termination, the most prominent changes at the top dose in both sexes included reduced RBC counts, Hb content and Ht values. In conjunction with increased reticulocyte counts, these were considered signs of regenerative anaemia. In the males, platelet count was also increased, but signs of functional blood clotting parameters were inconclusive. Leukocytosis, mainly by increased neutrophil content, was also observed in this dose group. No change was seen in the myelogram, excluding possible test article-related effects on bone marrow. The changes found at other doses were considered biologically irrelevant in the absence of a dose–responsive relationship.

In the top-dose animals, and at the two sampling times, both albumin and A:G ratios were consistently lowered. Activities of AST, ALP and GGTP were increased on these occasions. The decreased glucose level in the top-dose females was reflected in their malnourished state.

At the top-dose, absolute and relative liver and spleen weights were increased, but only significantly for the relative liver weight in males and the relative spleen weight in females. Lenticular degeneration of the lens (characterized by swelling and ballooning of the lens fibers) was evident in

all top-dose animals with females being more severely affected than males. Mottled or pale livers were observed in the top-dose animals. In histopathological examinations the findings corresponded to (mostly periportal) hepatocellular hypertrophy. Th espleen in females was enlarged. Also, residual blood was present in the spleen after exsanguination and haematopoiesis was more pronounced than in the controls. Liver and spleen findings were consistent with the clinical chemical and haematological changes. Additionally, renal tubular cell pigmentation (both sexes) and vacuolation (females) was increased at the top dose. However, primary nephrotoxicity was not suggested, as no clinicochemical parameters were altered and further histological signs were absent. Urinary bladder findings at the top dose were reported as being secondary to catheterization during urine sampling.

An increase in absolute and relative thyroid weight was observed in females at the top dose (6000 ppm). This finding was not corroborated by macroscopic or microscopic observations. In the absence of other substance-related pathological findings in this organ the increased thyroid weights are of unclear toxicological significance.

At 6000 ppm the adrenal weight was increased (but not with statistical significance) in both sexes and neither necropsy nor histopathological findings were indicative of adverse effects. The observed organ weight changes at the top dose were seen in the presence of marked reductions in food consumption and decreased body weight. Thymus involution, seen in 3/5 top-dose dogs, was considered a finding associated with stress as a consequence of decreased food intake and decreased body weight gain. Overall, the effects of *cis*/*trans*-metconazole on the organs of the endocrine system at the highest dose were considered to be a stress-induced secondary response to malnutrition, which was evident from the decreased body weight gain due to decreased food intake.

Increased relative testis weights (+26%) at the top dose were not statistically significant and in the absence of any macroscopic or microscopic correlates are not considered relevant.

Table 25. Key findings of 90-day dietary toxicity study in dog; statistically significant changes are shown as percentages of control value

Dose (ppm) **mg/kg bw per day**	**0** **0**		**60** **2.5**		**600** **24.3**		**6000** **206.6**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Food consumption								
weeks 1–4					-	**↓19%***	↓23%	**↓37%*****
weeks 1–13					-	**↓14%***	**↓14%***	**↓31%*****
Body weight week 13					-	↓8%	↓11%	↓21%
Body weight gain (kg)	2.01	2.26	2.31	1.94 (↓14%)	2.58	1.61 (↓29%)	**0.80****	**0.05*****
weeks 1–13								
Urinalysis (incidence /5)								
Bilirubin	0	0	0	0	0	0	3	0
Blood	1	2	1	2	2	1	3	2
Haematology								
RBC			**↓10%***	-	-	-	**↓11%***	**↓12%***
Haemoglobin, Hb							**↓12%****	**↓14%****
Haematocrit, Ht							**↓10%***	**↓12%****
Reticulocytes					**↑42%**§**	-	**↑33%**§**	↑31%
WBC							**↑51%***	↑21%
Neutrophils							↑53%	↑24%
Platelets							**↑72%***	-
PT							-	**↑24%****
APTT							-	**↓16%***

Dose (ppm) mg/kg bw per day		0 0		60 2.5		600 24.3		6000 206.6	
		M	F	M	F	M	F	M	F
Clinical chemistry									
Albumin								**↓14%***	**↓25%****
A:G ratio								**↓37%****	**↓24%****
Glucose								-	**↓13%***
Aspartate transaminase, AST								**↑76%*****	**↑46%****
Alkaline phosphatase, ALP								**↑2484%****	**↑3058%****
γ-Glutamyl transpeptidase, GGTP §								**↑120%****	↑60%
Organ weight									
Adrenals	absolute					↑11%	-	↑12%	↑17%
	relative							↑27%	↑50%
Liver	absolute							↑13%	-
	relative							**↑26%***	↑14%
Spleen	absolute							-	↑75%
	relative							-	**↑106%*****
Thyroids, (mean)	absolute						↑13%		↑20%
Thyroids/	relative						↑22%		↑50%

Statistically significant modification: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Regression analysis: significant trend: § $p < 0.05$

The NOAEL for this study was established at 60 ppm (equivalent to 2.6 mg/kg bw per day) based on decreased food consumption and body weight gain observed in females at 600 ppm (equivalent to 24.3 mg/kg bw per day) (Pickersgill, 1991c).

Study 3

Groups of four males and four females Beagle dogs were fed for 52 weeks with diets containing 30, 300, 1000, and 3000 ppm metconazole (WL 148271: purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01). Achieved doses for males were 1.1, 12.0, 38.5, and 110 mg/kg bw per day and for females 1.1, 10, 36.5, and 113.7 mg/kg bw per day.

The stability of the test substance and the homogeneous distribution, the stability and correct concentration of the test substance in the diet were confirmed by analysis.

One high-dose moribund male was euthanized following a history of diarrhoea (sometimes containing blood), serious feed consumption decreases and subsequent body weight loss. At necropsy, pale pancreas, ileo-caecal junction/colon mucosa redness and ileum mucosa thickening were observed, together with mesenteric lymph node enlargement and dark color. The dog showed severe chronic ileum enteritis and caecum/colon chronic inflammatory cell infiltration. However, body weight of this animal was 18% less than the mean group weight at commencement, thus it is not obvious if the poor general state was related to test substance intake alone.

Food consumption of high-dose animals decreased slightly in the first week, thereafter there was little difference between treated and control animals; body weight of 1000 ppm and 3000 ppm males was lower than control. However, the males showed a dose-dependent body weight gain reduction when calculated over the first 13 weeks at 1000 ppm and above. On week 13 lens opacity was increased in both sexes at top dose. At termination the increase compared to controls was evident in the males, but not the females, although the severity of lesions in the top-dose groups increased as the study progressed. In one male and one female the lesion was observed in association with uveitis.

Urinalysis revealed no relevant findings.

Platelet count was consistently elevated in top-dose animals on weeks 13, 26 and 52. The leukocyte count was also increased in males at all sampling times, sometimes in association with increased neutrophil counts. Other increases in RBC count, haematocrit of males at lower doses on weeks 13, 26 or 52 (reaching statistical significance occasionally) were not considered relevant, in the absence of a dose–response relationship, or when observed at predose sampling times.

There was a tendency towards decreased albumin and A:G ratio in the top-dose animals, although statistical significance in both sexes was only achieved on week 13. The effects seen at lower doses were spurious, in the absence of dose-dependency.

Alkaline phosphatase activity was very significantly and dose-dependently increased in both sexes and at all sampling times, attaining high significance at 1000 ppm and above.

On week 52 weak ALP increases were also observed at 30 ppm and 300 ppm, which were not considered of toxicological relevance since neither evidence of other enzymatic alterations, suggestive of hepatotoxic effects, nor histological findings in the liver were reported at those doses. Rather, the effect may have been secondary to induction phenomena.Thus, the small ALP increase at 300 ppm is an isolated finding and no other liver parameters were increased at that dose, thus the change is not considered adverse.

Finally, weakly increased GGTP (week 13, females) and creatinine phosphokinase (CPK) values (week 26, males) were noted at the top dose, but not at termination.

At the top dose, weights of liver, both absolute and relative, were increased (13% and 12% respectively for males, 26% and 32% respectively for females). Likewise for male spleen weights (14% and 17% increases respectively). Minimal weight increases (10–12%) were observed in kidney (males) and thyroid (both sexes), but these were probably irrelevant, in the absence of dose–response and other parameters indicating effects in these organs.

A slight increase at the top dose (3000 ppm) in both sexes (males 12%, females: 14%) was observed without any related macroscopic or histopathological observations.

Relative ovary weight was increased (45%) at the top dose (3000 ppm), but it was noted that considerable variations in weight were also observed at lower doses, probably reflecting various stages in estrus cycle of the individual animals: some ovaries contained large follicles while others contained corpora lutea.

Eye lens degeneration was detected in top-dose animals, varying from minor, multifocal degeneration (in two males and one female) to severe degeneration (lens swelling/liquefaction in males or adhesion and iris cyst in females) and apparent lens thinning (in one male and one female). The severe cases were reported in association with eye opacity at necropsy.

Liver lesions included hepatocyte hypertrophy and vacuolation at the top dose, and Kupffer cell pigmentation at 1000 ppm (females) and above (both sexes). Cell hypertrophy was predominantly midzonal or periportal, and the finding was in line with the observed liver weight increase. Evidence of treatment-related siderosis was found in Kupffer cell pigmentation, which appeared dose-related, both in incidence and severity.

The increased haematopoiesis and pigmentation (severity) in the spleen of top-dose animals was indicative of increased RBC turnover and breakdown.

Other findings included increased incidence of minor focal trachea metaplasia and increased caecum inflammatory lesions, although the aetiology of these findings was unexplained.

Results are summarized on the following page in Table 26.

Table 26. Key findings of one-year dietary toxicity study in dog; statistically significant changes are shown as percentages of control value

Dose (ppm) mg/kg bw per day		0 0		30 1.1		300 10		1000 36.5		3000 110	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Body weight gain	wk 1–13	1.16	0.51	0.96	0.78	1.08	0.44	**0.51**§§	0.6	**0.46**§§	0.28
	wk 1–52	1.38	0.65	1.69	1.39	1.25	0.64	1.21	1.35	0.72	0.79
Ophthalmology											
Cataract	wk 13	1	0	0	0	0	0	0	0	2	3
	wk 52	1	3	0	0	1	0	0	1	3	2
Haematology (% change relative to controls)											
Platelets	wk 52									**↑48%**§	**↑35%**§
WBC	wk 52									**↑35%**§	-
Clinical chemistry (% change relative to controls)											
Albumin	wk 52				**↓****					-	**↓13%****
A:G ratio	wk 52									↓22%	**↓20%***
ALP	wk 52			-	**↑35%***	↑76%	**↑45%***	**↑227%***	**↑378%***	↑1131%	**↑1454%***
GGTP	wk 13									-	**↑75%***
CPK	wk 26									**↑137%**§	↑42%
Organ weight (% change relative to controls)											
Liver	absolute									↑14%	↑26%
	relative									↑13%	↑28%
Spleen	absolute									↑14%	-
	relative									↑14%	-
Thyroids	absolute						↑5%		↑16%		↑11%
	relative						↑7%		↑11%		↑14%
Ovaries	absolute					↑16%					↑41%
	relative					↑21%		-		-	↑45%
Histopathology (incidence /4)											
Adrenal											
mineralization		0	0	0	0	0	0	0	0	1	0
vacuolation		0	0	0	0	0	1	1	0	0	1
Caecum											
Congestion/haemorrage		0	0	3	0	0	1	2	2	3	3
Eye											
Lens degeneration		0	0	0	0	0	0	0	0	3	2

Dose (ppm) mg/kg bw per day	0 0		30 1.1		300 10		1000 36.5		3000 110	
	M	F	M	F	M	F	M	F	M	F
Liver										
hepatocyte vacuolation	0	0							1	1
Kupffer cell pigmentation	1	1	1	1	1	0	0	3	4	4
erythrophagocytosis	0	0							1	0
hepatocyte hypertrophy	0	0							3	4
Spleen										
↑ haematopoiesis	0	0							4	2
Trachea										
squamous metaplasia	0	0	0	0	0	1	0	1	0	3
Ovary										
corpora lutea	-	2	-	3	-	3	-	3	-	4

WBC White blood cells

A:G ratio Albumin:globulin ratio

ALP Alkaline phosphatase

GGTP γ-glutamyl transpeptidase

CPK Creatinine phosphokinase

Statistically significant modification, Williams' or Wilcoxon test: * $p < 0.05$ ** $p < 0.01$

Statistically significant trend: § $p < 0.05$ §§ $p < 0.05$

The NOAEL for this study was established at 300 ppm (equivalent to 10 mg/kg bw per day) based on significantly increased alkaline phosphatase activity and decreased body weight gain observed at 1000 ppm (equivalent to 36.5 mg/kg bw per day) (Clay, 1992a).

(b) Dermal application

Metconazole (purity 98.7%; 84.3% *cis*, 14.4% *trans*; Batch AS 2122a) was applied by dermal application to groups of 10 male and 10 female Fisher 344 rats at dose levels of 0 (water), 250, 500 and 1000 mg/kg bw per day for 6 h per day during a period of 21 days under semi-occlusive conditions. General health, mortality and moribundity were checked twice daily and clinical sings of toxicity were checked daily. Detailed clinical observations (DCO) were performed on days 1, 7, 14 and 22. Body weight measurement were conducted prior to start of administration (day −1), and weekly thereafter on days 7, 14 and 21. Food consumption was determined on a weekly basis. Ophthalmoscopy was performed prior to application (on day −9) and at the end of the study period on day 21. As a previous range-finding study did not indicate any potential neurotoxicity at dose levels up to 1000 mg/kg bw per day using a functional observation battery (FOB) assessment, a FOB was not included in this study. Haematology and clinic chemistry examinations were carried out at the end of the study period. At termination, animals were subjected to gross necropsy and histopathology.

No test substance-related effects on food consumption were observed. Decreased body weight gain of 11% (not statistically significant) was reported for males at the top dose. No treatment-related changes of haematological parameters or clinical chemistry were observed. Although in the absence of reported gross necropsy and histopathology alterations, trends for relative liver weight modifications (+14%, statistically significative) were reported.

Based on the results of the study, the systemic NOAEL in both sexes was 1000 mg/kg bw per day, the highest dose tested (Bonnette, 2006).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

cis/*trans*-Metconazole (purity 95.3%; *cis*:*trans* 79.8:15.5; lot/batch 89-01) was administered for 22 months at dietary concentrations of 0, 30, 300, and 1000 ppm to groups of 51 Crl:CD-1 (ICR) BR mice per sex. Achieved test substance intake was 4.4, 43.6, and 144.9 mg/kg bw per day in males and 5.2, 53.0, and 179.2 mg/kg bw per day in females. Satellite groups (12/sex per group) were treated at the same concentrations and were sacrificed after 51 weeks of treatment. The stability of the test substance for two weeks at ambient temperature, its homogeneous distribution and correct concentration within a range ± 15% of nominal values in the diet, were confirmed by analysis.

Survival rates at week 91 for the animals receiving 0, 30, 300 and 1000 ppm were 47%, 44%, 42% and 43% respectively for males, and 51%, 54%, 69% and 56% respectively for females.Survival after 90 weeks was not adversely affected by treatment. In high-dose female decedents there was an increased incidence of liver tumours (in 52% of decedents). Of the 11 decedents with cause of death liver tumour, two had only adenoma, two had carcinoma and the rest (seven animals) showed both adenoma and carcinoma. As most animals showed both adenoma and carcinoma, a tumour promoting mechanism is likely.

During the second half of the study (weeks 62 to 90 for males, and weeks 52 to 90 for females) there was an increase in the occurrence of swollen abdomens in the top-dose animals only. At week 90 the incidence of swollen abdomen in the 1000 ppm group was 37.5% (9/24) for males and 43.8% (14/32) for females, as compared to 12.0% (3/25) for males and 11.5% (3/26) for females in the control group. There were no other treatment-related clinical observations.

Food consumption for males was decreased at the top dose in the first week of treatment. Small differences from the control group were observed until the end. For females, food consumption was statistically significantly decreased during weeks 1 to 13 and weeks 56 to 88 in the 1000 ppm group.

Mean body weights were statistically significantly decreased for males and females at 1000 ppm due to a body weight loss following one week of treatment. Slight and consistent (but not statistically significant) reductions in body weight were observed throughout the study in top-dose males (generally below 10%). In females slightly decreased body weights were observed at the intermediate dose and top dose from week 1 to week 88 (8% and 13%, respectively). Mean body weight gains were statistically significantly decreased due to a body weight loss following one week of treatment for males (−1.7 g, $p < 0.01$) and females (−1.0 g, $p < 0.001$) at 1000 ppm, compared to controls. Mean body weight gains during weeks 0 to 52 were significantly decreased; 23% for males and 32% for females at 1000 ppm, compared to controls. Overall body weight gain during the treatment period was significantly reduced by 25% for males and 32% for females in the 1000 ppm group when compared to controls.

At termination, an increased total leukocyte count was observed at 300 ppm (males) and above (both sexes). At the top dose a concomitant increase in both neutrophils and lymphocytes was noted in males and females. The findings at low and intermediate dose on week 52 (decrease of lymphocyte count) were considered irrelevant in the absence of dose–effect relationship.

Clinical chemistry parameters at week 52 and/or week 91 revealed highly increased ALT and AST activities (up to 4.4-fold of control in males and 11.4-fold in females) at the top dose of 1000 ppm in all animals (more pronounced in females), indicating marked hepatotoxicity at this dose level. At the mid dose of 300 ppm, only small increases in serum ALT and AST activity, under two-fold of the control, were induced (1.1–1.9-fold) in females, which is a similar response to that seen with other liver enzyme-inducing compounds in rats (Hall et al., 2012). This was also supported by the liver histopathology findings described below. Total cholesterol was decreased at intermediate sacrifice in males and females at 300 ppm and above, and triglyceride levels were decreased on weeks 52 and 91 at 300 ppm and above.

All top-dose animals showed markedly increased liver weights, and reduced spleen weights, both at interim and terminal kill. The liver weight increase was much more pronounced at the top dose and the effect was most prominent at terminal sacrifice. The slight liver weight increase observed at 300 ppm (mainly in females at interim kill) was also considered related to treatment, since body weight differences from controls were unremarkable at that dose.

The most notable necropsy findings in the liver of top-dose animals included enlargement and thickening or patchy/focal paleness, both at interim and final sacrifice. At termination, irregular surface appearance, and presence of multiple masses and nodules was obvious at top dose. Notably small spleens were observed at 300 ppm (females) and above (both sexes), while pale/mottled appearance of the spleen was noted in the top-dose group at week 52 and at week 91.

Liver histopathology findings consisted of increased incidences of vacuolation and hypertrophy in both sexes at 300 ppm and above. Parenchymal hypertrophy was consistent with the observed induction of CYP450 enzymes in the mouse. There was a dose-related increase in single cell necrosis/sinusoidal hypercellularity/pigmentation reported at the mid and top doses in both sexes, with females the more sensitive. In addition, increased hyperplastic proliferation of oval cells and biliary duct cells (both sexes), and multifocal parenchymal hyperplasia were observed in top-dose animals, with slightly increased prevalence in females.

The observed hepatic findings for oval cell hyperplasia, proliferation of biliary duct cells (both sexes), increased incidences of vacuolation and increase in single cell necrosis/sinusoidal hypercellularity/pigmentation at the top dose are considered clear evidence of liver toxicity. Oval cell proliferation is considered the one histological evidence, that together with further findings at this top dose, indicate a hepatotoxic effect (see Hall et al., 2012). The indication is that at this top dose the maximum tolerated dose had clearly been exceeded.

At 1000 ppm in males and females, decreased spleen weights at weeks 52 and 91 for males and females, spleen atrophy and concomitant prominent appearance of connective tissue (trabeculae and stroma) was observed, which may also be secondary to statistically significantly decreased terminal body weights, particularly at the top dose. Furthermore, pigment deposition in the corticomedullar part of adrenals was also observed at 300 ppm and above.

With regard to neoplastic findings, increases in liver adenoma were seen at the interim kill in the top-dose females. In the main study a dose-related increased incidence of liver adenoma was seen in females at 300 ppm, and above in males and females. Liver carcinomas were slightly increased at mid and top dose in males without a dose–response relationship; they were markedly increased in females only at the top dose. Liver tumour incidences in males were in all cases within the in-house historical control data for adenoma, and just outside the historical range for carcinoma at mid and high doses. In females, liver adenoma at 300 ppm and above and liver carcinoma at 1000 ppm were outside the historical control range. Therefore, metconazole was considered to be carcinogenic in female mice at dose levels of 300 ppm and above.

A non-statistically significant increase in skin sarcomas was observed in males at all doses tested (0, 3.9, 5.9 and 9.8% of the male animals at 0, 30, 300 and 1000 ppm, respectively). These findings were restricted to the unscheduled deaths, whilst at termination the subcutaneous sarcoma incidence was unremarkable.

The evidence does not support a treatment-related aetiology for skin/subcutis (subcutaneous) tumours observed only in male mice in the metconazole carcinogenicity study, but is rather related to randomly occurring skin lesions, wounds, infections (such as dermatitis, abscess, ulceration) from repeated injuries from in-cage fighting as a consequence of group housing (Creek & Hess, 2012). These reactive skin wounds most likely, with time and with recurrent injuries, became reactive, localized proliferation lesions/tumours. Additional data from Haseman, Bourbina & Eustis (1994) confirm that male mice are known to engage in more aggressive behaviour when housed together, compared to females, and demonstrate that group-housed male control B6C3F1 mice have a much higher incidence of skin/subcutaneous tumours, compared to singly-housed control male mice.

Skin tumour incidence at the high dose level exceeds the historical control range (see Creek & Hess, 2012), which mostly used individually-housed mice, but the remaining evidence does not support a carcinogenic mode of action for metconazole.

A further analysis of cell type in mouse skin sarcoma was performed; as a consequence, one sarcoma in group 4 and one sarcoma in group 3 were classified as other types of tumour. It is this reassessed sarcoma incidence that is provided with the summarized study results in the Table 27 on the following page.

Table 27. Key findings of 22-month dietary toxicity study in mice

Dose (ppm) mg/kg bw per day		0 0		30 4.4		300 43.6		1000 144.9		HC	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Survival (%)		47	51	44	54	42	69	43	56		
Nunber of decedents		28	25	30	26	31	15	32	21		
Cause of mortality: liver tumour		0	0	2	1	3	0	2	11		
Food consumption											
week 1								**↓14%*****	**↓10%*****		
weeks 2–13								-	**↓4%***		
weeks 56–88								↓3%	**↓6%**§		
Body weight						-	↓8%	↓7%	↓13%		
Body weight gain (g)											
weeks 0–1		1	0.8	0.9	0.4*	0.7	0.8	**−1.7****	**−1.0*****		
weeks 0–88		12.3	13.7	13	12.5 (↓9%)	11.2 (↓9%)	11.0 (↓20%)	**9.2*** **(↓25%)**	**9.3***** **(↓32%)**		
Haematology (% change relative to controls)											
White blood cells	wk 90					**↑35%***	-	**↑68%*****	**↑230%*****		
Neutrophils	wk 90							**↑91%****	**↑318%*****		
Lymphocytes	wk 90							**↑40%***	**↑264%***		
Clinical chemistry (% change relative to controls)											
Cholesterol	wk 52					**↓38%*****	**↓38%***	**↓64%*****	**↓46%****		
	wk 91					**↓***	**↓47%*****	-	-		
Triglycerides	wk 52					**↓24%**	**↓16%**§	**↓36%****	**↓31%**§		
	wk 91					**↓18%**§	**↓15%**	**↓31%**§	**↓35%****		
AST, aspartate transaminase	wk 52					-	**↑21%***	**↑63%*****	**↑209%*****		
	wk 91					-	**↑16%*****	**↑253%*****	**↑313%*****		
ALT, alanine transaminase	wk 52					-	-	**↑149%*****	**↑551%*****		
	wk 91					-	**↑94%*****	**↑342%*****	**↑1040%*****		
Organ weight (% change relative to controls)											
Liver (adjusted)	wk 52	1.72	-	1.62	-	1.77	-	**2.6*****	-		
	wk 91	1.45	1.31	1.72	1.34	1.77	1.67	**3.58*****	**4.38*****		
Spleen (adjusted)	wk 52	0.14	0.14	0.11	0.11	0.11	0.11	0.09	0.09		
	wk 91	0.12	0.15	0.1	0.12	0.12	0.1	**0.6***	0.1		

Dose (ppm) mg/kg bw per day		0 0		30 4.4		300 43.6		1000 144.9		HC	
		M	F	M	F	M	F	M	F	M	F
Histopathology: non-neoplastic findings											
Liver											
Number examined	wk 52	12	12	12	12	12	12	12	12		
	wk 91	51	51	51	51	51	51	51	51		
hepatocellular vacuolation	wk 52	3	5	4	4	5	10	6	10		
	wk 91	11	11	10	12	20	36	37	44		
hepatocellular hypertrophy	wk 52	0	0	0	0	9	3	11	10		
	wk 91	0	0	0	0	13	8	44	38		
single cell necrosis/sinusoidal	wk 52	0	0	0	0	2	3	9	11		
hypercellularity/ pigmentation	wk 91	0	0	1	0	10	18	37	35		
multifocal hepatocellular	wk 52	0	0	0	0	0	0	1	9		
hyperplasia	wk 91	0	0	1	0	0	0	31	44		

Neoplastic findings

Dose (ppm) mg/kg bw per day	0 0		30 4.4		300 43.6		1000 144.9		Historical controls (*Date range*; value range)	
	M	F	M	F	M	F	M	F	M	F
Liver										
Number examined	62	62	63	63	63	63	62	63	*88–91/86–96*; 407/276	*88–91/86–96*; 407/277
hepatocellular adenoma	11	0	17	1	16	4	35	50	*88–91/86–96*; 10–33%/17–37%	*88–91/86–96*; 0–2%/0–2%
hepatocellular carcinoma	4	0	4	1	7	0	7	20	*88–91/86–96*; 2–18%/2–6%	*88–91/86–96*; 0–2%/0%
hepatocellular adenoma and carcinoma	13	0	17	2	19	**4***	**38*****	**52*****		
Skin										
Number examined	51	51	51	51	51	51	51	51	*88–91/86–96*; 407/276	*88–91/86–96*; 407/277
subcutis sarcoma	0/63	1/62	2/63	2/63	3/63	1/63	5/62	0/63	*85–94*; 0–6.9%	*85–94*; 0–4%
reassessed subcutis sarcoma	0/63	1/62	2/63	2/63	2/63	1/63	4/62	0/63	*85–94*; 0–6.9%	*85–94*; 0–4%

Statistically significant modification: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

In the chronic toxicity study with metconazole in mice the liver (hypertrophic and hyperplastic events) and spleen (atrophy) were identified as the target organs. Based on these effects the toxicity NOAEL was 30 ppm (4.4 mg/kg bw per day). The presence of liver adenoma at medium and high doses, and of liver carcinoma at the high dose level in female mice was considered to be treatment-related and led to the NOAEL for carcinogenicity being set at 30 ppm (4.4 mg/kg bw per day) (Clay, 1992b).

Rat

Study 1

Metconazole (purity 95.3%; *cis*:*trans* 79.8:15.5, lot/batch 89-01) was administered at dietary concentrations of 0, 10, 100, 300, and 1000 ppm to Fischer 344 (SPF) rats. The number of treated rats per sex was 40 (vehicle control) and 20 (test article) for the terminal two-year sacrifice, or 20 (vehicle control) and 10 (test article) for the interim one-year sacrifice. Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. Achieved test article intake was 0.4, 4.3, 13.1, 43.9 mg/kg bw per day in males and 0.5, 5.3, 16.0, and 53.8 mg/kg bw per day in females. Analytical verification revealed dose levels being within 10% of nominal values. Compound stability for four weeks in the diet at ambient temperature, and compound homogeneity proved to be satisfactory.

There was no treatment-related increase in mortality. Survival rates after 104 weeks increased with increasing dose-levels. Lowest survival (even lower than historical controls) was noted in the study controls. Intermittent deaths were mostly caused by mononuclear cell leukemia, pituitary and uterine tumours, and chronic nephropathy, which commonly occur in old rats. There were no significant differences in death cause among the study groups. There were no notable treatment-related clinical observations. At interim sacrifice one male of the high-dose group was found dead. The cause of death was not determined.

All top-dose animals of the two-year sacrifice group showed an initial body weight drop, by 8–10% (males, weeks 1–10) and by 6–7% (females, weeks 1–7), which was sustained until week 80 at 6–8% and thereafter until week 104 at 4–5%. Overall, body weight gains during the 104-week treatment period were decreased by 9% for males and 6% for females at 1000 ppm, compared to controls. At the top dose, consistent decreases in food consumption were observed in the starting phase of the treatment (up to week 11 in females and week 3 in males), but differences during the last 20 weeks of treatment were slight or unremarkable. There were no treatment-related effects on body weight or food consumption in the other treatment groups.

Transient, slight reductions in RBC parameters (Hb concentration, erythrocyte mean diameter, and MCH were observed in females at 1000 ppm following 13 weeks of treatment in the 104-week study. Furthermore, slight (but statistically significant) decreases in platelet count and concentration were noted in males and females at 1000 ppm, compared to controls. These effects generally persisted and were seen at weeks 26 and 52. No treatment-related haematological effects were observed during the latter half of the study (weeks 77 and 104). The transient, slight reductions in RBC parameters are consistent with similar reductions RBC parameters observed for females at 1000 ppm in the 90-day study. Although these modifications were subtle, signs of anaemia were also described in the short-term studies and are therefore considered to be treatment-related. The mild monocytosis seen in the top-dose females (weeks 51, 77 and 104) was of questionable toxicological significance, as no abnormal cell types were reported, and no treatment-related leukemia was seen. An evaluation of clinical chemistry parameters showed a statistically significant increase in GGTP in males at week 26 and in females at weeks 26, 52, and 77 in the 1000 ppm group when compared to controls. At the top dose, statistically significant decreases in a number of parameters included: serum cholesterol and triglycerides in both sexes), bilirubin (both sexes), small decreases in albumin (females), and slight reductions in ALP (males) and ALT (both sexes). These were observed during one of the intermediate analysis intervals but not at termination. The increase of GGTP activity, as well as the decreased lipid parameters was consistent with observed hepatic lesions seen in histopathology. On the other hand, the (slight) decreases of the transaminase ALT and ALP activity remained unexplained. Increased urine osmolarity values and decreased urine volumes were observed in top-dose males during weeks 51, 77 and 104. However, the significance of these modifications was unclear due to the absence of nephrotoxicity both at intermediate and final sacrifice.

Statistically significant increases in relative liver weight were observed at 300 ppm (5%) and 1000 ppm (20%) in males at week 52, and at the top dose (1000 ppm) in females at termination (12%). Relative spleen weights were increased at 300 ppm (males 39%)) and 1000 ppm (males 56%; females 21%) at week 104. Already at 52 weeks a marginal but statistically significant relative spleen weight increase (9%) was observed in top-dose females.

At intermediate sacrifice, gross liver lesions (mottled appearance, enlargement, and on one occasion fatty appearance) were apparent in males at 300 ppm or 1000 ppm. Increased hepatocellular lipid vacuolation and centrilobular hypertrophy was observed in males at 300 ppm and in both sexes at 1000 ppm. Furthermore, at 52 weeks some evidence of cytotoxicity was suggested by increased slight mononuclear cell infiltrate in the liver of top-dose males and females, and the incidence of inflammatory necrotic foci was also slightly increased at this dose in males. However, no signs of necrotic inflammatory foci were observed at termination. At termination, lipid vacuolation and centrilobular hypertrophy was noted in females and males at the top dose. Top-dose males also showed increased hepatic pigment deposition in the liver, such as might be indicative of slight haemosiderosis taken along with subtle haematological disturbances that were noted, a finding that was also reported in earlier studies. In this study a slight increase in clear-cell hepatocellular foci was reported in top-dose male animals. This finding also occurs spontaneously in untreated Fischer rats, however at the same dose level in the carcinogenicity study, clear cell foci were much more prominently increased and were considered a consequence of the sustained liver activation. In correlation with the increased spleen weights an increased incidence of splenic histiocytic foci was noted for both sexes at the 1000 ppm dose level at termination. The findings in the liver (increased weights, centrilobular hypertrophy, vacuolation) were also reported in short-term studies and were associated with liver activation and the induction of metabolizing enzymes (see also mechanistic study in Section 2.6(c). The liver effects were more pronounced in males. There was an apparently increased incidence of 1, 2, 4, 5 and 6 masses/nodules seen in the pancreas at necropsy at termination in males of 0, 10, 100, 300 and 1000 ppm dose groups, respectively, which was not confirmed by the histopathology of this organ. Based on single animal sheets, most nodules in the pancreas correlated to islet cell adenomas, lymph nodes, or ectopic spleen. Thus, the macroscopic findings are considered unrelated to treatment and of no toxicological significance.

Table 28. Key findings of two-year dietary toxicity study in rat

Dose (ppm) **mg/kg bw per day**		**0** **0**		**10** **0.4**		**100** **4.3**		**300** **13.1**		**1000** **43.9**	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
						(% change relative to controls)					
Survival (%)	wk 104	48	75	55 ↑15%	60 ↓20%	55 ↑15%	60 ↓20%	60 ↑25%	65 ↓13%	90 ↑88%	75 0
	wk 108	38	65	45 ↑18%	55 ↓15%	45 ↑18%	60 ↓8%	50 ↑32%	55 ↓15%	**70*** ↑84%	75 ↑15%
Food consumption	wks 1–13									↓10%	↓5%
Body weight	wk 104									↓5%	↓5%
Body weight gain	wks 0–13									↓16%	↓4%
	wks 0–80									↓10%	↓9%
	wks 0–104							-	↓9%	↓9%	↓6%

(*Table 28 continued on the following page*)

Dose (ppm) mg/kg bw per day		0 0		10 0.4		100 4.3		300 13.1		1000 43.9	
		M	F	M	F	M	F	M	F	M	F
Clinical chemistry (% change relative to controls)											
Total cholesterol	wk 26							-	**↓11%***	**↓31%****	**↓8%****
	wk 51									**↓25%****	-
	wk 77									**↓35%****	-
Triglycerides	wk 26									**↓61%****	**↓30%****
	wk 51									**↓54%****	**↓44%****
	wk 77									**↓43%****	**↓30%***
Albumin	wk 26							-	**↓3%****	-	**↓4%****
	wk 51							-	**↓3%***	-	**↓3%****
Bilirubin	wk 26									**↓9%****	**↓11%****
	wk 51									-	**↓10%****
	wk 77									**↓15%****	-
Akaline	wk 26									**↓23%****	-
phosphatase, ALP	wk 51							**↓10%****	-	**↓26%****	-
	wk 77									**↓17%****	-
Alanine	wk 26							-	**↓26%***	-	**27%****
transaminase, ALT	wk 51							-	**↓17%***	-	**↓30%****
	wk 77							-	↓9%	**↓24%****	**18%****
γ-glutamyl	wk 26		0.1							**1.6***	**0.8****
transpeptidase,	wk 51		0.5								**1.4****
GGTP (IU)	wk 77		0.2								**0.9****
Organ weight (% change relative to controls)											
Terminal body weight	52									↓4%	**↓7%***
	104									↓5%	↓5%
Liver, wt, relative§	52							**↑5%***	-	**↑20%****	↑6%
Liver wt, relative§	104									-	**↑12%***
Spleen wt, relative§	52									-	**↑9%***
Spleen wt, relative§	104							**↑39%**	-	**↑56%***	**↑21%***
Histopathology											
Liver											
Mononuclear cell infiltrate, slight	52	3	4	0	1	1	1	4	2	**7****	**8****
Mononuclear cell infiltrate, moderate	52	0	0	0	0	0	0	0	0	2	1
Necrotic inflammatory focus	52	2		1		4		**5***		**5***	
Centrilobular hypertrophy	52	0	0					**3***	1	**10*****	**7*****
	104	2	0							**6***	1

Dose (ppm) mg/kg bw per day		0 0		10 0.4		100 4.3		300 13.1		1000 43.9	
		M	F	M	F	M	F	M	F	M	F
Macrovesicular vacuolation, CL	52	0	0							0	**7*****
Fatty vacuolation, CL	104	2	1	1	2	0	0	1	0	3	**9*****
Pigment deposit	104	2	0	2	0	0	0	1	0	**9*****	0
Clear-cell foci	104	4	3	2	2	3	0	4	2	**8***	5
Spleen											
Histiocytic foci	104	1	4	1	0	0	2	0	2	**8*****	**14*****
Adrenals (cortex)											
Vacuolation, total incidence$^{\#}$	104	15	1	5	1	9	1	9	2	10	0

CL Centrilobular

§ Relative weight here is not the proportion between organ and body weight, but covariate analysis with "terminal body weight as a covariate"

$^{\#}$ Total incidence for score-expanded finding

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

In this chronic toxicity study target organs were the liver and the spleen. The toxicity NOAEL was 100 ppm (4.3 mg/kg bwper day) based on increased liver and spleen weights, as well as hepatocellular hypertrophy and adrenal cortical vacuolation at 300 ppm, the lowest-observed-adverse-effect level (LOAEL, 13.1 mg/kg bw per day) (Taupin, 1992a).

Study 2

Metconazole (purity 95.3%; *cis*:*trans* 79.8:15.5; lot/batch 89-01) was administered at dietary concentrations of 0, 100, 300, and 1000 ppm to 50 Fischer rats per sex and group for 24 months. Samples were checked analytically to ensure accuracy, homogeneity and stability of the administered diet. Achieved test article intake was 4.6, 13.8, 46.5 mg/kg bw per day in males and 5.5, 16.6, 56.2 mg/kg bw per day in females. Analytical verification revealed dose levels within 10% of nominal values. Compound stability was proven for two weeks in the diet at ambient temperature, and compound homogeneity was shown to be satisfactory.

There were no treatment-related effects on mortality. Survival rates at study termination for the animals receiving 0, 100, 300 and 1000 ppm were 60%, 56%, 50% and 66% respectively for males, and 66%, 60%, 62% and 68% respectively for females. The most common causes of death in both treated and untreated animals were pituitary tumours, disseminated mononuclear cell leukemia and severe chronic nephropathy. All these findings commonly occur in ageing untreated control rats of the F344 strain and there were no significant differences in the causes of death between control and treated animals. There were no notable treatment-related clinical observations. Ophthalmology revealed a slightly increased incidence of keratitis in top-dose females (12/20) compared to controls (9/20).

Mean body weights were statistically significantly reduced during the initial 13 weeks of the treatment period for males at 1000 ppm and throughout the treatment period for females at 1000 ppm, compared to controls. Body weight gains were slightly reduced at both the week 0–13 interval and the week 16–104 interval for males and females at the top dose, with the largest differences occurring during the first week of treatment. Overall, body weight gains in the 1000 ppm group were decreased by 5% for males and 9% for females during the 104-week treatment period. Food consumption was statistically significantly decreased during treatment weeks 1 to 8 for males, and at numerous intervals during the first 72 weeks for females at 1000 ppm. There were no treatment-related effects on body weight or food consumption in the other treatment groups.

Haematological evaluations showed minimal (but consistent) statistically significant decreases in erythrocyte mean diameters in top-dose males at all sampling times, and in females at week 52. Changes in erythrocyte morphology (microcytosis, anisocytosis and spherocytosis) were observed for males in the 1000 ppm group, suggestive of a slight haemolytic anemia. Clinical chemistry and urinalysis were not performed in the study.

The organ weight analysis at terminal sacrifice showed statistically significantly increased weights of liver and spleen (males and females), as well as of kidneys and adrenals (males) in top dose animals only. Relative (to body weight) liver weights were increased for males and females, relative adrenal weights were increased for males, and relative spleen weights were increased for males and females at 1000 ppm, compared to controls. Relative kidney weight was increased compared to controls for males at 1000 ppm.

Macroscopic evaluation showed an increased incidence of pale kidneys in all treatment groups but only in males.

Histopathological evaluation showed increased incidences of hepatocellular vacuolation at 1000 ppm, which was more pronounced in males. Furthermore, increased hepatocellular (centrilobular) hypertrophy and pigment deposition was seen in males at 300 and 1000 ppm, as well as statistically significantly increased clear-cell (both sexes) and eosinophilic foci (males) in the liver at 1000 ppm, compared to controls. Liver hypertrophy and vacuolation were consistently observed in other rat feeding studies and are associated with liver activation and liver enzyme induction. Pigment deposition in the liver of mid- and high-dose males was also a change seen in the 90-day rat study by Esdaile (1991b) after exposure to 3000 ppm of *cis*/*trans*-metconazole, where it was demonstrated to be Fe^{3+}. This may be associated with haematological disturbances. Morphological observations of erythrocyte microcytosis in top-dose males would suggest anaemia in this dose group. No other evidence of increased erythrocyte destruction was seen histologically. Since no specific stain was performed to identify the origin of the pigments in this study they could also represent an aging pigment (lipofuscin). However, the evidence from the subchronic study provides quite a strong indication that it involves haemosiderin. The increased incidence of both clear cell and eosinophilic foci, more strongly observed in males in this study, may represent a hepatocellular proliferative response to the compound. The incidence of adrenal cortical vacuolation was increased in males at 300 and 1000 ppm. This change is considered to be reflected by the increased adrenal weights in males at 1000 ppm. In this study observed forestomach lesions were consistent with irritation and inflammation and are considered to induce pain and distress. In the forestomach of treated male rats an increased incidence of histological changes associated with erosion and ulceration was seen which was not dose-related; this incidence was not considered to be related to treatment by the study's author. An increase in inflammatory and hyperplastic changes in the non-glandular stomach of males at 100 ppm and above was reported without a clear dose–response. The relative incidence of erosions and ulcers at the intermediate doses (but not top dose) exceeded slightly those of the in-house historical controls.

It has to be noted that this carcinogenicity study was run in parallel with the rat chronic toxicity study, which did not report adrenal changes at similar dose levels and in which no stomach lesions were detected. However, similar stomach lesions were present in both subacute and subchronic rat studies, hence, a causal relationship with treatment is probable. In the kidney, a slight increase in the severity of chronic renal nephropathy was observed in males at 300 ppm and 1000 ppm. Parathyroid hyperplasia was observed in males at 300 ppm and above and corresponded to secondary hyperparathyroidism, probably subsequent to the renal insufficiency. All top-dose animals showed focal histiocytes in the spleen, which can be a sign of increased erythrocyte breakdown. Thus, the effect may be associated with the haematological disorders since an accumulation of pigmented macrophage aggregates has been described in short-term feeding studies. A marginal increase of focal interstitial cell hyperplasia in the testes was reported at the top dose, but the effect is not necessarily adverse since no reproductive impairment was described in the two-generation rat studies (see section 2.5(b)) and no gonadal tumours appeared. Furthermore, the spontaneous rate of testis interstitial cell tumours is quite high in the Fisher 344 rat strain used and the observed incidence of testis interstitial cell tumours was within the range of historical controls as reported by National Toxicology Program/NTP (Haseman, Arnold & Eustis, 1990). Focal interstitial cell hyperplasia commonly forms part of the continuum leading to interstitial cell adenoma. However, in this two-year rat study while the incidence of testis interstitial cell hyperplasia was

increased, the number of interstitial cell adenomas was decreased. Furthermore, in the second two-year rat chronic study (Taupin, 1992a), no increase in focal hyperplasia was observed. Thus, it is doubtful that hyperplasia on its own is a relevant effect without an increase in tumours. The observation of increased arteritis of the testes (6, 15, 18 and 22%* at 0, 100, 300 and 1000 ppm, respectively) is to be considered independent of the interstitial cell hyperplasia. Polyarteritis in F344 rats is most frequently seen in the pancreatic, mesenteric and spermatic arteritis (Mitsumori, 1990). In these other organs, no increased arteritis was found after treatment with metconazole. In addition no such effect was seen in the former two-year chronic toxicity study (Taupin 1992a). Therefore, it is unlikely that metconazole has a general effect on arteries and the overall relevance of the increased arteritis in the testis is unclear/questionable.

There were no treatment-related neoplastic findings. At 300 ppm, an increased incidence of pituitary adenoma (pars distalis) was observed in males. At the top dose, the incidence was similar to that of controls, and the incidence at all doses was within the incidence range which could be expected for F344 rats of this age (Haseman, Hailey & Morris, 1998). An increased incidence of mononuclear cell leukemia was observed at 100 ppm and 300 ppm (both sexes) and at the top dose (females, 15/50 or 30%), compared to controls (5/50 or 10%). The incidence was slightly outside the range of in-house historical controls, but within the historical control incidence as reported by National Toxicology Program/NTP, where the incidence for this neoplasm in untreated Fischer 344 rats ranges from 10% to 72% (mean 34%) for males, and 6 to 31% (mean 20%) for females (Haseman, Arnold & Eustis, 1990). Considering the high spontaneous incidence of both pituitary and haematopoietic tumours in this rat strain and the lack of a dose–response relationship, metconazole is regarded as devoid of carcinogenic properties in the rat.

Table 29. Key findings of two-year dietary toxicity study in rat

Dose (ppm) **mg/kg bw per day**		**0** **0**		**100** **4.6**		**300** **13.8**		**1000** **46.5**	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Survival (%)	week 104	60	66	56	60	50	62	66	68
Food consumption	weeks 1–13							↓6%	↓5%
Body weight	week 104							↓4%	**↓6%****
Body weight gain	weeks 0–13							↓7%	↓5%
	weeks 0–104							↓5%	↓9%
		Haematology (% change relative to controls)							
Red blood cell,	week 52							-	**↓1.7%****
mean diameter	week 78							**↓1.7%***	
	week 101							**↓1.8%****	
		Organ weight (% change relative to controls)							
Adrenals	relative							**↑7%***	-
Liver	relative							**↑12%****	**↑13%****
Kidney	relative							**↑13%***	-
Spleen	relative							-	**↑21%***
		Gross pathology							
Kidney	pallor	5	2	**10**	3	**14**	2	**14**	0
Liver	discoloration	6	2	2	1	1	2	4	**6**
Spleen	enlargement	16	6	18	9	18	9	13	**14**

Dose (ppm) mg/kg bw per day		0 0		100 4.6		300 13.8		1000 46.5	
		M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
		Histopathology: non-neoplastic findings							
Adrenal[1]	cortical vacuolation	13	11	**17**	7	**25***	8	**38*****	7
	%	26%	22%	**34%**	14%	**50%**	16%	**76%**	14%
	medullary foci, hyperplastic	4	4	**13***	2	**18****	3	10	1
	%	8%	8%	26%	4%	36%	6%	20%	2%
Brain	ventral indentation (pituitary adenoma)	5	10	5	**11***	**10****	**15****	1	13
Kidney	chronic nephropathy severe	7	5	6	3	**15**	4	10	2
	very severe	3	0	**7**	3	**10**	1	**11***	1
Liver	fatty centrilobular vacuolation[1]	3	0	3	1	4	2	**13***	**7***
	%	6%	0%	6%	**2%**	8%	**4%**	**26%**	**14%**
	fatty vacuolated foci/areas	8		1*		11		**18***	
	basophilic foci	32	36	27	34	34	33	38	**23***
	clear-cell foci	4	1	**7**	3	**9**	3	**28*****	**15*****
	%	8%	2%	**14%**	6%	**18%**	6%	**56%**	**30%**
	eosinophilic foci	1	0	3	1	4	0	**12****	2
	%	2%		**6%**		**8%**		**24%**	**4%**
	centrilobular hypertrophy	0	0	2	0	**9****	0	**31*****	1
	%			**4%**		**18%**		**62%**	**2%**
	pigment deposit	2	0	**6**	0	**14****	1	**35*****	**4**
	%	4%	0%	**12%**	0%	**28%**	2%	**70%**	**8%**
Parathyroids,									
	No. examined	***49***	***50***	***25***	***21***	***30***	***18***	***48***	***50***
	hyperplasia	8	1	10	3	**14****	3	11	0
Spleen	histiocytic foci	2	11	1	5	1	7	**20*****	**31*****
	%	4%	22%	16%	10%	2%	14%	40%	62%
Glandular stomach,									
	No. examined	***50***	***50***	***29***	***26***	***31***	***22***	***50***	***50***
	focal erosions	3	1	3	**6****	4	**4***	1	5
	mucosal hypertrophy	6	4	5	7*	7	5	10	5

Dose (ppm) mg/kg bw per day	0 0		100 4.6		300 13.8		1000 46.5	
	M	F	M	F	M	F	M	F
Nonglandular stomach								
No. examined	***50***	***50***	***28***	***23***	***33***	***21***	***50***	***50***
erosions/ulcers	5	8	**13*****	7	**15*****	8	8	8
%	10%	16%	46%	30%	45%	38%	16%	16%
HCD§ (%)	Male: range 4–46%; mean 25%				Female: range 5–31%; mean 13%			
acanthosis/ epithelial hyperplasia[a]	7	12	**14****	8	**17*****	**13****	**17***	11
%	14%	24%	50%	35%	52%	62%	34%	22%
HCD§ (%)	Male: range 16–65%; mean 25%				Female: range 8–46%; mean 20%			
focal submucosal inflammation	3	12	**11*****	6	**15*****	11*	**12***	9
%	6%	24%	39%	26%	45%	52%	24%	18%
HCD§ (%)	Male: range 10–40%; mean 26%				Female: range 4–39%; mean 17%			
Neoplastic findings								
Pituitary[b],								
No. examined	***50***	***49***	***42***	***42***	***40***	***44***	***49***	***50***
anterior lobe adenoma	17	25	21	21	**26****	30	18	24
%	34	51	50	50	65	68	37	48
Haematopoietic tissue[b],								
No. examined	***50***	***49***	***32***	***22***	***31***	***20***	***50***	***50***
mononuclear cell leukaemia	17 /50	5 /50	**22**** /50	**8*** /50	**21**** /50	7* /50	14 /50	**15*** /50
%	34	10	44	16	42	14	28	30

HCD§ Historical control data for F344 rats; incidences (% of animals with stomach lesions); data test facility, seven studies, $N = 336$ for males, $N = 338$ for females

[a] Total incidence for score expanded finding

[b] F344 rat historical control incidences (% of animals with tumours) of:

Mononuclear cell leukaemia:	*Test-facility historical control*:	♂, range 5–44% (mean 12%); ♀, range 5–28% (mean 10%)
	NTP historical control (1991)	♂, range 10-72% (mean 34%); ♀, range 6-31% (mean 20%)
	NTP historical control (1998)	♂, range 32-74% (mean 51%); ♀, range 14-52% (mean 28%)
Pituitary adenoma, pars distalis	*NTP historical control (1991)*	♂, range 5-52% (mean 23%); ♀, range 18-70% (mean 45%)
	NTP historical control (1998)	♂, range 14-60% (mean 30%); ♀, range 30-74% (mean 53%)

Statistically significant modification; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

In this two-year carcinogenicity and toxicity study, target organs were the liver (pigment deposit and centrilobular hypertrophy) and the adrenals (cortical vacuolation). Based on these findings the NOAEL for chronic toxicity was 100 ppm (4.6 mg/kg bw per day). Because no treatment-related effects on the types or incidences of neoplasia were observed at any concentration, the NOAEL for carcinogenicity was set at 1000 ppm (46.5 mg/kg bw per day), the highest dose tested (Taupin, 1992b).

2.4 Genotoxicity

The genotoxic potential of metconazole (*cis*:*trans* 80:15) was investigated in vitro using the Ames test and a chromosomal aberration assay in Chinese hamster ovary (CHO) cells, and in vivo in the mouse bone marrow micronucleus test and in an unscheduled DNA synthesis (UDS) in rat liver cells. The genotoxic potential of *cis*-metconazole (95% *cis*) was also investigated in a battery of both in vitro (Ames test, gene mutation assay in lymphoma cells, chromosome aberration assay in human lymphocytes) and in vivo tests (mouse bone marrow micronucleus assay, rat UDS assay).

Metconazole (*cis*/*trans* mix) tested negative in the Ames assay using bacterial cells. Results of an in vitro chromosomal aberration assay performed with a *cis*:*trans* 80:15 mixture in mammalian CHO cells were equivocal, as structural chromosomal aberrations were induced in the presence of S9 mix but not in its absence, in both main and repeat experiments. However, the non-clastogenicity of the metconazole *cis*/*trans* mix was confirmed in an in vivo micronucleus assay in mouse bone marrow cells, whilst an in vivo UDS test using rat hepatocytes was also negative.

All available genotoxicity studies with the *cis*-metconazole, including an Ames test, a mouse lymphoma assay in mammalian cells, an in vitro chromosome aberration in human lymphocytes, an in vivo UDS assay in rat hepatocytes, and an in vivo micronucleus test in mouse bone marrow, were negative.

A summary of results for genotoxicity studies of metconazole is shown below in Table 30.

Table 30. Summary of results for genotoxicity studies of metconazole

Type of study, strain, species	**Purity, *cis*/*trans* ratio, batch number**	**Test conditions**	**Results**	**Reference**
		In vitro: *cis*:*trans* ca 80:15		
Ames mutagenicity test	95.3%, *cis*:*trans* 79.8:15.5 Batch 89-01	Plate incorporation, Solvent: acetone, ± S9 mix, up to 5000 µg/plate	**Negative**	Brooks & Wiggins, 1990
Chromosome aberration in CHO-K1 cells	95.3%, *cis*:*trans* 79.8:15.5 Batch 89-01	Solvent: acetone –S9 mix, 24 h/24 h: 1.56–12.5 µg/mL, 48 h/48 h: 0.625–5 µg/mL +S9 mix, 3 h/24 h: 6.25–50 µg/mL, 3 h/48 h: 4.375–35 µg/mL	– S9 mix, **Negative** + S9 mix **Positive** 24 h, 50 µg/mL **Negative** 48 h	Brooks & Wiggins, 1991b
		Ex vivo: *cis*:*trans* ca 80:15		
UDS, rat (Sprague Dawley) hepatocytes	97.9%, *cis*:*trans*: 83.7:13.7 Batch AC 9339-114	400, 1000,2000 mg/kg bw in 0.5% CMC by gavage Treatment: 4 h and 16 h	**Negative**	Pant, 1995

Type of study, strain, species	Purity, *cis*/*trans* ratio, batch number	Test conditions	Results	Reference
In vivo: *cis*:*trans* ca 80:15				
In vivo chromosome aberration: Mouse (CD-1) micronucleus test	97.9%, *cis*:*trans*: 83.7:13.7 Batch AC 9339-114	400, 1000,2000 mg/kg bw in 0.5% CMC by gavage Sacrifice: 24 h, 48 h, 72 h	**Negative**	Xu, 1995
In vitro: *cis* ca 95%				
Ames mutagenicity test, TA 98, TA 100, TA 1535, TA 1537, TA 1538, and WP2 uvrA	95.29%, *cis*:*trans* 95.2:0.1 Batch 12	Plate incorporation, Solvent: acetone, ± S9 mix, up to 5000 µg/plate	**Negative**	Brooks & Wiggins, 1991a
Gene mutation assay in mouse lymphoma cells L5478Y/TK$^{+/-}$	95.29%, *cis*:*trans* 95.2:0.1 Batch 12	Solvent: acetone ± S9 mix, 12.5–90 µg/mL	**Negative**	Clements, 1991
In vitro cytogenetics: Chromosome aberration in human lymphocytes	95.29%, *cis*:*trans* 95.2:0.1 Batch 12	Solvent: acetone ± S9 mix (3 h/24 h): 93.75–750 µg/mL − S9 mix (24 h/24 h): 5.86–93.75 µg/mL − S9 mix (48 h/48 h): 5.86–46.88 µg/mL	**Negative**	Brooks & Wiggins, 1991c
Ex vivo: *cis* ca 95%				
UDS, rat (Sprague Dawley) hepatocytes	95.29%, *cis*:*trans* 95.2:0.1 Batch ST 91/106	443 and 1400 mg/kg bw in 0.5% CMC by gavage treatment: 12–13 h, 2–3 h	Negative	Dean, 1991
In vivo: *cis* ca 95%				
In vivo chromosome aberration: Mouse (CD-1) micronucleus test	95.29%, *cis*:*trans* 95.2:0.1 Batch 12	Two doses/day: 75, 150, 300 mg/kg bw in 0.5% CMC by gavage Sacrifice: 24 h, 48 h after second dosing	Negative	Marshall, 1991

CMC Carboxymethyl cellulose

UDS Unscheduled DNA synthesis

Overall, both *cis*:*trans*-metconazole 80:15 mixture and 95% *cis*-metconazole are considered not genotoxic.

2.5 Reproductive toxicity

Metconazole was tested in several 1-generation reproduction studies and in two 2-generation studies in rats, as well as in several developmental toxicity studies in rats and rabbits using *cis*-metconazole and/or the *cis*/*trans* mixture.

(a) One-generation studies

Study 1
Rats (Crl: CD(SD) BR VAF/Plus)in groups of 10/sex per dose were administered metconazole (WL148271; purity 95.3%; *cis*:*trans* 83.7:16.3, nominally 85:15; batch 89-01) in the diet at levels of 0, 50, 500 and 1500 ppm (equivalent to 0, 2.9, 28.0, 89.9 mg/kg bw per day in males, 0, 3.6, 35.8, 116 mg/kg bw per day in females) in a preliminary one-generation study. F0 animals were treated for four weeks prior to pairing, continuing throughout to termination which occurred at or around the time of weaning of the F1 offspring (approximately 11 weeks for F0 males and pregnant females). The F1 offspring remained with the dam until weaning (day 21 post partum), the dam was then sacrificed, and selected offspring retained until six weeks of age in order to assess susceptibility of weanling rats to dietary inclusions of *cis*/*trans*-metconazole. Selection of an F1 generation was impossible at 1500 ppm, since only one litter survived weaning.

F0 generation
At 1500 ppm, there were seven mortalities; these all occurred during the perinatal period. Six of these occurred after signs of delayed parturition (presumed day 23 of pregnancy) and poor condition (pallor, piloerection, lethargy, cold and loss of body tone). The seventh died on day 2 post partum after the birth of a stillborn litter. In addition, one dam which totally resorbed her litter showed signs consistent with the mortalities at a similar stage of pregnancy. At 500 ppm, one pregnant female was also sacrificed after signs of delayed parturition (presumed day 24 of pregnancy), with clinical signs similar to those seen at 1500 ppm. In addition, one dam which reared young to weaning showed piloerection at a similar stage of pregnancy to the mortalities. There were no mortalities or specific signs of reaction to treatment observed among any male animals of the F0 generation, nor among females at 50 ppm.

For males, mean water consumption at 1500 ppm was lower than controls during weeks 1–4. For females at 1500 ppm, intake was consistently lower than controls. At the lower doses, consumption values were only slightly lower than controls with no clear dose relationship. Mean feed consumption was decreased in top-dose animals during the first week of treatment. Recovery occurred thereafter, and intake regained parity with the controls. At 500 ppm, feed intake was marginally lower in all animals during week 1, but again showed recovery to regain parity with the controls. A dose-related decreased body weight gain was observed at 500 ppm (females) and above (both sexes).

Body weights were most noticeably affected during the first week of treatment when overall weight loss was recorded for both males (−11 g) and females (−15 g) at 1500 ppm, compared with gains of 34 g and 21 g in the respective controls. Although mean weight gain was restored, parity with control mean overall weight was never regained at either 500 or 1500 ppm. For all pregnant dams at 1500 ppm, body weight gain during pregnancy was retarded and continued to diverge from the controls through to day 20 of pregnancy. At 500 ppm, body weight gain was slightly retarded during days 0–14 of pregnancy, although good recovery occurred thereafter with overall gain comparable to that of control. Calculated food conversion ratios were indicative of inferior efficiency of food utilization for males at 1500 ppm and females at 1500 and 500 ppm, the effect being most marked in the first week of treatment.

Terminal examinations revealed no relevant findings.

F1 generation
There were no clinical signs observed and no effects on mortality. For males at 500 ppm, there was an indication of a slight reduction in mean food consumption during weeks 5 and 6. Although mean body weight gains for both males and females at 50 and 500 ppm were essentially similar or superior to controls from birth to day 21 post partum, thereafter values were slightly (but consistently) retarded and continued to diverge from controls in a dose-related manner up to day 41 (week 6). Food conversion ratios were essentially similar to controls. Terminal examinations revealed no relevant findings.

Reproductive parameters

F0 generation
No relevant findings were recorded during pre-mating. A 100% pregnancy rate was achieved in all groups, and mating performance, as assessed by the type of vaginal smear recorded on the day of conception and the median precoital time, showed no consistent treatment- or dose-relationship. At 1500 ppm, 6/10 dams were sacrificed/found dead with signs of prolonged pregnancy (presumed day 23) and at 500 ppm, 1/10 dams was sacrificed with signs of prolonged pregnancy (day 24).

Offspring data
At 1500 ppm, in addition to the six dams which died or were sacrificed around parturition, two dams suffered total litter loss on day 0/1 post partum, of which one subsequently died on day 2 post partum. The ninth dam totally resorbed her young. No litter losses were observed in the other groups.

For the one surviving litter at 1500 ppm, litter and mean pup weights from birth to day 21 post partum were lower than controls. However, the group size is too low for any meaningful comparison. Apart from a slightly higher percentage cumulative pup loss at 500 ppm on day 4 post partum (before the cull), there were no clear, consistent effects of treatment on litter values or sex ratios at 500 or 50 ppm. A slight retardation in body weight gain was observed at 50 ppm, which was more pronounced at 500 ppm (statistically significant in both sexes). Terminal examinations revealed no relevant findings.

Table 31. Key findings of one-generation dietary toxicity study in rat

Dose level (ppm)	**0**	**50**	**500**	**1500**
			Parental data F0	
Intake F0 (mg/kg bw per dayay) M/F	0/0	2.9/3.6	28.0/35.3	89.9/116
Number of animals mated	10	10	10	10
Died/sacrificed	0	0	1 (dystocia, GD24)	6 (dystocia, GD23), 1 (PND2, total litter loss prior to death)
Total resorptions				1
Total litter loss at birth				1
Rearing young to weaning	10	10	9	1
Body weight gain – males (g)				
week 0–4	112	115	107	**60** (−46%)**
week 0–10	181	194	177	**148** (−18%)**
Body weight gain – females (g)				
week 0–4	69	64	**45** (−35%)**	**20** (−71%)**
week 0–10	126	138	118	**57** (−55%)**
Food consumption – males (g)				
week 1	198	496	180	**171* (−14%)**
Food consumption – females (g)				
week 1	134	143	124	**105* (−22%)**
Gestation length	21.6	22.1	22.4	22.3

Dose level (ppm)	0	50	500	1500
		Litter observation		
Intake F1 (mg/kg bw per dayay) M/F	0/0	6.8/6.5	67.3/66.2	§
Implantations (F0)	16	17.4	16.7	15.0§
Live litter size				
At birth	14.5	15.9	14.1	11.0§
Day 4 pre cull (% cumulative pup loss)	14.3 (2.9%)	15.7 (4.4%)	13.3 (9.4%)	11.0§
Fetal weight (F1):				
At birth	6.1	5.8	6.2	5.7§
Day 4 (pre cull)	9.9	10.1	9.9	9.0§
Day 4 (post cull)	9.8	10.1	9.8	9.1§
Body weight gain (% of control)				
Day 0–42, M/F		−4%/−4%	**−12%*/−7%***	§
Day 21–42, M/F		−10%/**−10%****	**−16%*/−10%****	§

GD Gestation day PND Postnatal day

§ Mean values not applicable; only one litter survived to weaning

Statistical evaluation; * $p < 0.05$ ** $p < 0.01$

Administration in the diet of 1500 ppm *cis*/*trans*-metconazole induced clear adverse effects on food and water consumption and body weight gain in both sexes of the F0 generation, particularly in early treatment stages. This dose also had profound effects on the ability of dams to successfully complete parturition and led to the sacrifice or death of 7/10 pregnant animals. It was considered that this dose would be inappropriate for further investigation. At 500 ppm, similar responses to those seen at 1500 ppm were evident in the F0 adults, although much milder in degree, exemplified by only 1/10 mortalities in pregnant females.

The parental/reproduction NOAEL for this study was established at 2.9 mg/kg bw per day (50 ppm) based on decreased food and water consumption, decreased body weight gain, slightly delayed gestation and dystocia (1/10 mortalities) observed at 28 mg/kg bw per day (500 ppm).

The offspring NOAEL was established at 2.9 mg/kg bw per day (50 ppm) based on prebirth fetus loss, increased pup loss at birth, decreased body weight gain during postnatal days 0–42 (PND 0-42) observed at 28 mg/kg bw per day (500 ppm) (Masters, Jones & Parker, 1991, part 1).

Study 2

Rats (Crl: CD(SD) BR VAF/Plus), 10/sex per dose were administered in the diet either metconazole WL148271 (*cis*:*trans* 83.7:16.3, nominal 85:15; batch 89-01) or WL136184 (purity 95.3%; isomer mix not specified, assumed 95% *cis*; batch ST 89/411) at levels of 0, 500 and 750 ppm. F0 animals were treated from day 6 of pregnancy throughout to termination, at or around the time of weaning of the F1 offspring (approximately 11 weeks for F0 males and pregnant females). On day 22, parents and pups were sacrificed and examined for abnormalities. Histopathological examination was not performed.

Parental data

During days 22 to 23 of pregnancy there were several mortalities in treated groups, the highest incidence occurring at 750 ppm of the *cis*/*trans* mixture. Signs included piloerection, cold and pale extremities, lethargy and hunched posture; these were generally noted for animals just prior to death/sacrifice (from day 22) and were considered to be associated with delayed parturition. The single mortality at 500 ppm of the *cis*/*trans* mixture was comparable (incidence and signs) to that in the previous preliminary study at this dose. Similar signs of reaction to treatment were also observed amongst surviving dams at 750 ppm of the 95% *cis* isomer.

Both the isomer mix and 95% *cis* isomer provoked a consistent decrease of water intake at all doses after day 7 of pregnancy; showing a slight dose–effect relationship by days 16–19.

Feed intake was slightly lower than controls and tended to follow a dose–effect relationship with comparability between test materials. For both materials, there was a noticeable dose-related decrease of mean body weight gain during days 6–20, with the *cis*/*trans* mixture showing the greatest divergence from controls at the same dose as the *cis* material. On day 8, top-dose animals had the same weight (*cis*) or even lost weight (*cis*/*trans*) compared to day 6. For all test groups, the difference from the control was still clearly evident at day 20 of pregnancy and continued throughout lactation, although body weights tend to converge for both materials.

The duration of pregnancy tended to increase with dosage and appeared slightly longer in the groups treated with the *cis*/*trans* mixture than in those treated with the 95% *cis* isomer. Three out of five mortalities at 750 ppm of the *cis*/*trans* mixture had been sacrificed on day 23 of pregnancy with signs of dystocia. For the five dams with total litter loss, the duration of pregnancy was 23 or 24 days.

It was stated in the report that the occasional macroscopic changes detected at necropsy of F0 animals were not related to treatment (data not shown).

Offspring data

Incidences of total litter loss seemed to follow a dose-related pattern and were comparable for both materials at the same levels. The losses occurred before or by day 2 post partum. The small number of litters reared to weaning limits the confidence with which conclusions can be drawn from the data, particularly at 750 ppm for both materials. Although the implantation rate was similar for all groups, mean live litter size at birth for treated dams was noticeably lower than the controls and was due to both higher prebirth losses and pup losses at birth. This higher pup mortality continued to day 4 post partum in a dose-related pattern with the 95% *cis* isomer showing a marginally higher rate than the *cis*/*trans* material; after culling, further pup loss was negligible for all groups through to weaning. However, litter sizes for treated groups remained lower than for controls with the 95% *cis* isomer groups showing slightly inferior values compared to the *cis*/*trans* group.

For both materials at 750 ppm, mean pup weight at birth was slightly lower than controls; from day 8 to weaning, pup weight at both 500 and 750 ppm showed a dose-related decrease with the *cis* groups marginally lower than the *cis*/*trans* groups. Reflecting the slightly lower litter sizes and mean pup weights, litter weights for the *cis* material were consistently lower than the mix at the same dose from day 4 post cull and tended to follow a dose–effect relationship.

At 750 ppm with the 95% *cis* isomer there was one occurrence of hydrocephalus. Whereas, in isolation, this and other occasional findings observed at macroscopic examination of F1 offspring (data not shown) did not indicate a clear effect of treatment, it was remarked that this malformation was present in a further developmental study.

The results obtained generally showed a dose-related toxicological response at 500 and 750 ppm for both metconazole WL148271 (85:15 *cis*/*trans* mixture) and metconazole WL136148 (the 95% *cis* isomer). However, when comparing these materials, the mixed isomers tended to exhibit a greater response for maternal parameters, while the *cis* isomer seemed to show a more pronounced effect on the F1 offspring.

The results of the above study are summarized on the following page in Table 32.

Table 32. Key findings of one-generation dietary toxicity study in rat

		Control	Metconazole 95% *cis*		Metconazole 85:15	
Dose (ppm)		**0**	**500**	**750**	**500**	**750**
			Maternal data			
Number mated		10	10	10	10	10
Number of mortalities		0	0	1	**1**	**5**
Number not pregnant		0	2	1	0	0
Number of total resorptions		0	1	0	0	0
Number of total litter losses		0	0	2	1	2
Number rearing young to weaning		10	**7**	**6**	**8**	**3**
Water intake (%) [§]	GD 6−19	100	**90**	**87**	92	**86**
Food intake (%) [§]	GD 6−19	100	**84**	**79**	**87**	**77**
Body weight gain (g)	GD 6−8	8.5 -	**4.0** (↓53%)	**0.4** (↓95%)	**2.3** (↓73%)	**−2.8** (↓133%)
	GD 6−20	119.7 -	**98.4** (↓18%)	**77.0** (↓36%)	**90.7** (↓29%)	**67.6** (↓44%)
	days 6−21 post partum	64.2 -	**57.0** (↓11%)	**46.5** (↓28%)	**51.8** (↓19%)	**39.0** (↓39%)
Duration of pregnancy (days)		22 -	22.3 (↑1%)	22.8 (↑4%)	22.4 (↑2%)	23.2 (↑5%)
			Offspring data			
Pre-birth loss (%)		4.8	**14.1** (↑202%)	**21.3** (↑348%)	**26.1** (↑444%)	**20.0** (↑317%)
Pup loss (%)	day 0 (birth)	0.8 -	**14.1** (↑1663%)	**15.1** (↑1788%)	**2.8** (↑250%)	**7.4** (↑825%)
	day 4 (pre-cull)	1.6 -	**19.0** (↑1088%)	**22.9** (↑1331%)	**5.2** (↑225%)	**11.1** (↑594%)
Live litter size	day 0 (birth)	11.3 -	**8.7** (↓23%)	**7.7** (↓32%)	**8.5** (↓25%)	**8.7** (↓23%)
	day 21	8 -	**6.6** (↓18%)	**6.8** (15%)	**7.0** (↓13%)	**7.3** (↓9%)
Litter weight (g)	day 0 (birth)	72.2 -	**55.8** (↓23%)	**46.7** (↓35%)	**56.8** (↓21%)	**53.4** (↓26%)
	day 21	422.5 -	**321.7** (↓24%)	**303.7** (↓28%)	**353.4** (↓16%)	**344.4** (↓18%)
Pup weight (g)	day 0 (birth)	6.4	6.5 (↑2%)	6.1 (↓5%)	6.7 (↑5%)	6.1 (↓5%)
	day 21	52.8 -	**47.4** (↓10%)	**44.4** (↓16%)	50.5 (↓4%)	**46.3** (↓12%)

GD Gestation day

[§] Expressed as a percentage of control

The results obtained generally showed a dose-related toxicological response at 500 and 750 ppm for both metconazole WL148271 (85:15 *cis*/*trans* mixture) and metconazole WL136148 (95% *cis* isomer). However, when comparing these materials, the *cis* isomer appeared to show a more pronounced effect on the F1 offspring (pup loss at day 0 and day 4).

The parental/reproduction NOAEL was < 500 ppm, based on decreased food and water consumption, decreased bodyweight gain, slightly delayed gestation observed at 500 ppm (*cis*/*trans*).

The offspring NOAEL was < 500 ppm, based on prebirth loss, decreased live litter size, increased pup loss at birth and decreased pup weight (PND 21) observed at 500 ppm (for *cis* only and *cis*/*trans*). (Masters, Jones & Parker, 1991, part II).

Study 3
Rats (CD strain of Sprague Dawley origin) 6/sex per dose were administered metconazole WL148271 (purity 95.3%; *cis:trans* 83.7:16.3, nominal 85:15; batch B 89-01) in the diet at levels that were adjusted at intervals to maintain calculated chemical intakes of 1, 2, 8 or 32 mg/kg bw per day. The actual dietary level during the nine week feeding period (in ppm) ranged as follows: 12–18, 25–37, 100–149 and 394–570 for males, 4–12, 9–24, 35–93 and 141–378 for females. The achieved chemical intakes were usually in the range of ± 10% of target values. The F0 animals were treated for 15 days prior to pairing. Treatment was continued throughout mating, gestation and lactation and until the F1 litters were eight weeks old. Control animals received untreated diets throughout the same period. The study is considered to provide additional information.

Parental toxicity

F0 generation
There were no relevant findings observed on clinical signs or mortality. Body weight gain for females in all treated groups was slightly lower than that of controls during the gestation period, with females receiving 32 mg/kg bw per day being the most affected (−16%). Food consumption was similar in all groups during the lactation period. Food conversion efficiency of females during the second week of treatment tended to be slightly reduced at 8 and 32 mg/kg bw per day.

Water consumption was occasionally slightly increased in males in all treated groups, but no dose relationship was apparent, thus the finding was not relevant. Water consumption in females was unaffected by treatment. There were no relevant findings noted during terminal examinations.

F1 generation
There were no relevant findings observed on clinical signs and mortality. Body weight gain and food consumption were not affected by treatment. Water consumption in females showed a slight increase in all treated groups, but the effect was not dose-related. Necropsy of F1 animals at eight weeks of age revealed no effects that were considered to be related to treatment.

Reproduction parameters

F0 generation
No effect was observed on estrus cycles, mating performance or fertility up to the highest dose tested. Gestation length was within the normal range of 22–23 days for all females. At the top dose, mean gestation length tended to be increased by approximately half a day. Parturition was unaffected and gestation index was maximal in all groups.

Offspring data

F1 generation
There was no indication of a treatment-related effect on the number of implantation sites or total numbers of offspring at birth from day 1 post partum to weaning. The lowered live birth index (−6% of control) at the top dose was mainly attributable to one litter. Pup body weights and sex ratios were unaffected by treatment. There were no relevant findings noted during terminal examinations.

Key findings of this study are shown on the following page in Table 33.

Table 33. Key findings of preliminary one-generation dietary toxicity study in rat

Dose levels (mg/kg bw per day)	Generation	0	1	2	8	32
Reproduction parameters						
Number of females/pregnants		6/6	6/6	6/6	6/6	6/6
Mating index (%)	F0 (M+F)	100/100	100/100	100/100	100/100	100/100
Fertility index (%)	F0 (M+F)	100/100	100/100	100/100	100/100	100/100
Gestation index (%)	F0	100	100	100	100	100
Estrus cycle, regular (%)[a]	F0	100	83	100	100	100
Estrus cycle, irregular (%)[b]		0	17	0	0	0
Mean gestation length (days)	F0	22.3	22.2	22.3	22.2	22.8
Offspring data						
Implantation sites	F1	17.2	16.7	15.7	16.3	16.3
Litter size (day 1 post partum)	F1	15.8	15.3	14.5	15	15
Postimplantation survival index (%)	F1	92	91	91	92	92
Live birth index (%)	F1	98	99	100	99	92
Viability index (day 4 post partum)	F1	100	97	97	99	99

[a] Regular: 4 or 5 days of estrous cycle

[b] Irregular: at least one cycle of two, three or six to nine days

It was concluded from this preliminary study that dietary concentrations of metconazole WL148271 (*cis*/*trans*), resulting in test article intakes of up to 32 mg/kg bw per day would be suitable for use in a main two-generation study in the rat. This was based upon the slight body weight changes during gestation and slightly increased gestation lengths as the only effects.

The parental/reproductive NOAEL was 8 mg/kg bw per day (35 ppm), based on decreased food consumption and decreased body weight gain during gestation and slightly increased gestation length, observed at 32 mg/kg bw per day (100 ppm).

The offspring NOAEL was 32 mg/kg bw per day (100 ppm), the highest dose tested (Willoughby, 1991).

(b) Two-generation studies

Study 1

Rats (CD strain of Sprague Dawley origin) were administered *cis*-metconazole (WL136184; purity 95.29%; *cis*:*trans* 95.2:0.09); batch 12) in the diet at levels that were adjusted at intervals to maintain calculated chemical intakes of 2, 8, 32 or 48 mg/kg bw per day throughout two generations. The actual dietary level ranges (in ppm) during the 20 week feeding period were, for males 13–49, 54–195, 219–780, 336–1173 and 10–32, 41–127, 160–511, 238–755 for females. The achieved chemical intakes were usually in the range of ± 10% of target values.

Control animals received untreated diets throughout the same period. The F0 generation, which comprised 32 rats/sex per dose, received 14 weeks treatment before pairing to produce F1 litters, from which 32 male and 32 female offspring were selected to form the F1 generation. Treatment of F1 animals continued until termination after the breeding phase. Both sexes received 14 weeks treatment after selection but before pairing to produce F2 litters. All adult animals were subjected to a detailed necropsy on PND 25 (weaning): for the control and highest treatment levels animals, reproductive organs were weighed and retained, and histological examination performed on these reproductive organs.

Parental toxicity

F0 generation

The clinical condition and appearance of F0 animals were unaffected by treatment. Two males and three females died. One control male died on week 7 (severely cannibalized) and one top-dose male

was found dead during week 23 (cause of death unknown). One female of group 8 mg/kg bw per day was sacrificed on day 3 post partum (parturition difficulties, bleeding from vagina and vagina dilated). At the top dose two females were sacrificed, one on day 3 post coitum (teeth and nasal damage) and another one on day 24 post coitum (parturition difficulties, bleeding from vagina with subsequent loss of six fetuses). It was uncertain if the deaths of the top-dose animals on week 23 and day 24 post coitum were treatment-related.

Body weights and body weight gain of males showed no statistically significant differences between groups. In top-dose females body weight gain decreased during the maturation and gestation periods but animals recovered during the lactation period. Food consumption and food conversion efficiency was unaffected by treatment. There were no relevant findings observed in reproductive organ weights, gross pathology or histopathology.

F1 generation
The clinical condition and appearance of F1 animals were similar in all groups. Two males and four females died or were sacrificed, but necropsy findings indicate no treatment-related effects. One control female was sacrificed on day 25 post coitum after prolonged parturition. Two males from the 8 mg/kg bw per day group were sacrificed during week 12 (poor condition) and week 15 (teeth overgrown, underactive). One female of 8 mg/kg bw per day group and one of the 32 mg/kg bw per day group were sacrificed on day 23 post coitum, each because of prolonged parturition. Another female of the 32 mg/kg bw per day group was found dead on day 3 post partum (accidental).

At selection (week 0), F1 animals receiving 32 or 48 mg/kg bw per day were lighter than the controls. This difference persisted until the end of treatment at least at the top dose in males. In the females, the difference was remarkable throughout gestation, and until day 7 of lactation. While intergroup differences in body weight gain were insignificant in the males, decreased body weight gain was observed in top-dose females during maturation (weeks 0–14), but not during gestation or lactation. There was no relevant effect on food consumption in males or females. Food conversion efficiency was unaffected by treatment.

At necropsy of F1 adults terminal body weights were significantly reduced in females at 32 mg/kg bw per day and in males and females at the top dose. Relative and absolute ovarian weights were increased at 32 and 48 mg/kg bw per day, but histopathology revealed no corroborative findings.

There were no macroscopic abnormalities observed that were considered to be related to treatment, except for four top-dose males, that showed small/dark testes or epididymes. In histology, reduced sperm content (epididymis) and degeneration of tubular germinal epithelium were observed. However, the finding was not corroborated by modifications in organ weight, and fertility was not altered, thus the toxicological relevance of the finding is questionable.

Reproduction parameters

F0 generation
There was a slight increased incidence of irregular estrus cycle (16%) or acyclicity (13%) at the top dose compared to controls (6% and 3%, respectively). The incidences were higher than historical control mean rates, but within the ranges. At this dose and at the next lowest dose (32 mg/kg bw per day) gestation length was slightly prolonged with values for gestation length > GD 23–25, which is outside historical control ranges. The female mating index at 8 mg/kg bw per day (84%) was lower than usually achieved and at the top dose (90%) just outside the range of historical control data from the same laboratory. The male and female fertility index (72%) for the F0 8 mg/kg bw per day group was marginally outside the historical control data range. However, in the absence of a clear dose–response relationship these effects were not considered to be related to treatment. Parturition difficulties associated with mortality (see above) were observed in one female in the 8 mg/kg bw per day group and one female at the top dose. Due to these single incidences at isolated dose levels this finding was not considered relevant. Furthermore, gestation indices were similar in all groups.

F1 generation
The estrus cycle of F1 adults appeared to be normal. Gestation length of females receiving 32 and 48 mg/kg bw per day was slightly, but statistically significantly, increased with values at the top dose being outside the historical control range.

The F1 male and female mating index and fertility index were slightly lower in the control group and the lowest dose group than in the higher dose groups. In the absence of a dose–response relationship this finding was considered incidental. Other litter parameters were not affected. Dystocia was observed in single animals from the 8 and 32 mg/kg bw per day groups but also in the control group and was therefore not treatment-related. Furthermore, gestation indices were unaffected by treatment. Ovary weights were significantly increased in F1 females. However, in the absence of any histopathological correlate, the weight changes were not considered to be relevant.

Table 34. Parental and reproductive data of a preliminary two-generation dietary toxicity study in rat

Dose levels (mg/kg bw per day)	Generation	0	2	8	32	48	HCD mean	HCD range
Parental data								
Number of females/pregnant		32/27	32/23	32/26	32/26	32/25		
Deaths/sacrificed	F0	1 (M)	-	1 (F)	-	1 (M), 2 (F)		
	F1	1 (F)	-	2 (M), 1 (F)	2 (F)	-		
Body weight gain (% of control)								
weeks 7–14	F0 (F)					**−16%***		
GD 0–20						**−11.8%***		
weeks 0–14	F1 (F)					**−9.5%****		
Terminal body wt (g)	F1 (M/F)	763/417	736/414	789/411	774/**390***	**710*/382****		
Organ weight								
Ovary weight	F1 (F)							
absolute (g)		0.132	0.133	0.145	**0.155***	**0.158****		
relative (%)		0.0322	0.0325	0.0357	**0.0398****	**0.0419****		
Reproduction Parameters								
Mating index (%)	F0 (M/F)	97/97	84/84	97/97	94/94	90/90	96.3/97.3	83–100%/92–100%
	F1 (M+/F)	81/91	84/90	90/97	100/100	97/100	96.5/97.3	83–100%/92–100%
Fertility Index (%)	F0 (M/F)	84/84	72/72	81/81	81/81	81/81	84.7/85.6	75–96%/75–96%
	F1 (M/F)	53/63	61/68	70/75	84/84	78/81	84.2/85	68–96%/65–96%
Gestation Index (%)	F0	100	96	96	100	96	97.9	87–100%
	F1	90	100	96	93	100	98	87–100%
Estrus cycle (%)								
regular §	F0	88	91	97	88	72	80.8	63–96%
regular §	F1	91	74	84	78	88	81.7	63–96%
irregular §	F0	6	3	0	6	16	7.3	0–18%
irregular §	F1	0	13	6	6	6	7.9	0–18%
extended §	F0	3	6	3	0	0	1.2	0–8%
extended §	F1	3	0	0	3	3	1.4	0–8%
acyclic §	F0	3	0	0	6	13	10.7	0–25%
acyclic §	F1	6	13	9	13	3	9.1	0–25%
Mean gestation length (days)	F0	23.1	23	23	23.2	23.4		
	F1	22.9	22.8	22.7	23.2	23.3		

Dose levels (mg/kg bw per day)	Generation	0	2	8	32	48	HCD mean	HCD range
				Gestation length (%)				
< GD 23	F0	15	41	28	12	4	0–45%	
= GD 23		59	32	40	46	42	16–67%	
> GD 23		26	27	32	42	**44***	0–32%	
< GD 23	F1	37	47	46	4	0	0–45%	
= GD 23		53	48	46	67	42	16–67%	
> GD 23		10	5	8	**30****	**58*****	0–32%	

HCD Historical control data as presented in the study report

GD Gestation day

§ Regular estrus: 4 or 5 days of estrus cycle
Irregular estrus: at least one cycle of two, three or six to nine days
Extended estrus: at least four consecutive days of estrus
Acyclic: at least ten days without estrus

Statistical evaluation: * $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Offspring data

F1 generation

The numbers of implantation sites were similar in all groups. Post implantation survival at the top dose was significantly reduced, resulting in a slight (not significant) reduction in litter size. Live birth index was slightly reduced (not statistically significant), but subsequent survival of offspring was unaffected by treatment. The sex ratio was unaffected by treatment.

While body weights of offspring at birth were unaffected by treatment, subsequent body weight gain of pups dosed at 8, 32 or 48 mg/kg bw per day were significantly decreased when calculated until PND 25. The reductions in body weight gain at 8 mg/kg bw per day and above were small (5–7%).

Physical development was assessed by timing of onset and completion of pinna unfolding, hair growth and tooth eruption, which were unaffected by treatment. The onset of eye opening in offspring receiving 32 or 48 mg/kg bw per day occurred slightly earlier than in the control group, but was just outside the lower limit of the background data which was: range 12.6–14.0 days, mean 13.5 days (n = 21 studies). During necropsy at termination slightly increased unilateral or bilateral hydronephrosis or unilateral hydroureter was observed at the top dose.

F2 generation

The numbers of implantation sites were similar in all groups. Post implantation survival at 32 and 48 mg/kg bw per day was statistically significantly reduced compared to the controls, resulting in slightly smaller mean litter sizes (not significant). The live birth index was slightly, but not significantly, reduced at 48 mg/kg bw per day, but subsequent survival of offspring was unaffected by treatment. The sex ratio was unaffected by treatment.

The pup body weights at birth were unaffected by treatment, but body weight gain up to PND 25 at 48 mg/kg bw per day was statistically significantly reduced in both sexes compared to control animals. With regard to the measures of physical development parameters, the onset of eye opening occurred slightly but statistically significantly earlier at 32 and 48 mg/kg bw per day compared with the control group. However, the effect was within the lower limit of background control data. During necropsy at termination in F2 offspring, unilateral hydronephrosis was observed occasionally in control animals and in the treated dose groups. Thus, the effect of ureter or kidney dilatation was not considered treatment-related.

Offspring data are summarized on the following page in Table 35.

Table 35. Offspring data of preliminary two-generation dietary toxicity study in rat

Dose levels (mg/kg bw per day)		0	2	8	32	48
Offspring data						
Implantation sites	F1	15	15	15.1	15.1	15.1
	F2	14.7	15.7	15.3	15.7	15.7
Live litters born	F1	27	22	25	26	24
	F2	18	21	23	25	26
Litter size, day 1 post partum	F1	13.6	13.6	14.4	13.8	12
	F2	14.3	14.4	14.2	13.1	12.6
Post implantation survival index (%)	F1	89	89	94	90	**78****
	F2	93	90	92	**83****	**79****
Live birth index (%)	F1	97	100	98	99	93
	F2	100	95	98	97	**94***
Viability index day 4 post partum (%)	F1	99	98	99	99	98
	F2	99	90	100	98	98
Body weight						
Pup body weight (g)						
day 1 post partum	F1 (M/F)	7.3/6.8	7.2/6.6	7.1/6.7	6.9/6.6	6.9/6.6
day 4 post partum		11.1/10.5	10.6/9.8	10.7/10.1	10.7/10.1	10.6/9.9
day 25 post partum		91.7/86.7	89.5/84.6	86.9/82.6	86.5/82.3	85.7/80.4
day 0 post partum	F2 (M/F)	7.2/6.8	6.6/6.4	7.0/6.5	7.0/6.6	6.7/6.5
day 4 post partum		11.1/10.7	10.3/9.9	10.8/10.1	11.1/10.4	10.3/10.1
day 25 post partum		94.1/88.8	90.1/85.6	90.9/85.0	92.2/85.8	**85.6/83.1**
Pup body weight gain (% of control)	F1 (M/F)			**−5.5%*/ −5.1%***	**−5.7%**/ −5.3%***	**−6.6%***/ −7.6%****
day 1–25 post partum	F2 (M/F)					**−9.2%***/ −6.6%****
Physical development						
Onset eye opening (days)	F1	13.1	13	13.1	**12.6***	**12.5***
	F2	13.3	13.4	13	**12.8***	**12.8***
Necropsy selected:						
Unilateral hydronephrosis	F1	0.7 (1)	1.0 (1)	2.5 (3)	2.8 (2)	7.4 (6)
	F2	3.5 (4)	3.3 (4)	2.7 (5)	3.2 (4)	1.6 (3)
Bilateral hydronephrosis	F1	0.7 (1)	0.0 (0)	0.0 (0)	0.0 (0)	1.1 (1)
Unilateral hydroureter	F1	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	3.2 (2)

Statistical evaluation: * $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Metconazole WL136184 (95% *cis*) caused alterations of some reproduction parameters and slight fetal systemic toxicity at maternal toxic doses.

The NOAEL for parental toxicity was 8 mg/kg bw per day based on decreased body weights and ovarian weight changes at 32 mg/kg bw per day in the F1 generation. Likewise, the NOAEL for

reproductive toxicity was 8 mg/kg bw per day based on increased gestation length and decreased post implantation survival in the F2 generation at 32 mg/kg bw per day. The offspring NOAEL, at 8 mg/kg bw per day, was based on decreased body weight gain of F1 pups until weaning seen at 32 mg/kg bw per day (Willoughby, 1992a).

Study 2. Preliminary study

The dosages used in this study were selected based on the results of a preliminary study (Teramoto, 2002 pilot study). In the study, eight male and eight female Crj:CD(SD)[IGS] rats per group were given diet containing the test substance at a concentration of 0, 30, 100, 300, or 1000 ppm for three weeks before mating and until weaning of F1 pups. The doses correspond with measured intakes (overall weeks 1–10 in the male) of 0, 1.8, 5.8, 18.0 or 58.4 mg/kg bw per day.

No effects due to test substance treatment were noted in parental animals or pups in the 30 and 100 ppm groups. In the 1000 ppm group, food consumption, body weight, and body weight gains of parental animals were affected adversely, and two parental females died at parturition. In the 300 ppm group, the duration of gestation in parental females was slightly prolonged. The number of pups delivered and mean pup body weight for both sexes were significantly decreased in the 1000 ppm group. Therefore, considering that a dose level of 1000 ppm of the test substance is too high for parental rats and pups, a dietary concentration of 750 ppm was selected for the high-dose group in the main study. The low and intermediate dietary levels of 30 and 150 ppm, respectively, represent a five-fold interval in concentration.

Table 36. Key findings of pilot two-generation dietary toxicity study in rat

		Dose level (ppm)									
		Males					Females				
Parameter		**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
		F0 Generation									
Number of animals		8	8	8	8	8	8	8	8	8	8
Mortality		0	0	0	0	0	0	0	0	0	2 (dystocia)
Body weight and body weight gain (g)											
Body weight	week 3	391	394	388	390	388	248	259	254	245	**224** (↓10%)**
Body wt gain,	week 0–1	45	45	47	43	**35* (↓22%)**	19	24	21	16 (↓16%)	**2*** (↓89%)**
Body wt gain	week 0–2	88	89	87	85	82	37	45	39	32 (↓14%)	**14*** (↓62%)**
Body wt gain	week 0–3	121	124	118	120	118	47	**59***	54	44 (↓6%)	**24*** (↓49%)**
Gestation:	day 0	-	-	-	-	-	250	262	263	250	**229* (↓8%)**
	day 7	-	-	-	-	-	293	302	302	289	**259*** (↓12%)**
	day 14	-	-	-	-	-	329	339	343	329	**288*** (↓13%)**
	day 20	-	-	-	-	-	410	415	424	409	**349*** (↓15%)**
Lactation:	day 0	-	-	-	-	-	305	308	310	282	**261** (↓14%)**
	day 7	-	-	-	-	-	330	337	343	323	**278 (↓16%)**
	day 14	-	-	-	-	-	346	357	360	350	**301*** (↓13%)**
	day 21	-	-	-	-	-	322	328	332	321	304 (↓6%)

		Dose level (ppm)									
		Males					Females				
Parameter		**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
Food consumption (g/animal/day)											
	week 1	23.5	24.4	24.3	24.7	22.5	19.9	17.3	16.2	15.6	**12.2*** (↓39%)**
	week 2	24.5	25.1	24.7	24.7	24.9	16.5	18.3	17.1	16.4	**14.2 (↓14%)**
	week 3	24.6	25.7	24.5	24.8	25.9	16.2	17.5	17.2	16.1	**14.6 (↓10%)**
Gestation:	day 0–7	-	-	-	-	-	20.9	21.6	21.8	20.3	**16.6*** (↓21%)**
	day 7–14	-	-	-	-	-	22.9	24.5	24.1	22.4	**18.2*** (↓21%)**
	day 14–20	-	-	-	-	-	24.7	25.9	24.3	24.2	**18.3*** (↓26%)**
Gestation											
Gestation index (%)							100	100	100	100	**75**
Duration of gestation (days)							22	22	22.2	22.5	**22.7***
Body weight-related organ weights (%)											
Liver		2.89	3	3.11	3.07	3.17 (↑10%)	3.92	4.09	4.1	4.1	**5.25** (↑34%)**
Offspring data											
Body weight at necropsy (g)		78	77	81	75	**65* (↓17%)**	73	72	74	71	**63* (↓14%)**

Statistical evaluation: * $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Study 2. Main study

Groups of 24 male and 24 female rats were fed dietary concentrations of 0, 30, 150 or 750 ppm of metconazole (purity 98.99%; *cis*:*trans* 83.13:15.86); lot/batch 9Z521) for 10 weeks before pairing, throughout pairing, gestation and lactation and until termination after F1 litters were weaned. In each group, 24 males and 24 females were selected from F1 weanlings at 21–27 days of age to become the F1 parental animals. The selection was performed using litters born during a six day period including the day of the largest number of parturitions. The number of F1 weanlings selected at random from each litter was one or two per sex. Selected F1 parental animals were fed the relevant concentration of test diet continuously from the start of the F1 generation, for 10 weeks prior to pairing and until termination after F2 litters were weaned. F2 offspring received treated diets from weaning until termination on day 25–27 of age. Control groups of each generation and their offspring were given untreated diet throughout.

Table 37. Achieved test material intakes for all generations

	Parental males		Parental females		F1 males		F1 females	
Dose (ppm)	**Premating wks 1–10**	**Postmating wks 12–17**	**Premating wks 1–10**	**GD 0–20 and LD 0–21**	**Premating wks 1–10**	**Postmating wks 12–17**	**Premating wks 1–10**	**GD 0–20 and LD 0–21**
30	1.97	1.33	2.14	3.22	2.13	1.29	2.2	3.04
150	9.79	6.34	10.78	16.39	10.63	6.41	11.21	15.1
750	49.4	32.9	53.2	80	53	33.5	55.5	72.9

GD Gestation day LD Lactation day

The analyses for homogeneity demonstrated that the coefficients of variation of mean test substance concentrations in each treated group diet ranged from 1.4% to 5.7%, indicating that the test substance was homogeneously distributed in the test diets. In the analyses for concentration, the test substance was detected in the samples from the treated groups at levels ranging from 93% to 107% of the nominal concentrations, indicating that the test diets were properly prepared. No test substance was detected in any control diet.

Parental toxicity

F0 generation

There were no deaths among males in the F0 generation. Five F0 females in the 750 ppm group died during delivery, but animals showed no treatment-related signs.

In the 750 ppm group, mean body weights of F0 males at treatment weeks 1 and 2 were significantly lower than those in the control group (−5%). Mean body weights of F0 females at treatment weeks 6–10 (up to −5.3%) and on GDs 14 and 20 (up to −6.4%) in the 750 ppm group were significantly lower than those in the control group. Mean body weight gains of F0 males and females in the 750 ppm group were significantly lower than those in the control group at treatment weeks 1 and 2 (up to −16.7% for F0 males and −12.1% for F0 females) and of F0 females at treatment weeks 1–10 (−8.4%) and on during the gestation period (−13%). During the lactation period (days 0–21), the mean body weight gain of F0 females in the 750 ppm group was significantly higher than that in the control group, which may be explained by lower body weights on the day of delivery (−6%), which showed a recovery thereafter.

Male food consumption in the F0 generation was unaffected by treatment. For F0 females receiving 750 ppm, food consumption was significantly reduced during GDs 14–20 (−7.9%).

In the 750 ppm group, two F0 females repeatedly failed to show estrus. Moreover, the mean estrus cycle length in the 750 ppm group was significantly prolonged compared to controls (4.4 days compared to 4.1 days). Historical control data from four studies conducted between 2002 and 2012 (see Takahashi, 2015c) reported a mean estrus cycle length of 4.0–4.1 days for F0 females and from 4.0–4.3 for F1 females.

[It should be noted that data on estrus cycle length, days until mating, preputial separation and vaginal opening had not been collected before 2002 because the test guidelines from regulatory authorities did not require these parameters at that time. Therefore, the time period of the historical data set for the mentioned parameters (2002–2012) deviates from the other parameters that were collected between 1997 and 2012.]

Mating performance and fertility were unaffected by treatment. The number of days until mating was also unaffected by treatment. Gestation index in the 750 ppm group was significantly lower than that in the control group (66.7% compared to 100%) due to reduced number of live litters (14/21 compared to 24/24). The reduction in number of live litters at 750 ppm is due to increased number of dams that died during delivery. Duration of gestation in the 750 ppm group was significantly prolonged compared to controls (23.0 days compared to 22.2 days). Historical control data from a total of nine studies conducted from 1997–2012 (see Takahashi, 2015c) reported mean values for duration of gestation of 22.1–22.4 days. There were no treatment-related effects on sperm.

At 750 ppm, the relative liver weight of F0 males was significantly higher than that in the control group (6%). F0 females in the 750 ppm group showed statistically significant increases in the absolute (11.7%) and relative (12.8%) liver weights when compared with the corresponding controls. Compared with the control groups F0 females in the 750 ppm group showed significantly increased absolute (51.4 g compared to 60.5 g) and relative (0.01648% compared to 0.01991%) ovary weights, which was, however, within the historical control range. Historical control data for nine studies conducted from 1997–2012 (see Takahashi, 2015c) for mean absolute ovary weights ranged from 49.3–61.1 g for individual weights and from 119.5–132.3 g for total weight of bilateral ovaries. The historical control range for the mean relative ovary weight to body weight was as follows: 0.01551% to 0.0199% for individual weight, and 0.0369% to 0.0409% for total weight of bilateral ovaries.

There were no macroscopic findings attributable to treatment with metconazole. Five females found dead during delivery also had no treatment-related gross abnormalities.

In histopathological examination of 10 randomly selected pairs of F0 parental males and females in the control and 750 ppm groups, no treatment-related abnormalities were noted in the reproductive organs, pituitary or adrenals. Examination of animals that did not yield successful reproduction results also showed no treatment-related abnormalities. Livers of F0 males in the 750 ppm group showed a significantly increased incidence of centrilobular fatty change of hepatocytes, and livers of F0 females in the same group showed a significantly increased incidence of centrilobular hypertrophy of hepatocytes when compared with the corresponding controls.

Table 38. Key findings for the F0 generation of a two-generation dietary toxicity study in rat

		Dose level (ppm)							
		Males				Females			
Parameter		**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
		F0 Generation							
Number of animals/group		24	24	24	24	24	24	24	24
Mortality		0	0	0	0	0	0	0	5*
Clinical signs		No treatment-related effect							
Body weight/gain (g)									
Body weight,	week 0	146	146	146	146	117	117	117	117
Body weight,	week 1	206	206	202	**196*** **	150	150	149	146
Body weight,	week 2	264	263	259	**251****	175	174	171	168
Body weight,	week 6	430	416	421	424	243	245	236	**230***
Body weight,	week 7	453	437	447	448	252	259	249	**240***
Body weight,	week 8	473	457	467	468	261	267	257	**249***
Body weight,	week 9	488	474	484	487	266	271	263	**255***
Body weight,	week 10	500	488	500	502	272	279	267	**259***
Body weight gain,	week 0–1	60	60	56	**50*** **	33	33	31	**29***
Body weight gain,	weeks 0–2	118	117	113	**105****	58	57	54	**51****
Body weight gain,	weeks 0–4	215	210	210	208	97	99	92	**88***
Body weight gain,	weeks 0–6	284	270	275	278	126	128	119	**113****
Body weight gain,	weeks 0–7	307	291	301	302	135	142	132	**123***
Body weight gain,	weeks 0–8	327	311	321	322	144	150	140	**132***
Body weight gain,	weeks 0–10	354	342	354	356	155	162	150	**142***
Body weight,	GD 0	-	-	-	-	275	284	274	265
Body weight,	GD 14	-	-	-	-	336	341	330	**321***
Body weight,	GD 20	-	-	-	-	410	411	400	**384***
Body weight gain,	GDs 0–20	-	-	-	-	135	127	127	**118***
Body weight,	LD 0	-	-	-	-	300	302	290	282
Body weight gain,	LDs 0–21	-	-	-	-	12	8	17	**29****
Food consumption (g/animal per day)									
Gestation,	days 0–7	-	-	-	-	17.9	18.3	17.9	17.7
	days 14–20	-	-	-	-	21.6	21.6	20.9	**19.9***
Lactation,	days 0–7	-	-	-	-	34.1	35.3	36.9	35.9
	days 7–14	-	-	-	-	51.8	51.7	52.6	50.2
	days 14–21[§]	-	-	-	-	64.5	65.3	65.7	62.9

	Dose level (ppm)							
	Males				Females			
Parameter	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Gestation								
Estrus cycle length (days)	-	-	-	-	4.1	4.1	4.1	**4.4***
Gestation index (%)	-	-	-	-	100	100	100	**66.7****
Duration of gestation (days)	-	-	-	-	22.2	22.3	22.4	**23.0****
Organ weights								
Liver (mg)	17746	17295	17550	18750	10336	10426	10602	**11542***
Ovaries[#] (mg)	-	-	-	-	51.4	50.7	53.7	**60.5****
Liver (%)	3.07	3.07	3.04	**3.25***	3.36	3.37	3.5	**3.79*****
Ovaries[#] (%)	-	-	-	-	0.017	0.016	0.018	**0.01991*****
Microscopic findings								
Liver; centrilobular fatty change of hepatocyte	0	0	1	**19****	0	0	0	0
Liver; centrilobular hypertrophy of hepatocyte	0	0	0	0	0	0	0	**18****

GD Gestation day LD Lactation day

[§] Includes food consumption of offspring

[#] Mean weight of both sides

* $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

F1 generation

There were no deaths among males in the F1 generation. Four F1 females in the 750 ppm group died during delivery. There were no treatment-related signs.

In the 750 ppm group, mean body weights of F1 males at treatment weeks 0 to 10 were significantly lower than those in the control group, a feature most prominent during the first four weeks (0–4; up to −12.5%). Mean body weights of F1 females were significantly lower than those in the control group at treatment weeks 0 to 10 (up to −11.1%), on gestation days 0, 7, 14 and 20 (up to −8.3%), and on lactation days 0 and 7 (up to −8.8%) in the 750 ppm group. Mean body weight gains of F1 males in the 750 ppm group were significantly lower than those in the control group at treatment weeks 1 to 4 and in females of this group at treatment week 10.

Mean food consumption of F1 males in the 750 ppm group was significantly lower than that in the control group at treatment weeks 1 to 4 and week 10, with the strongest effect at week 1 (−15.1%).

No significant differences were noted in the parameters of sexual development, preputial separation in F1 parental males and vaginal opening in F1 parental females, between the control group and any of the treated groups and both parameters were well within the range of the historical control data from the four studies conducted between 2002 and 2012 that provide a range of 40.8–42.0 days for preputial separation and 30.4–31.1 days for vaginal opening (see Takahashi, 2016).Therefore, measurement of anogenital distance in F2 pups was not conducted.

In contrast to the F0 generation, estrus cycles of F1 females in the 750 ppm group were unaffected by treatment. Mating performance and fertility were unaffected by treatment. Although a statistically significant difference was observed in the mean number of days until mating in the 750 ppm group (1.2 days), this change was not due to a lower value in the 750 ppm group, but to slightly higher values in the control (2.0 days), in the 30 ppm and 150 ppm groups (1.7 and 1.4 days respectively). Gestation index in the 750 ppm group was significantly lower than that in the control group (75% compared to 100%) due to reduced number of live litters (18/24 compared to 22/22). The reduction in the number of live litters at 750 ppm is based on the increased number of dams that died during delivery. Duration of

gestation in the 750 ppm group (22.7 days) showed no statistically significant difference but a tendency to be prolonged compared to the controls (22.4 days). However, historical control data means for the duration of gestation ranged from 22.1 to 22.4 days (nine studies from 1997–2012; see Takahashi, 2015c). There were no treatment-related effects on sperm.

At 750 ppm, the relative weight of seminal vesicles in F1 males was significantly higher than that in the control group. In addition, significantly lower values were observed in the absolute brain, pituitary, and kidney weights of F1 males in the 750 ppm group, but no significant differences were noted in the relative weights of these organs. F1 females in the 750 ppm group showed a statistically significant increase in the relative liver weight when compared with the control (11.5%). The absolute and relative ovary weights of F1 females in the 750 ppm group were increased significantly when compared with the controls (absolute, 66.6g compared with 53.3g; relative, 0.02071% compared with 0.01549%). Besides this increase in ovary weight, significantly lower values were observed in the absolute brain and kidney weights of F1 females in the 750 ppm group, however, no significant differences were noted in the relative weights of these organs. Relative spleen weights of F1 females in the 750 ppm group showed a tendency to increase compared to controls.

There were no macroscopic findings attributable to treatment with metconazole. Four females found dead during delivery similarly had no treatment-related gross abnormalities. In histopathological examination of 10 randomly selected pairs of F1 parental males and females from the control and 750 ppm groups, no treatment-related abnormalities were noted in the reproductive organs, pituitary, or adrenals.

Examination of animals that did not yield successful reproduction results also showed no treatment-related abnormalities. Livers of F1 males and females in the 750 ppm group showed significantly increased incidences of centrilobular fatty change in hepatocytes, and centrilobular hypertrophy of hepatocytes, respectively, when compared with the corresponding controls. Examination of spleens revealed a significant increase in the incidence of congestion in the F1 females in the 750 ppm group. No treatment-related histopathological changes were noted in seminal vesicles of F1 males or in the ovaries of F1 females from any of the treated groups. Ovarian cysts occur with a total incidence of six in concurrent F1 control animals, while up to five cysts were observed in the top-dose F0 animals and 11 cysts in the top-dose F1 females. Overall, the occurrence of ovarian cysts is considered to be unrelated to treatment. Furthermore, no correlation was seen between the occurrence of cysts and the ovary weight changes based on the individual animal data. No statistically significant difference was noted in the mean number of primordial follicles in the ovaries from F1 parental females between the control group and the 750 ppm group (low and intermediate doses not evaluated), and the values displayed high standard deviations in both the control and top-dose groups.

Table 39. Key findings for the F1 generation of a two-generation dietary toxicity study in rat

		Dose level (ppm)							
		Males				Females			
Parameters		**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
		F1 Generation							
Number of animals/group		24	24	24	24	24	24	24	24
Mortality		0	0	0	0	0	0	0	4
Body weight/gain (g)									
Body weight,	week 0	86	86	86	**77****	81	81	79	**72****
Body weight,	week 1	140	139	137	**123****	122	121	118	**110****
Body weight,	week 2	205	202	199	**181****	163	160	158	**150****
Body weight,	week 3	272	267	263	**238****	193	191	188	**178****
Body weight,	week4	334	329	325	**299****	222	215	214	**204****
Body weight,	week 5	381	379	373	**355****	242	238	238	**224****

		Dose level (ppm)							
		Males				Females			
Parameters		**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Body weight,	week 6	422	420	412	**397***	260	254	256	**238****
Body weight,	week 7	455	452	445	**427***	274	273	271	**252****
Body weight,	week 8	484	480	473	**452****	288	284	283	**264****
Body weight,	week 9	508	503	494	**474****	296	294	291	**272****
Body weight,	week 10	529	522	512	**491****	305	301	300	**277*****
Body weight gain,	weeks 0–1	54	53	52	**47*****	41	40	39	38
Body weight gain,	weeks 0–2	119	116	114	**104*****	81	79	79	78
Body weight gain,	weeks 0–3	185	181	178	**162*****	112	111	109	106
Body weight gain,	weeks 0–4	248	243	239	**223*****	141	135	135	132
Body weight gain,	weeks 0–10	443	436	427	415	223	220	221	**204****
Body weight,	GD 0	-	-	-	-	305	304	304	**281***
Body weight,	GD 7	-	-	-	-	336	333	334	**308****
Body weight,	GD 14	-	-	-	-	366	364	364	**338****
Body weight,	GD 20	-	-	-	-	434	437	438	**404***
Body weight gain,	GDs 0–20	-	-	-	-	129	132	134	124
Body weight,	LD 0	-	-	-	-	337	328	327	**309***
Body weight,	LD 7	-	-	-	-	363	357	360	**331***
Body weight gain,	LDs 0–21	-	-	-	-	1	4	11	14
Food consumption (g/animal per day)									
Pre-pairing,	week 1	13.9	14	13.9	**11.8*****	11.1	11.5	11.5	10.5
	week 2	19.4	19.3	19.1	**17.2*****	15.3	15.4	15.2	**14.5***
	week 3	23.7	23.5	23.1	**21.1*****	16.2	16.1	16.2	15.4
	week 4	26.1	26	25.7	**23.6****	17	16.6	17	16
	week 10	25	24.7	24.3	**23.5***	16.4	16.6	17.2	15.7
Gestation,	days 0–7	-	-	-	-	18.4	17.5	18.6	17.4
	days 7–14	-	-	-	-	22.4	21.3	21.5	20.8
	days 14–20	-	-	-	-	22.8	22.9	23.4	21.6
Lactation,	days 0–7	-	-	-	-	33.9	35.6	36.2	31.8
	days 7–14	-	-	-	-	50.3	54.3	53.6	47.3
	days 14–21[§]	-	-	-	-	61.1	64.8	64.4	57.2
Sexual maturation (preputial separation and vaginal opening)									
Age in days at completion		40.8	40.8	41	41.7	31.1	30.9	31.1	31.2
Body weight at completion		217.5	216.3	218.2	208.3	111.5	111.6	112.8	108.9
Estrus cycles		-	-	-	-	4.3	4.6	4.1	4.1
Time until mating (days)		-	-	-	-	2	1.7	1.4	**1.2***
Gestation									
Gestation index (%)		-	-	-	-	100	100	100	**75.0***
Duration of gestation (days)		-	-	-	-	22.4	22.3	22.3	22.7

	Dose level (ppm)							
	Males				Females			
Parameters	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Organ weights								
Brain; absolute (mg)	2221	2194	2205	**2083*** **	2034	2004	2007	**1896*** **
Pituitary; absolute (mg)	13.4	13.1	12.5	**12.2***	15.3	15.1	15.4	14.4
Kidneys; absolute (mg)	1802	1803	1736	**1635****	1117	1083	1079	**1000****
Ovaries; absolute (mg)	-	-	-	-	53.3	55.2	57.9	**66.6*** **
Liver; relative (%)	3.25	3.25	3.13	3.42	3.46	3.4	3.58	**3.86****
Spleen; relative (%)	0.153	0.154	0.154	0.156	0.188	0.186	0.185	0.208
Seminal vesicles; relative (%)	0.405	0.408	0.424	**0.460****	-	-	-	-
Ovaries[#]; relative (%)	-	-	-	-	0.015	0.016	0.017	**0.02071*** **
Microscopic findings								
Liver – centrilobular fatty change	0	0	1	**20****	0	0	0	0
Liver – centrilobular hypertrophy	0	0	0	0	0	0	0	**20****
Spleen – congestion	0	0	0	0	0	1	1	**16****
Ovarian follicle counts	-	-	-	-	437	NS	NS	362

GD Gestation day LD Lactation day

[§] Includes food consumption of offspring

[#] Mean weight of both sides

* $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Offspring data

F1 litters

There were no treatment-related clinical signs.

Litter size on day 0 was slightly but not significantly decreased. Offspring survival was unaffected by treatment. Sex ratios were unaffected by treatment. In the 750 ppm group, mean body weights of both F1 male and female pups were slightly but not significantly lower on lactation days (LDs) 14 and 21 than those of the control group (up to –6.5%). Male and female body weights of the 750 ppm group were well within historical control data (total of nine studies conducted between 1997 and 2012; see Takahashi, 2015c).

In detail, male body weights on LD 14 were 39.0 g and 37.5 g in the control and 750 ppm groups respectively. Females yielded body weights of 38.1 g and 35.9 g in the control and 750 ppm group respectively. Historical control data reported mean values for F1 body weights of 35.1–40.5 g for males and 33.7–39.0 g for females on LD 14 of lactation.

Male body weights on LD 21 were 63.3 g and 60.5 g in the control and 750 ppm groups respectively. Females yielded body weights of 61.4 g and 57.4 g in the control and 750 ppm groups respectively. Furthermore, historical control data reported mean values for F1 body weights of 58.0–68.3 g for males and 55.3–65.4 g for females on LD 21.

In the 750 ppm group, relative spleen weights of F1 male and female pups were significantly higher than those in the control group. Absolute brain weights of F1 male and female pups were significantly lower than those in the control group, however no significant differences were noted in the relative weights of their brains.

There were no macroscopic findings attributable to treatment with metconazole. There were no treatment-related microscopic findings in the spleen of F1 male or female weanlings.

F2 litters
There were no treatment-related signs.

Implantation sites and litter size were unaffected by treatment. Offspring survival (viability index) in the 750 ppm group was significantly decreased on day 0 when compared with that in the control group. The reduced viability index in the 750 ppm group on day 4 was due to a total litter loss in two litters during LDs 1–4.

Sex ratios were unaffected by treatment.

In the 750 ppm group, mean body weights of both F2 male and female pups were significantly lower on LDs 0, 14 and 21 than those in the control group (–10.4% for F2 males and −11.6% for F2 females) at weaning. However, mean male and female body weights in the 750 ppm group were well within historical control data.

In detail, male body weights on LD 21 were 68.0 g and 60.9 g in the control and 750 ppm groups respectively. Females yielded body weights of 65.7 g and 58.1 g in the control and 750 ppm groups respectively. Historical control data (nine studies, 1997–2012; see Takahashi, 2015c) reported mean values for F2 body weights of 58.6–69.8 g for males and 55.8–66.7 g for females on LD 21.

Nevertheless, due to a clear dose-dependency and the sustained nature of this body weight deviation, and since the same observation albeit not statistically significant, was already noticed in F1 animals, the effect is considered treatment-related.

For F2 female pups in the 750 ppm group final body weight was significantly lower and relative spleen weight significantly higher than weights in the control group. Absolute brain weights of F2 male and female pups in the 750 ppm group were significantly lower than those in the control group, however, no significant differences were noted in the relative weights of their brains. There were no macroscopic findings attributable to treatment with metconazole. There were no treatment-related microscopic findings in the spleens of F2 male and female weanlings.

Table 40. Key findings for the F1/F2 generations of a two-generation dietary toxicity study in rat

	Dose level (ppm)							
	Males				Females			
Parameters	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Litter data (F1 litters)								
Live litters born	-	-	-	-	24	23	24	14
Litter size; implantation sites	-	-	-	-	14.4	14.3	14.3	13.4
Litter size; day 0 (total)	-	-	-	-	13.3	13.4	13.3	11.8
Viability indices (%) [a]								
Day 0	-	-	-	-	97.3	98.9	99.7	96.3
Day 4	-	-	-	-	99.7	99.5	99.7	96.3
Day 21	-	-	-	-	94.8	97.8	97.4	98.2
Sex ratio [b]	-	-	-	-	0.5	0.52	0.44	0.491
Body weight (g)								
Day 0	7	6.9	7	6.8	6.6	6.6	6.7	6.4
Day 14	39	39.9	39.4	37.5	38.1	38.2	38	35.9
Day 21	63.3	64.8	65.2	60.5	61.4	61.7	62.2	57.4
Organ weights								
Brain (mg)	1632	1616	1588	**1517*****	1587	1562	1538	**1481*****
Spleen (%)	0.38	0.38	0.4	**0.456*****	0.38	0.37	0.38	**0.430***

	Dose level (ppm)							
	Males				Females			
Parameters	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Litter data (F2 litters)								
Live litters born	-	-	-	-	22	23	20	18
Litter size; implantation sites	-	-	-	-	13.5	14	14.9	13.5
Litter size; day 0 (total)	-	-	-	-	11.7	13	13.9	10.3
Viability indices (%) [a]								
Day 0	-	-	-	-	98.6	99.7	99	**89.1***
Day 4	-	-	-	-	100	95.4	98.5	88.5
Day 21	-	-	-	-	100	100	97.5	100
Sex ratio [b]	-	-	-	-	0.51	0.5	0.49	0.508
Body weight (g)								
Day 0	7.4	7	6.9	**6.4*****	7.1	6.7	6.5	**6.3***
Day 14	41.4	40.7	40.3	**37.7****	40.4	38.9	38.7	**36.5****
Day 21	68	67.1	66.6	**60.9****	65.7	63.5	63.6	**58.1*****
Terminal observations								
Number examined	22	22	20	16	22	22	20	16
Body weight at necropsy (g)	94	95	93	87	89	89	86	**79****
Organ weights (mg)								
Brain (mg)	1607	1616	1606	**1520*****	1571	1567	1557	**1465*****
Spleen (%)	0.39	0.39	0.4	0.426	0.37	0.36	0.37	**0.418****

[a] Viability indices are calculated for each litter from the following formulae and given as mean values for each group:

Day 0 = (no. of pups alive on lactation day 0/no. of pups delivered) × 100

Day 4 = (no. of pups alive on lactation day 4/ no. of pups alive on lactation day 0) × 100

Day 21 = (no. of pups alive on lactation day 21/ no. of pups selected on lactation day 4) × 100

[b] Sex ratio = total number of male pups/total number of pups delivered

* $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Based on the results of this two-generation study in the rat, the NOAEL for parental toxicity in the F0 and F1 males and females was 150 ppm (9.79, 10.78, 10.63, and 11.21 mg/kg bw per day for F0 males, F0 females, F1 males and F1 females, respectively), based on increased mortality, decreased body weight gain and increased liver weights associated with hepatocyte fatty change at the highest dose tested, 750 ppm (49.4, 53.2, 53.0, and 55.5 mg/kg per day for F0 males, F0 females, F1 males and F1 females, respectively).

The NOAEL for offspring was 150 ppm based on decreased live litters born (in F1 and F2), decreased viability index (F2) and decreased body weight of F2 at 750 ppm.

The NOAEL for reproduction is 150 ppm based on increase in gestation length, reduced gestation index in the F1 generation at 750 ppm (Teramoto, 2006a; Takahashi, 2015a).

(c) Developmental toxicity

Rats

Study 1

Five groups of 10 mated female Sprague Dawley rats were used in this preliminary study. Metconazole technical (WL148271; purity 95.3%; *cis*:*trans* 83.7:16.3, nominal 85:15; lot/batch 89-01) was administered by gavage in 1% CMC. Animals were treated from day 6 to day 15 of pregnancy at 0, 37.5, 75, 150 and 300 mg/kg bw per day. Dosing volume was 10 mL/kg bw. On GD 20 the females were sacrificed and subjected to postmortem examination. Litter parameters were determined but fetuses were not examined in detail.

A dose-related adverse response was observed in the dams treated at 150 and 300 mg/kg bw per day, including two and four mortalities, respectively. Increased water intake throughout the dosing period, persistent suppression of food consumption from initiation of treatment, initial body weight loss coinciding with the start of dosing followed by incomplete recovery, clinical signs (post-dosing salivation and brown stained fur), and total resorption in 3/8 and 3/5 instances was observed at 150 and 300 mg/kg bw per day, respectively. At 75 mg/kg bw per day maternal effects were still apparent but much less marked and were confined to slightly increased water intake on GDs 6–7, slightly decreased food consumption and body weight gain from the start of treatment through to termination. No clear effects were recorded in animals treated at 37.5 mg/kg bw per day, but possible marginal disturbances of food intake and body weight gain could not be discounted.

At 300 mg/kg bw per day only two pregnancies were maintained to GD 20. At all dose levels investigated there was evidence of a dose-related reduction in litter size concomitant to an increasing risk of embryo-fetal death involving some or all concepti in the litter. Despite lower litter sizes, mean fetal weight was also impaired. However, at dosages where there were sufficient fetuses available for valid comparison there was no obvious increase in incidence of gross morphological defects.

Severe maternal toxicity (increased mortality) and embryo toxicity (100% intrauterine deaths) were observed at dose levels of 150 and 300 mg/kg bw per day. Significant maternal toxicity (reduced body weight gain) and embryo/fetotoxicity (increased number of resorptions and decreased fetal weight) was noted at 75 mg/kg bw per day. Therefore, from the results of this preliminary study 75 mg/kg bw per day was considered to be sufficiently high a top dose for the main study.

Four groups of mated female Sprague Dawley rats (25 females/group) were used in the main study. Metconazole technical (WL148271; purity 95.3%; *cis*:*trans* 83.7:16.3, nominal 85:15; lot/batch 89-01) was administered by gavage as a suspension in 1% CMC. Animals were treated on GDs 6–15 at dose levels of 0, 12, 30 or 75 mg/kg bw per day. Dosing volume was 10 mL/kg bw. On day 20 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined and fetuses fixed prior to examination for skeletal and visceral abnormalities.

No mortalities occurred. Post-dose salivation was noted in 20/25 dams at the highest dose of 75 mg/kg bw per day during late gestation (GDs 12–15). The late onset of this sign was associated with irritation of the GIT due to the repeated gavage of the compound. Clinical signs were not evident at the other dose levels.

Water consumption appeared to be slightly elevated at 75 mg/kg per day during the first four days of the dosing period. Food consumption was slightly reduced on the first two days of treatment at the top dose (−10%). Related to the early reduction in food consumption, body weight gain was statistically reduced at 75 mg/kg per day at both the beginning of the dosing period (GDs 6–8; −67%) as well as from the beginning of dosing throughout gestation (GDs 6–20; −11%). Body weight at GD 20 appeared to be reduced at this dose (−4%) but was not statistically different from controls. Body weight gain was statistically reduced at the beginning of dosing at 30 mg/kg per day as well (GDs 6–8; −29%). No macroscopic changes related to treatment were observed in the dams at necropsy.

Treatment with metconazole technical at dose levels up to and including 75 mg/kg bw per day did not affect pregnancy status as the pregnancy rates were comparable in control and treated groups. There was no incidence of total litter loss or effects on mean corpora lutea or implantation parameters. Pre-implantation loss was not affected. However, post-implantation loss and resorptions (both early and late) were significantly increased (four-fold from controls), at the high dose of 75 mg/kg per day. As a result, the mean number of live fetuses per litter appeared to be reduced at this dose compared with

controls, albeit the difference was not statistically significant. Mean fetal weights and total litter weights were also significantly reduced at the highest dose. Sex ratios were unaffected. A statistically significant reduction in mean fetal weight was also observed at 30 mg/kg per day; this effect could well be an indirect consequence of the slightly increased litter size (11%) and slightly increased litter weight (6.5%). It should be noted, that total litter weights were increased at 12 and 30 mg/kg per day; these increases appear to be a function of the increased mean number of fetuses per litter at these doses compared to controls.

A low incidence of malformations was observed in this study. Malformations were observed in 4/281, 1/315, 0/340, and 7/239 fetuses, respectively at 0, 12, 30 and 75 mg/kg per day; with regard to litters this corresponds to 3/22, 1/22, 0/24 and 6/22 litters affected respectively. No one fetus displayed more than a single malformation.

Two incidences of hydrocephalus were observed at the top dose in separate litters. Hydrocephalus was not observed in any control fetus from studies conducted at the testing facility with Sprague Dawley (SD) rats received from the same supplier (Charles River, France). Historical control data from the testing facility (1990–1992; see Charles River, 1990–92) indicated that hydrocephalus in SD rats was observed in three studies conducted with rats received from Charles River, UK, and in one study with rats from Charles River, USA (historical incidence range for fetuses [litters]: 0 [0] – 1 [1]). The fetal and litter incidences of hydrocephalus in this study, albeit low and not statistically significantly different from controls, are above the in-house historical control data and were considered treatment-related. Other malformations at the top dose included one brachygnathia, and one umbilical hernia.

A single case of interventricular septal defect (VSD) was reported at both the low and high dose. This finding was designated as a malformation in this study, whereas in the Fulcher study (2002) both VSDs and small VSDs are classified as variations.

The significant increase of visceral variations at the top dose may be of a general nature rather than due to specific abnormalities. Slight increases in the incidence of dilated renal pelvis and ureter were observed at 75 mg/kg bw per day compared to controls. Historical controls per separate end-point were not provided, but figures were presented for the combination (renal pelvis and/or ureter dilatation) and were in the ranges 0–5.67% (fetal incidence) and 0–24% (litter incidence). In comparison with the combined incidences in this study 6/116 = 5.17% (fetal incidence), and 4/22 = 18.18% (litter incidence) the findings appeared coincidental. It should be remarked that the findings were also observed in other developmental studies. Likewise, the observed increased incidence of testis displacement at the top dose was within historical control ranges and was therefore considered not treatment-related.

Small interventricular septal defects (small VSDs) were reported in all dose groups, including controls. The incidence of small VSDs at 75 mg/kg per day was slightly above the historical control range. Both VSDs and small VSDs are likely to be related to developmental delay (evidenced by lower fetal body weights) in the high-dose group and are assumed to resolve over time postnatally. It was attempted to evaluate further the relationship between VSDs and developmental delay by examining the fetal weights of litters with fetuses reported to have VSDs or small VSDs. Unfortunately, individual fetal weights were not reported in this study except for one very small fetus with a VSD.

Other visceral variations, such as the incidences of haemorrhage of the brain, although above control in the treated groups, were within the historical control range. Data for haemorrhage of the eyes showed a similar trend.

There was a dose-related increase in the incidence of fetuses with skeletal variations at 30 and 75 mg/kg bw per day. This was mainly due to increased incidences showing delay of ossification in the axial skeleton, especially the incidence of lumbar ribs, and to a lesser degree the incidences of cervical ribs, extra pre-sacral vertebrae, and variant sternebrae.

The incidence of lumbar ribs in particular was statistically increased at both 30 and 75 mg/kg per day. At 12 mg/kg bw per day the overall incidence of skeletal abnormalities was similar to the control but the number of fetuses with lumbar ribs was twice that found in the concurrent control group. The relationship of this intergroup difference to treatment was considered uncertain, as the difference was not statistically significant.

The fetal incidence for additional lumbar ribs in historical controls ranges from 8.4–19.6% and the litter incidence from 40–50%. The incidences of all other ossification variations in the treated groups were comparable to controls.

Table 41. Key findings of developmental toxicity study in rat

Dose level (mg/kg bw per day)	**0**	**12**	**30**	**75**	**HCD** Number of fetuses (litters) affected; fetal incidence in %
Number of pregnant animals	22/25	22/25	24/25	22/25	
Litter observations					
Corpora lutea	16.5	17.1	16.5	17	
Implantations	13.6	15.1	15.1	14.5	
Pre-implantation loss	16.6%	11.5%	8.4%	13.8%	
Post-implantation loss	6%	5.2%	7.2%	**24.5%*****	
Total resorptions	0.8	0.8	1	**3.5*****	
Early resorptions (Number of deaths/dam)	0.7	0.7	0.7	**1.6*****	
Late resorptions (Number of deaths/dam)	0.1	0.1	0.3	**2.0*****	
Litter size	12.8	14.3	14.2	10.9	
Fetal weight (g)	3.82	3.84	**3.65***	**3.51****	
Total litter weights (g)	48.64	**54.70***	51.78	**38.49***	
Placental weight (g)	ND	ND	ND	ND	
Malformations – No. fetuses/(litters) (% fetuses affected)					
All malformations					
Fetuses	4/281	1/315	0/0	7/239	
Fetuses (%)	1.4%	0.3%	0.0%	2.9%	
Litters	3/22	1/22	0/0	6/22	
Litters (%)	13.6%	4.5%	0.0%	27.3%	
Hydrocephalus					
No. fetuses (litters)	-	-	-	**2 (2)**	0 (0)–1 (1); 0–0.4%
Fetal (litter) incidence	-	-	-	**0.8% (9.1%)**	
VSD	-	1(1)/0.32%	-	1(1)/0.42%	0 (0)–2(2); 0–0.74%
Umbilical hernia	-	-	-	1(1)	
Brachygnathia	-	-	-	1(1)	
Distortion affecting ribs	2(2)	-	-	-	
Forelimb flexure[+]	2(1)/0.71%	-	-	2(1)/0.84%	0 (0)–3(1); 0–1.19%
Visceral variations					
% skeletal variations					
Fetuses	5.8	6.9	5.6	**16.3***	
Litters	27.3	40.9	25	63.6	
Dilatation renal pelvis					
Fetuses	0/139 (0.0%)	1/156 (0.6%)	1/171 (0.6%)	2/116 (1.7%)	0 (0)–8(6); 0–5.67%[#]
Litters	0/22 (0.0%)	1/22 (4.5%)	1/24 (4.2%)	2/22 (9.1%)	0–24%[#]

Dose level (mg/kg bw per day)	0	12	30	75	HCD Number of fetuses (litters) affected; fetal incidence in %
Dilatation ureter					
Fetuses	0/139 (0.0%)	0/156 (0.0%)	0/171 (0.0%)	4/116 (3.4%)	(0)–8(6); 0–5.67%[#]
Litters	0/22 (0.0%)	0/22 (0.0%)	0/24 (0.0%)	2/22 (9.1%)	0–24%[#]
Displaced testis					
Fetuses	0/139 (0.0%)	0/156 (0.0%)	0/171 (0.0%)	2/116 (1.7%)	
Litters	0/22 (0.0%)	0/22 (0.0%)	0/24 (0.0%)	2/22 (9.1%)	
Hemorrhage of the brain/spinal cord					
Fetuses	0/139 (0.0%)	3/156 (1.9%)	3/171 (1.8%)	3/116 (2.6%)	0–5.41%
Litters	0/22 (0.0%)	2/22 (9.1%)	3/24 (12.5%)	3/22 (9.1%)	
Hemorrhage of the eyes					
Fetuses	1/139 (0.7%)	0/156 (0.0%)	0/171 (0.0%)	2/116 (1.7%)	0–2.29%
Litters	1/22 (4.5%)	0/22 (0.0%)	0/24 (0.0%)	2/22 (13.6%)	
Small interventricular septal defects					
Fetuses	4/139 (2.9%)	2/156 (1.3%)	3/171 (1.8%)	6/116 (5.2%)	(0)–4(4); 0–3.15%
Litters	4/22 (18.2%)	2/22 (9.1%)	2/24 (8.3%)	6/22 (27.3%)	
Skeletal Variations					
% skeletal variations					
Fetuses	21.9	23	**42.2****	**72.2*****	
Litters	77.3	77.3	95.8	95.5	
Additional cervical rib (s)					
Fetal incidence	1/138 (0.7%)	1/158 (0.6%)	4/169 (2.4%)	**9/116 (7.8%)**	0–2.56%
Litter incidence	1/22 (4.6%)	1/22 (4.6%)	2/24 (8.3%)	7/22 (31.8%)	
Additional lumbar rib (s)					
Fetal incidence	11/138 (8%)	28/158 (18%)	**48/169 (28%)**	**69/116 (59%)**	0–17.42%
Litter incidence	8/22 (36%)	14/22 (64%)	18/24 (75%)	19/22 (86%)	
One Extra Pre-Sacral Vertebra					
Fetal incidence	0/138 (0.0%)	1/158 (0.6%)	**6/169 (3.6%)**	**9/116 (7.8%)**	0–2.56%[§]
Litter incidence	0/22 (0.0%)	1/22 (4.6%)	6/24 (25.0%)	5/22 (22.7%)	
Unossified sternebra					
Fetal incidence	33/138 (26.3%)	31/158 (19.7%)	**82/169 (47.3%)***	**55/116 (49.7%)****	
Litter incidence	15/22 (68%)	15/22 (68%)	22/24 (92%)	21/22 (95%)	
Thoracic distorted ribs					
Fetal incidence	0/138 (0.0%)	1/158 (0.6%)	0/169 (0.0%)	5/116 (4.3%)	
Litter incidence	0/22 (0.0%)	1/22 (4.5%)	0/24 (0.0%)	5/22 (22.7%)	
Caudal vertebra/disc irregularity					
Fetal incidence	0/138 (0.0%)	0/158 (0.0%)	0/169 (0.0%)	4/116 (3.4%)	
Litter incidence	0/22 (0.0%)	14/22 (0.0%)	18/24 (0.0%)	3/22 (13.6%)	

[§] Based on historical control range reported for 'one additional thoracolumbar vertebra' ND Not determined

[#] Based on historical control data reported for combined 'dilation renal pelvis/ureter'

Metconazole (WL148271; *cis*:*trans* 80:15) administered to pregnant rats from GDs 6–15 induced maternal toxicity (reductions in body weight gain) and developmental toxicity (increases in skeletal ossification variations) at 30 and 75 mg/kg bw per day. At the top dose, embryo/fetal toxicity (post-implantation loss, reductions in fetal/litter weights) was evident. A slight but significant increase in hydrocephalus incidence was considered potentially treatment-related, but this teratogenic finding could be subsequent to the high maternal toxicity demonstrated. The presence of skeletal anomalies (variations) at 12 mg/kg bw per day was ambiguous but a relationship to treatment could not be dismissed. Nevertheless, globally, the lowest dose could be considered a no-adverse effect level.

Therefore, the NOAEL for maternal and developmental toxicity was 12 mg/kg bw per day, the NOAEL for fetal toxicity was 30 mg/kg bw per day (Masters et al., 1991a).

Study 2

Groups of 22 mated CD female rats were given daily oral doses by gavage of KNF-474m (lot/batch 9Z521; purity 98.99%; *cis*:*trans* 83.13:15.86) suspended in 1% aqueous methylcellulose. Administration was during days 6–19 of gestation at a dose volume of 4 mL/kg body weight. Controls received the vehicle alone.

Study 2. Preliminary study

KNF-474m was administered once daily by oral gavage at dosages of 4, 16 or 64 mg/kg per day to groups of six mated female rats from GD 6 to GD 19 inclusive. Control animals received the vehicle, 1% methylcellulose, through the same period. Females were killed on GD 20 for examination of their uterine contents, and fetuses were examined externally for any abnormalities. Lower maternal body weight gain and slightly lower food intake were seen at 64 mg/kg per day. Litter data was not obviously affected but increased placental weight and decreased fetal weight were observed at this dosage, these findings being supported by observations of external fetal necropsy. At lower dosages there was no evidence of maternal toxicity, however, at 16 mg/kg per day there was a suggestion of a similar effect on placental and fetal weight to that observed at the highest dosage, and two litters contained fetuses with swollen placentae

Study 2. Main study

Dosages of 0, 1, 4, 16 and 64 mg/kg per day were based on a contemporary preliminary study where Individual doses were calculated based on body weight on the nearest day of dosing; the final dose volume adjustment was based on body weight on day 17 after mating.

One female receiving 64 mg/kg bw per day was killed on GD 18 after showing body weight loss (14 g) from GD 16, hunched posture, pallor, pale eyes and piloerection. Necropsy revealed firm and reduced caecal contents; there were 14 implantations, two of which were early resorptions and another four were late deaths. The remaining eight live fetuses had a pale swollen placenta.

Treatment at 64 mg/kg per day was associated with body weight stasis or slight mean body weight loss (−6 g) from GD 6 to GD 8. Thereafter, some recovery was evident to termination although body weight gain remained lower than for controls. This lower gain was most noticeable between GDs 8 and 15 (−25.6% compared with controls; $p < 0.01$). Overall mean body weight gain, after adjustment by subtracting gravid uterus weight, was very markedly lower than for controls (mean of 11 g compared with 38 g for the controls; $p < 0.01$), indicating that this was a maternal effect.

Means for body weight and body weight change at dosages of 1, 4 and 16 mg/kg pere day were essentially similar to controls. Slightly low overall mean adjusted body weight change at 16 mg/kg per day was associated with slightly higher (not statistically significant) gravid uterus weight.

At 64 mg/kg per day, group mean food consumption was significantly lower (−20% for GDs 6–19) than for concurrent controls, consistent with the lower body weight gain. Group mean food consumption at 1, 4 and 16 mg/kg per day was unaffected by treatment.

Two females receiving 64 mg/kg bw per day had blood discharge or red staining at the vagina at termination. With this exception, maternal necropsy findings showed no adverse effect of treatment with KNF-474m.

All females surviving until scheduled termination had live young on GD 20. All females at 64 mg/kg bw per day had swollen placentas, many also being pale and/or mottled. At 16 mg/kg bw per day, all placentas of one female were swollen and mottled, and seven of 15 placentas were swollen in a second

female. Occasional swollen placentas were also recorded in both the control and 4 mg/kg bw per day groups, indicating a low background incidence. This increased post-implantation loss resulted in a significantly lower live litter size (12.8 compared with 15.0 for controls; $p < 0.05$), but sex ratio was unaffected. At dosages of 1, 4 and 16 mg/kg per day, the number of in utero deaths, live young and sex ratio were unaffected by treatment.

At 64 mg/kg per day there was a reduction in mean fetal weight (males and females combined were −17.6% compared with controls; $p < 0.01$) and increased mean placental weight (45.5% compared with controls; $p < 0.01$). All placentas in this group were noted at necropsy as being swollen and many were pale or had a mottled appearance. Mean litter weight and gravid uterus weight in this group were markedly lower than for controls.

There was a minimal increase (5.5% compared with control) in mean placental weight at 16 mg/kg per day; in the two females with swollen placentas; litter mean placental weights were slightly high (0.66 g and 0.69 g) but litter mean fetal weights (3.40 g and 3.55 g) and other fetal developmental parameters were unaffected. With this exception, placental, litter and fetal weight at dosages of 1, 4 or 16 mg/kg per day were essentially similar to controls.

Overall, the aetiology of enlarged placenta and increased placental weight is unclear. No further investigations were performed on the placenta. Enlarged placentas had however, no effect on the survival of the fetuses at the dose levels in the absence of resorptions.

There were 0, 2, 2, 2 and 3 fetuses showing malformations (0, 2, 1, 2 and 2 litters affected) in the 0, 1, 4, 16 and 64 mg/kg groups respectively. However, neither type nor incidence of these changes indicated any obvious adverse effect of treatment with KNF-474m.

The incidence of the external malformations rudimentary/threadlike tail and imperforate anus was low and within the background incidences seen in the historical control data for this laboratory.

At 64 mg/kg/day, a dosage where fetal weight was significantly low, there was a notable increase in the incidence of minor skeletal and visceral anomalies compared with the control, as follows.

There was an increased incidence of small ventricular septal defect and of ventricular septal defect, which may both be associated with delayed development (eight of the 11 affected fetuses were much smaller than the average), and of cervicothoracic artery anomalies (variation in origin of subclavian and rudimentary/absent innominate arteries) at this high dosage. One or more of these minor anomalies occurred in 10/21 litters at 64 mg/kg bw per day, compared to 2/22 litters for both the control and the 4 mg/kg bw per day groups. Such findings occur sporadically in the historical control data.

The increased interventricular septal defects (VSD) and small VSD found at the top dose in this study have been reviewed in an expert opinion by Dr Willoughby in 2005 (see p10 in Davies, 2015). Dr Willoughby was Head of Reproductive Studies Group at the laboratory where the metconazole rat study was conducted); in these extracts from his expert opinion he states:

> "None of the ventricular septal defects or small ventricular septal defects recorded in this study were considered large enough to be incompatible with postnatal life, and as such have been classified as visceral anomalies rather than as malformations. In man, simple ventricular septal defects (VSD) occur in approximately 12/10,000 births but it is thought that approximately 10–25% of these undergo spontaneous closure after birth (Warkany J., 1971, Congenital Malformations: notes and comments: Chapter 53, pages 489–494: Ventricular septal defects, ISBN 0-8151-9098-0). The incidence of VSD is much higher among children with a birth weight of less than 2500 g. In this rat strain at these laboratories, the incidence of VSD is very low in control animals but it does occur sporadically in association with other malformations and in cases of low fetal weight, suggestive of growth retardation. In the rat, the ventricular septum is normally closed on the 17th day of gestation (Warkany 1971). In the study with KNF-474m, 8 of the 11 affected fetuses were much smaller than the average, falling below the 95th percentile for fetal weight on day 20 of gestation. The three most affected fetuses averaged only 53% of expected bodyweight. These findings suggest that failure of the ventricular septum to be fully closed on day 20 of gestation may be associated with delays in fetal development. It seems likely that the developmental delay was established before the VSD would be apparent as this anomaly is generated at a late stage of development."

Furthermore, VSDs were detected only in fetuses of prenatal developmental toxicity studies. In the 2-generation studies from 1992 and 2002 which involved visceral examination of necropsied pups and adult offspring, VSDs were not reported in any animal.

At this high dose (64 mg/kg bw per day), minor skeletal bone and/or cartilage anomalies included a marked increase in the incidence of cervical ribs, a variation in false/floating rib/cartilage configuration compared with controls, and a marked increase in the incidence of lumbar ribs (13/14 or 14/14 ribs) compared with controls. There were also a few occurrences of 20 thoracolumbar vertebrae and of offset alignment of the pelvic girdle. This array of minor skeletal findings was considered indicative of increased fetal and/or maternal stress. The incidence of incomplete ossification of the sternebrae at 64 mg/kg bw per day was also higher than in controls, in accordance with low fetal weight.

Visceral examination also revealed at 64 mg/kg bw per day a high incidence of fetuses (20/21 litters affected) with green "deposits" in the renal papilla/renal pelvis and, to a lesser extent, ureters. The slightly higher incidence of hydroureter at the top dose was well within the historical control range and was considered most likely to represent a sign of delayed development as a consequence of reduced fetal weight at this dose. The finding of green "deposits" was not observed in the control or any other treatment group and has not been seen previously within this laboratory. Subsequent microscopic examination of selected fetuses indicated that these deposits were lipofuscin (breakdown products resulting from the oxidation of lipids and lipoproteins). The architecture of the fetal kidney appeared unaffected. It was considered possible by the study's authors that the enlarged placentas were less effective at removing waste products, resulting in accumulation of debris in the renal pelvis, or that the material was placental in origin.

Table 42. Key findings of developmental toxicity study in rat

Parameter	Dose (mg/kg bw per day)					
	0	**1**	**4**	**16**	**64**	**HCD**
Maternal data						
Number mated	22	22	22	22	22	
Number pregnant	22	22	22	22	22	
Killed prematurely	0	0	0	0	1	
Bodyweight (g) day 0	256	259	256	255	256	
day 6	292	292	291	290	288	
day 20	420	422	424	418	**380****	
At necropsy, minus gravid uterus	329	333	334	323	**299****	
Gravid uterine weight (g)	85.7	85.6	85.5	91.6	**77.0***	
Bodyweight change (g)						
days 6–8	8	9	8	7	**−6****	
days 8–15	43	42	44	41	**32****	
days 6–20	128	130	133	128	**92****	
days 6 to necropsy, minus gravid uterus	38	41	42	33	**11****	
Food consumption (g/rat per day)						
Days 0–2	29	29	29	29	29	
Days 3–5	30	29	29	30	29	
Days 6–9	30	30	30	30	**23****	
Days 10–13	31	31	31	30	**26****	
Days 14–17	33	32	34	32	**26****	
Days 18–19	32	33	32	31	**25****	
Approx. total consumption (g/rat)						
Days 6–19	410				327	

Parameter	Dose (mg/kg bw per day)					
	0	1	4	16	64	HCD
Litter mean values						
Number of litters examined	22	22	22	22	21	
Corpora lutea	15.9	16	15.7	16.6	15.5	
Implantations	15.7	15.5	15.5	15.9	15.4	
Pre-implantation losses (%)	2.9	4.7	3.3	4.2	2	1.9–9.6
Post-implantation losses (%)	4.4	6.1	3.3	3.6	**17.7****	2.9–10.1
Total resorptions,	0.7	1	0.5	6	**2.6****	
early	0.7	1	0.5	0.5	**1.6***	0.4–1.4
late	0	0	0	0	**1.0*****	0.0–0.1
Number of live fetuses	15	14.5	15	15.3	**12.8***	13.2–16.2
Sex ratio (male fetuses/live fetuses %)	48.1	51.1	49.7	49.3	50.8	
Litter weight (g)	54.2	54.64	54.66	56.39	**38.55****	50.17–59.83
Fetal weight (g)						
males	3.73	3.87	3.75	3.79	**2.99****	3.66–4.05
females	3.55	3.63	3.58	3.58	**2.95****	3.45–3.86
combined m/f	3.63	3.75	3.66	3.68	**2.99****	3.56–3.96
Placental weight (g)	0.55	0.55	0.55	**0.58***	**0.80****	0.51–0.57
Placental findings – number of placentas (number of litters involved)						
Number examined	329 (22)	320 (22)	329 (22)	337 (22)	268 (21)	
Swollen placentas	3 (2)	-	5 (3)	23 (2)	260 (21)	
Placenta pale and/or mottled	1 (1)	-	1 (1)	16 (1)	106 (9)	
Malformations – number of fetuses (number of litters involved)						
Number examined	329 (22)	320 (22)	329 (22)	337 (22)	268 (21)	
Number affected	-	2 (2)	2 (1)	2 (2)	3 (2)	
External malformations:						
Agenesis of tail, imperforate anus				1[a](1)		(0)–1 (1); 0–0.3%
Rudimentary/threadlike tail, imperforate anus					2[bc](2)	0 (0)–2 (2); 0–0.6%
Visceral malformations:						
Transposition of ascending aorta and dorsally displaced pulmonary trunk; ventricular septal defect				1(1)		
Retro-esophageal aortic arch		1(1)				
Duplicated inferior vena cava		1(1)				
Diaphragmatic hernia					1[d](1)	

Parameter	Dose (mg/kg bw per day)					
	0	1	4	16	64	HCD
Skeletal malformations:						
Exencephaly; brachygnathia with fused mandibles; misshapen basisphenoid; split/partially split basioccipital, cervical to 4th thoracic vertebra/ cartilage			1(1)			
Termination of normal vertebral column lumbar region; malrotated hind limbs					1[b](1)	
Termination of normal vertebral column lumbar region				1[a](1)	1[c](1)	
Anomalies and variants						
Visceral examination – fetuses (litters) [%fetal incidence]						
Number examined	163 (22)	159 (22)	163 (22)	168 (22)	136 (21)	
Innominate artery: absent/rudimentary	3 (2) [1.84%]	-	2 (2) [1.23%]	-	**5 (3) [3.68%]**	0(0)–2(1); 0–1.22%
Subclavian artery: variation in origin	-	-	-	-	2 (2) [1.47%]	0(0)–2(2); 0–1.22%[§]
Heart:						
ventricular septal defect	-	-	-	-	3 (3) [2.21%]	0(0)–3(2); 0–1.83%[#]
ventricular septal defect (small)	-	-	-	-	8 (6)	0(0)–1(1); 0–0.68%
Ureter(s): dilated	1 (1) [0.61%]	1 (1) [0.63%]	-	-	4 (2) [2.94%]	0(0)–6(4); 0–3.90%
Kidney(s)/ureter(s): green deposits	-	-	-	-	61 (20) [44.85%]	
Skeletal examination – fetuses (litters) [%fetal incidence]						
Number examined	166 (22)	159 (22)	165 (22)	167 (22)	129 (21)	
Cervical rib	2 (2) [1.20%]	-	-	1 (1) [0.60%]	**25 (12) [19.38%]**	0(0)–4(3); 0–2.41%
Complete 14th rib with associated costal cartilage	-	-	-	-	2 (2) [1.55%]	0(0)–4(2); 0–2.45%
1 extra false/1 less floating rib/ costal cartilage	7 (5) [4.22%]	5 (4) [3.14%]	4 (4) [2.42%]	4 (4) [2.40%]	2 (1) [1.55%]	
1 less false/1 extra floating rib/ costal cartilage	1 (1) [0.60%]	-	2 (2) [1.21%]	1 (1) [0.60%]	7 (5) [5.43%]	
20 thoracolumbar vertebrae	-	-	-	-	3 (2) [2.33%]	
Offset alignment of pelvic girdle	-	-	-	-	2 (2) [1.55%]	

Parameter	Dose (mg/kg bw per day)					
	0	1	4	16	64	HCD
Skeletal variants – fetuses (litters) [%fetal incidence]						
Ribs: number with 13	144	146	136	139	67	
Supernumerary lumbar ribs: number with 13/14 or 14/14	22 (10) [13.25%]	13 (8) [8.18%]	29 (15) [17.58%]	28 (13) [16.77%]	**62 (18) [48.06%]**	5 (3)–35 (12); 3.25–21.41%
Sternebrae: incompletely ossified/unossified						
5th and/or 6th	109 (21) [65.66%]	100 (22) [62.90%]	105 (21) [63.64%]	93 (21) [55.69%]	109 (21) [84.50%]	56 (20)–123 (22); 36.36–77.12%
Other	8 (6) [4.82%]	2 (1) [1.26%]	3 (2) [1.82%]	1 (1) [0.60%]	16 (11) [12.40%]	1 (1)–16 (9); 0.65–9.85%
Total	110 (21) [66.27%]	100 (22) [62.86%]	105 (21) [63.64%]	93 (21) [55.69%]	109 (21) [84.50%]	56 (20)–123 (22); 36.36–77.12%
% total (mean of means)	65.1	63.4	64.1	54.4	**84.9***	

HCD Historical control data from 30 studies (May 2000–July 2004) as given in the study summary (2015/1087909): presented as range of affected fetuses (litters), % fetal incidence

§ Based on historical control range reported for "systemic/pulmonary vessel abnormality/retroesophageal subclavian artery" under major abnormalities

Based on historical control range reported for "atrial/ventricular septal defects" under major abnormalities.

a,b,c Superscripts indicate observations relating to the same fetus

c,d Superscripts indicate fetuses from the same litter

Statistical significance: * $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Maternal toxicity was evident at 64 mg/kg per day, manifested as mean body weight loss (−6 g) in the period GD 6 to 8, then body weight gain at a reduced rate (ca 26% less compared to controls for GDs 8–15) and lower food intake (ca −20% for GDs 6–19, as compared to controls). At the high dose (64 mg/kg per day), there was an increased incidence of post-implantation loss, reduced live litter size, increased placental weight, reduction in mean fetal weight and increased incidence of minor fetal variations. At 16 mg/kg bw per day, increased incidences of swollen placentas occurred in two females, but fetal weight was unaffected. Apart from that, at the lower dosages there was no evidence of any adverse effect on the pregnant female, and there were no effects on survival, development or morphology of the fetus.

It was concluded from the results of this study that the NOAEL for KNF-474m in the rat was 16 mg/kg per day both for the pregnant female and the developing fetus (Fulcher, 2002, 2006; Davies, 2015).

Study 3. Preliminary studies

Two preliminary studies where performed in order to select the dose levels for the main study. In these preliminary studies six pregnant rats (CD strain of Sprague Dawley origin) were treated with the test substance metconazole WL136184 (purity 95.2%; 95% *cis* isomer; batch 12) in 1% methylcellulose by gavage from GD 6 to GD 15. Investigations comprised daily observation for clinical signs, regular determination of maternal body weight, and food and water consumption. All females were sacrificed on GD 20 for examination of their uterine contents. The second preliminary study was conducted in order to establish a suitable upper dosage level, in the absence of effects at the top dose of the first pilot study.

In the the first preliminary study rats were treated with 0, 6, 12, 24 and 48 mg/kg bw per day of *cis*-metaconazole. General condition of the females was unaffected by treatment and no deaths occurred. Body weight gain, feed and water consumption of females during gestation were unaffected by treatment. No treatment-related macroscopic abnormalities were observed at necropsy. Placental weight was increased at 48 mg/kg bw per day (21.2% of control) and there was an increased incidence of large placentas. Fetal weight was unaffected by treatment in all groups. No treatment-related abnormalities were observed on examination of fetuses. Oral administration of 95% *cis*-metconazole to pregnant rats during organogenesis produced no clear maternal response at doses up to 48 mg/kg bw per day, although at this dose there was an increase in placental weight. It was concluded the highest dose level for use in a main teratology study in the rat should be higher than 48 mg/kg bw per day.

In the second preliminary study, rats were treated with 0, 6, 60 and 80 mg/kg bw per day of *cis*-metconazole. The general condition of females was unaffected by treatment and no deaths occurred. Body weight gain was depressed during the treatment period at 60 mg/kg (−10%) and above (−32%); at both dose-levels, the effect was visible until termination (14–17% decrease). Both feed and water consumption were unaffected by treatment. No treatment-related macroscopic abnormalities were observed at necropsy. The number of implantations was similar in all groups, but there were increased incidences of early and late resorptions at 60 and 80 mg/kg bw per day (3.17 and 2.67, respectively compared with 0.50 in controls for early resorptions; 0.83 and 1.00, respectively for late resorptions compared with 0.0 for controls). The incidence of post-implantation loss was increased at 60 and 80 mg/kg bw per day (25.5% and 23.4%, respectively compared to 3.3% in controls). Consequently, the numbers of viable young was reduced (11.7 and 12.0, respectively compared to 14.8 in controls). At 60 mg/kg bw per day fetal weight was slightly lower than that of the controls (4.6%) and a more marked effect was observed at 80 mg/kg bw per day (15.2% lower than controls). The incidence of small fetuses (less than 2.80 g) was increased at 60 and 80 mg/kg bw per day and a small number also showed shiny skin and/or domed head. These observations mainly occurred in association with reduced fetal weight and were probably signs of fetal immaturity at these doses. Placental weight was increased at 60 and 80 mg/kg bw per day (9.6% and 11.5% respectively) and there was an increased incidence of thickened placentas. There was no clear indication of an adverse effect of treatment upon fetal morphology. Maternal toxicity (reduced body weight gain) and embryotoxicity (increased number of resorptions and decreased fetal weight) were evident at dose levels of 60 and 80 mg/kg bw per day. Based on the results of this range finding study a dose level of 60 mg/kg bw per day was selected as a suitable high dose level in the main teratology study in the rat.

Study 3. Main study

In the main study 22 pregnant rats (CD strain of Sprague Dawley origin)/dose received metconazole WL136184 (purity 95.2%; 95% *cis* isomer; batch 12) in 1% methylcellulose by gavage at dose levels of 0, 6, 24 and 60 mg/kg bw per day from GD 6 to GD 15. Dosing volume was 10 mL/kg bw. On day 20 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses fixed prior to examination for skeletal or visceral abnormalities.

Maternal data

All animals survived to scheduled sacrifice and no treatment-related clinical signs were observed during the study. A number of animals in all treated groups developed brown staining of the fur, particularly in the head region. The distribution of this finding between the groups did not suggest any toxicologically significant dose-related response.

Food consumption was slightly but statistically significantly lower during the treatment period (7% lower during GDs 6–8 and 13% lower on GDs 12–15) for females in the 60 mg/kg bw per day group. During the post-treatment period (GDs 16–20) food consumption was again comparable to controls in this group. Top-dose females showed slightly but significantly increased water consumption immediately after completion of treatment. Body weight gain of top-dose females was significantly reduced during the treatment period by 21% (GDs 6–16), particularly during the second half of the treatment period (23% lower). This led overall to a statistically significantly reduction in body weight gain of 12% during GDs 6–20. No treatment-related macroscopic changes were noticed in maternal animals at necropsy.

Litter data

Treatment with *cis*-metconazole did not affect pregnancy status as the pregnancy rate was 100% for the control and treated groups. The mean numbers of corpora lutea and total implantation sites for the treated groups were comparable to the controls and there was no treatment-related increase in pre-implantation loss. No female aborted prior to scheduled sacrifice and no female exhibited total litter loss.

The number of early and late resorptions was increased at 60 mg/kg bw per day with a consequent increase in post-implantation loss and a decrease in viable litter size. Fetal weight was significantly lower at 60 mg/kg bw per day compared to the control group. In agreement with the fetal weight effect, the placental weights were slightly increased at the top dose. However, if in any way treatment-related, it should be regarded as an adaptive effect (see also study above by Fulcher, 2002).

There were no treatment-related increases in the incidence of fetal external, visceral or skeletal malformations. At the top dose, one incidence of upturned snout and posterior cleft palate was found. Despite the fact that the latter malformation incidence (0.3%) was outside the historical control range (0–0.2%), the singularity of the event does not a priori suggest a relationship with compound exposure. External fetal examinations indicated one fetus (from litter no. 68) in the 60 mg/kg bw per day group with a domed head. However, neither dilated lateral ventricles nor unossified cranial bones were observed in any fetus from this litter. As such, there was no confirmed case of hydrocephalus in this study. In agreement with the effects on fetal and placental weight, the incidences of small fetuses (below 2.8 g) and of large, thickened placentas were increased at the top dose. Skeletal ossification data indicated that the fetal and litter incidences of the following ossification variations were increased at 60 mg/kg bw per day when compared to controls, most of which were above the historical control range:

- large anterior fontanelle,
- fetuses with an additional 14th thoracic rib or ribs,
- incomplete ossification of 3 or 4 sternebrae,
- the presence of cervical ribs.

The slight delay in ossification was possibly secondary to the reduction in pup weight.

Slight increases (compared to study control incidence) of large anterior fontanelle (fetal incidence) was also detected at 24 mg/kg bw per day. Fetal incidences were slightly outside the historical ranges and displayed dose-dependency. However, litter incidences were comparable to controls. This finding could be indicative of general retardation of skeletal development (showing a broad variability), and not necessarily localized bony defects, and was thus not taken into account for the determination of the NOAEL.

At necropsy slight developmental effects (bilateral dilatation of ureter at 24 mg/kg bw per day and above, and bilateral dilatation of the kidney at the top-dose) were observed. However, these effects were not confirmed during serial sectioning examination.

Table 43. Key findings of developmental toxicity study in rat

Dose level (mg/kg bw per day	**0**	**6**	**24**	**60**	**HCD**
No. of pregnant animals	*22*	*22*	*22*	*22*	
Litter observation					
Corpora lutea	17	18	17.2	17.7	
Implantations	16	16.9	16.1	16.2	
Pre-implantation loss (%)	6.6	6.5	6.8	8.5	*4.5–27.2*
Post-implantation loss (%)	6	3.8	6.5	**17.1****	*1.7–10.9*
Total resorptions	0.95	0.64	1.05	**2.77****	*0.26–1.54*
early resorptions	0.95	0.55	1	**2.09****	*0.26–1.46*
late resorptions	0	0.09	0.05	**0.68**	*0.00–0.30*
Litter size	15	16.2	15	**13.5***	
Fetal weight (g)	3.74	3.71	3.68	**3.36*****	*3.19–3.84*
Small fetus (< 2.80 g)	1/330 (0.3%)	2/357 (0.6%)	2/331 (0.6%)	33/296 (11.1%)	
Placental weight (g)	0.51	0.51	0.53	**0.58**** (+13.7%)	*0.48–0.62*

(*Table 43 continues on the following page*)

Dose level (mg/kg bw per day	0	6	24	60	HCD
Skeletal variation					
Large anterior fontanelle					
Fetal incidence	14/223 (6.3%)	11/241 (4.6%)	23/226 (10.2%)	**35/201 (17.4%)**	*0.0 – 8.4*
Litter incidence	10/22 (45.5%)	9/22 (40.9%)	9/22 (40.9%)	12/22 (54.6%)	
Incomplete ossification of 3rd sternebrae					
Fetal incidence	30/223 (13.5%)	32/241 (13.3%)	20/226 (8.8%)	43/201 (21.4%)	*7.5–29*
Litter incidence	15/22 (68.1%)	17/22 (77.3%)	12/22 (54.6%)	16/22 (72.7%)	
Incomplete ossification of 4th sternebrae					
Fetal incidence	3/223 (1.3%)	6/241 (2.5%)	6/226 (2.7%)	**16/201 (8.0%)**	*0.4–6.3*
Litter incidence	3/22 (13.6%)	4/22 (18.2%)	5/22 (22.75)	11/22 (50.0%)	
Additional cervical rib/s					
Fetal incidence	0/223 (0.0%)	0/241 (0.0%)	1/226 (0.4%)	**5/201 (2.5%)**	*0–2.2*
Litter incidence	0/22 (0.0%)	0/22 (0.0%)	1/22 (4.6%	2/22 (9.1%)	
Additional 14th rib – unilateral					
Fetal incidence	22/223 (9.9%)	28/241 (11.6%)	21/226 (9.3%)	**35/201 (17.4%)**	*0–11.5*
Litter incidence	12/22 (54.6%)	13/22 (59.1%)	12/22 (54.6%)	18/22 (81.8%)	
Additional 14th rib – bilateral					
Fetal incidence	14/223 (6.3%)	16/241 (6.6%)	25/226 (11.1%)	**42/201 (20.9%)**	*0–14.0*
Litter incidence	7/22 (31.8%)	9/22 (40.9%)	10/22 (45.4%)	12/22 (54.6%)	
Necropsy findings					
Hydronephrosis – unilateral					
Fetal incidence	2/223 (0.9%)	4/241 (1.7%)	3/226 (1.3%)	1/201 (0.5%)	*0–1.3*
Litter incidence	2/22 (9.1%)	4/22 (18.2%)	1/22 (4.5%)	1/22 (4.5%)	
Hydronephrosis – bilateral					
Fetal incidence	0/223 (0.0%)	1/241 (0.4%)	1/226 (0.4%)	5/201 (2.5%)	*0–2.5*
Litter incidence	0/22 (0.0%)	1/22 (4.5%)	1/22 (4.5%)	4/22 (18.2%)	
Hydroureter – unilateral					
Fetal incidence	7/223 (3.1%)	5/241 (2.1%)	10/226 (4.4%)	2/201 (1.0%)	*0–4.2*
Litter incidence	5/22 (22.7%)	4/22 (18.2%)	9/22 (40.9%)	2/22 (9.1%)	
Hydroureter – bilateral					
Fetal incidence	3/223 (1.3%)	4/241 (1.7%)	12/226 (5.3%)	**13/201 (6.5%)**	*0–2.5*
Litter incidence	2/22 (9.1%)	4/22 (18.2%)	6/22 (27.3%)	9/22 (40.9%)	
Serial section examination					
Bilateral hydronephrosis					
Fetal incidence	0	0	0	4/95 (4.2%)	0.0–7.3
Litter incidence	0	0	0	2/22 (9.1%)	
Bilateral hydroureter					
Fetal incidence	1/107 (1.93%)	0	1/105	6/95 (6.3%)	0.0–21.9
Litter incidence	1/22 (4.5)%	0	1/22 (4.5%)	6/22 (27)%	

HCD Historical control data

Statistical significance: * $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

cis-Metconazole (WL136184; 95% *cis*) induced maternal and developmental toxicity at the top dose of 60 mg/kg bw per day. The NOAEL for maternal toxicity was 24 mg/kg bw per day based on increased water consumption, decreased food consumption and decreased body weight gain.

The NOAEL for developmental/fetal toxicity was 24 mg/kg bw per day based on increased placental weight, decreased fetal weight and decreased litter size/viability, and increased post-implantation loss (early and late resorptions) (Willoughby, 1992b, c, d).

Rabbit

Study 1

Rabbits of strain Hra: (NZW) SPF and presumed pregnant, in groups of 10/dose, received metconazole (WL148271; purity 98.3%; *cis*:*trans* 80:15; lot/batch AC 10575-61 in main study or AC 9339-114 in the preliminary study) in 0.5% CMC by gavage at dose levels of 0, 1, 5, 10, 20 and 40 mg/kg bw per day from GD 6 to GD 28 inclusive. Dosing volume was 10 mL/kg bw. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses fixed prior to examination for skeletal and visceral abnormalities.

Study 1. Preliminary study

Maternal data

There were three non-pregnant females, one each in the 0, 20, and 40 mg/kg bw per day groups. No mortalities, abortions or premature deliveries were noted. No relevant clinical findings were observed. Absolute food consumption values (g/day) tended to be reduced at the top dose on days 12–15, and days19–24 (by 5–6%), as well as for the entire dosage period (days 6–29, by 3%), but relative values (g/kg bw per day) were comparable in all dosage groups. At the top dose, maternal body weights tended to be reduced at 40 mg/kg bw per day after GD 9; when calculated for days 6–29, difference with controls amounted to a reduction of about 10%. Gravid uterine weights were slightly reduced at the top dose. Placentas appeared normal for all animals in control and treated groups. During necropsy 2/10 animals showed red areas in the stomach at the top dose.

Litter data

When compared to controls (control values in parentheses) top-dose animals showed altered litter averages for late resorptions: 1.1 (0.0), percentage of resorbed conceptuses/litter: 14.3% (1.2%), and an increased number of does showing any resorptions: 55.6% (11.1%). A reduction of live litter size at 6.9 was noted at the top dose, compared to 8.1 for controls. No relevant effect on fetal weight was observed. The litter incidence of fetuses with any alterations was elevated compared to controls, but a dose–response relationship was lacking. Analyses of the litter averages for ossification sites per fetus did not identify any dose-related or statistically significant differences. With regard to variations, one fetus in the 1mg/kg bw per day group had an absent spleen. Also an absent intermediate lobe of the lungs occurred in 2, 1 and 1 fetuses (from a single litter in each case) in the 1, 5 and 10 mg/kg per day groups, respectively. These variations are a common variation in this strain of rabbit. The incidences of malformed fetuses in this study were 0/73, 1/82 (1.2%), 0/85, 0/96, 0/81, and 2/62 (3.2%) in the control, 1, 5, 10, 20, and 40 mg/kg bw per day dose groups, respectively. During gross and soft tissue examination (or skeletal examination), at the top dose one fetus was found with a domed head associated with extreme dilation of lateral ventricles and one fetus with absent hindlimb digits associated with reduced metatarsal bones. Moderate to extreme dilation of the cerebral lateral ventricles is usually equated with hydrocephalus. Missing digits were reported in the available control data for the laboratory (available for 1994–1996, presented in the study report) at a low rate with a maximum of one per study, and dilation of the lateral ventricles (moderate to extreme) ranged as high as three fetuses per study.

Key findings for this preliminary developmental toxicity study are shown on the following page in Table 44

Table 44. Key findings of developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	0	1	5	10	20	40
Maternal data						
Number pregnant	9	10	10	10	9	9
Body weight GD 29 (kg)	3.94 ± 0.36	3.93 ± 0.28	3.98 ± 0.35	3.94 ± 0.32	4.02 ± 0.44	3.84 ± 0.22
Gravid uterine weight (g)	474 ± 110	494 ± 120	500 ± 148	537 ± 83	534 ± 88	449 ± 81
Body weight change GDs 0–29 (kg)	0.69	0.66	0.72	0.66	0.73	0.61
Food consumption GDs 6–29 (g/day)	157 ± 20	168 ± 6	157 ± 26	159 ± 9	164 ± 16	152 ± 19
Placenta appeared normal	9 (100%)	10 (100%)	10 (100%)	10 (100%)	9 (100%)	9 (100%)
Litter observation						
Corpora lutea	9.0 ± 1.7	9.4 ± 2.1	9.4 ± 2.3	10.1 ± 1.5	10.4 ± 1.7	9.1 ± 1.2
Implantations	8.2 ± 1.9	8.5 ± 2.0	8.9 ± 2.5	9.7 ± 1.5	9.6 ± 1.4	8.1 ± 1.6
Total resorptions	0.1 ± 0.3	0.3 ± 0.7	0.4 ± 1.3	0.1 ± 0.3	0.6 ± 1.0	1.2 ± 1.4
early resorptions	0.1 ± 0.3	0.1 ± 0.3	0.4 ± 1.3	0.1 ± 0.3	0.4 ± 1.0	0.1 ± 0.3
late resorptions	0	0.2 ± 0.6	0	0	0.1 ± 0.3	1.1 ± 1.3
Litter size	8.1 ± 1.9	8.2 ± 2	8.5 ± 3.2	9.6 ± 1.6	9.0 ± 1.5	6.9 ± 1.7
Fetal weight (g)	42.13 ± 6.25	43.15 ± 4.35	43.84 ± 9.15	40.15 ± 2.95	42.92 ± 6.54	41.65 ± 5.27
Fetal gross external alteration						
Fetuses evaluated	73	82	85	96	81	62
Litters evaluated	9	10	10	10	9	9
Fetuses (litter) with any alteration observed	1 (1)	6 (5)	8 (7)	4 (4)	4 (3)	5 (4)
head domed	0	0	0	0	0	1 (1)
forepaws and/or hindpaws: digit absent	0	0	0	0	0	1 (1)

GD Gestation day

The top dose of 40 mg/kg bw per day was minimally toxic to the dams (small reductions in body weight gain and food consumption) and showed slight embryo/fetal toxicity in the form of increased incidences of late (but not early) resorptions with a corresponding decrease in live litter size. Only one craniofacial malformation was seen in this study, that is one case of hydrocephalus at 40 mg/kg bw per day. Across the study, all fetal morphological changes were considered unrelated to treatment. Based on the results of this range-finding study doses of 5, 10, 20 and 40 mg/kg bw per day were recommended for the main study

Study 1. Main study

In the main study, four groups (25 females/group) of mated female New Zealand White rabbits (Hra: (NZW) SPF) received metconazole (purity 98.3%; *cis*:*trans* 80:15; batch AC 10575-61) in 0.5% CMC by gavage at dose levels of 0, 5, 10, 20 and 40 mg/kg bw per day from GD 6 to GD 28. Dosing volume was 10 mL/kg bw. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses fixed prior to examination for skeletal and visceral abnormalities. In addition to standard examinations, blood samples had been obtained before sacrifice in order to perform both haematological and clinical chemistry analysis.

Maternal data

Mortality observed in this study (one case each in the control and 40 mg/kg bw per day group) was not related to treatment but attributed to intubation accidents. Single incidences of abortions (one in the control and one at 20 mg/kg bw per day) were not related to treatment. Regarding clinical signs, localized alopecia on the back or limbs, a red substance in the cage pan and ungroomed coat were considered unrelated to treatment as incidences were not dose-related, and the observation occurred only in one or two does in any dosage group. Dams in the 40 mg/kg bw per day group lost weight on GDs 24–29. As a result of this body weight loss, body weight gain for the entire dosage period (days 6–29) was reduced by 15%. Slight (but non-statistically significant) reductions in gravid uterine weights were noted for the 40 mg/kg bw per day group. This observation was associated with a slightly smaller live litter size that, in turn, reflected a small but statistically significant increase in post-implantation loss (early and late resorptions) and a small reduction in fetal body weight. After correction of maternal body weight gains for gravid uterine weights, maternal body weight gains for the entire dosing period and maternal body weight on day 29 were unaffected at any dose. Food consumption values were reduced at 40 mg/kg bw per day on days 24–29 without statistical significance. Animals in the 40 mg/kg bw per day group exhibited slight, but statistically significant reductions in Hb, Ht and MCV values compared to controls. Additionally, the values for platelet counts and ALP activity were significantly increased at 40 mg/kg bw per day when compared to control values. These findings were consistent with effects observed in short-term toxicity studies in other species and a relationship with metconazole administration is thus probable. Top-dose females showed slight, but not statistically significant increases in both absolute (13%) and relative (15%) liver weight. This effect was considered test substance-related. Necropsy observations included pale liver, but association with treatment is uncertain in the absence of dose–response relationship. There were no treatment-related microscopic changes in the maternal livers from the control and 40 mg/kg bw per day groups.

Litter data

The mean numbers of corpora lutea and total implantation sites for the treated groups were comparable to the controls and no increase in pre-implantation loss was observed in any treated group when compared to controls. No doe exhibited total litter loss. The top-dose was associated with a small increase in post-implantation loss as evidenced by statistically significant increases in the number of does with any resorptions and the rate of dead/resorbed conceptus per litter. A total of six resorptions (three early, three late) in five litters was observed in the control group and a total of 22 resorptions (11 early, 11 late) from 13 litters were observed in the 40 mg/kg bw per day group. Corresponding to the slight increase in post-implantation loss, a slight, but non-statistically significant reduction in mean litter size was observed at the top dose. Fetal weights were slightly (not statistically significant) reduced by 5–7%. Placentas appeared normal throughout all groups, placental weights were not determined in this study. The incidence of malformed fetuses in this study was low and consisted 0/202, 1/219 (0.5%), 2/212 (0.9%), 3/183 (1.6%), and 3/177 (1.7%) fetuses in the control, 5, 10, 20 and 40 mg/kg/day dose groups, respectively. The malformations included: zero in the control; one fetus with fused ribs at 5 mg/kg/day; one fetus with extreme dilatation of the lateral ventricles (hydrocephalus) plus one fetus with vertebral and rib malformations at 10 mg/kg per day; two fetuses with fused ribs as well as one fetus with spina bifida and fused and malformed lumbar vertebrae at 20 mg/kg/day; and at the top dose one fetus with a short tail, one fetus with vertebral and rib malformations, and one fetus with marked dilatation of the lateral ventricles/hydrocephalus. A variety of malformations was reported, all of which were also seen in the historical control studies. There were in total two craniofacial malformations observed in this study: one hydrocephalus at 10 mg/kg/day and one hydrocephalus at 40 mg/kg/day. Since this malformation occurs spontaneously in control animals (up to three fetuses were recorded with marked/extreme dilated lateral brain ventricles in the historical control data from 1994–1996), and there was no clear dose–response relationship it was not considered related to treatment in this study.

Key findings for this developmental toxicity study are shown on the following page in Table 45.

Table 45. Key findings of developmental toxicity study in rabbit

Dose level (mg/kg bw/day)	0	5	10	20	40	HCD range	HCD mean
			Maternal data				
Number/pregnant	25/25	24/25	24/25	24/25	24/25		
Mortality	1 (intubation error)	-	-	-	1 (intubation error)		
Abortions	1	0	0	1	0		
Food consumption (g/day)							
GDs 6–29	159±14	158±19	160±15	154±19	151±19 (−5%)		
GDs 24–29	113±31	112±45	117±63	105±47 (−7%)	95±55 (−16%)		
Body weight GD 29 (kg)	4.16±0.33	4.16±0.35	4.20±0.29	4.04±0.35	4.09±0.36		
Gravid uterine weight (g)	523±104	530±97	518±94	477±132 (−9%)	462±141 (−12%)		
Corrected maternal body weight	3.64±0.28	3.63±0.32	3.68±0.30	3.58±0.32	3.63±0.30		
Body weight gain (kg)							
GDs 0–29	0.49±0.14	0.50±0.15	0.54±0.22	**0.36±0.15***	0.42±0.20		
GDs 24–29	0.03±0.13	0.05±0.12	0.01±0.09	0.01±0.15	−0.03±0.15		
GDs 6–29	−0.41±0.12	−0.41±0.20	−0.42±0.14	−0.37±0.16	−0.35±0.21		
Liver weights							
absolute	96.6±13.0	96.7±18.9	96.3±15.8	98.4±21.5	108.8±24.8 (+13%)		
relative	2.32±0.28	2.32±0.38	2.29±0.31	2.44±0.50	2.66±0.58 (+15%)		
Haematology/clinical chemistry							
Haemoglobin, Hb	12.10±0.84	11.99±0.97	12.4±0.7	12.33±1.15	**11.3±1.08* (↓6%)**		
Haematocrit Ht	34.80±2.67	34.76±3.20	35.7±2.1	35.83±3.57	**32.6±3.50* (↓6%)**		
MCV	66.11±2.17	66.22±2.95	65.6±2.1	65.49±2.00	**63.8±2.9** (↓4%)**		
Platelets	369.9±111.9	373.7±109.1	410.2±117	365.8±66.7	**459±123* (↑24%)**		
ALP	29.4±13.6	35.1±19.8	32.1±12.3 (↑9%)	37.2±18.2 (↑27%)	**63.0±34.1** (↑114%)**		

Dose level (mg/kg bw/day)	0	5	10	20	40	HCD range	HCD mean
Litter observations							
Fetuses (litters) examined	202 (23)	219 (24)	212 (24)	183 (23)	177 (23)		
Litter mean corpora lutea	9.3 ± 2.2	9.4 ± 1.7	9.2 ± 2.1	9.1 ± 2.1	8.9 ± 2.3	7.8–11.8	9.6
Implantations	9.0 ± 2.4	9.2 ± 1.8	9.2 ± 2.2	8.5 ± 2.7	8.7 ± 2.4	5.0–10.5	8.6
Resorptions							
Total	0.3 ± 0.5	0.1 ± 0.3	0.3 ± 0.6	0.5 ± 0.8	1.0 ± 1.4	0–3.2	0.4
Early resorptions							
fetuses (litters)	3 (3)	3 (3)	5 (4)	6 (4)	11 (6)		
mean ± SD	0.1 ± 0.3	0.1 ± 0.3	0.2 ± 0.5	0.3 ± 0.6	0.5 ± 1.1	0–2.8	0.2
Late resorptions							
fetuses (litters)	3 (3)	0 (0)	3 (3)	6 (5)	11 (9)		
mean ± SD	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3	0.3 ± 0.5	0.5 ± 0.7	0–0.8	0.2
Does with any resorptions	5 (21.7%)	3 (12.5%)	7 (29.2%)	8 (34.8%)	**13 (56.5%)****	0–60%	27.20%
Dead or resorbed conceptus/litter	2.5%	1.5%	3.8%	7.2%	**12.4%****		
Litter size/live fetuses	8.8 ± 2.2	9.1 ± 1.9	8.8 ± 2.3	8.0 ± 2.7	7.7 ± 2.9	4.8–10.4	8.2
Fetal weight (g)	42.72 ± 4.52	41.73 ± 4.10	42.08 ± 3.79	42.64 ± 5.95	40.41 ± 5.77	31.85–50.27	38.65
Litter weights (g)	368.6	372.6	367.34	327.39	303.14		
Placenta appeared normal	100%	100%	100%	100%	100%		
Fetal alterations							
Fetuses with any alteration							
Fetuses (%)	6.4	9.6	8	8.2	7.3		
Litters (%)	47.8	45.8	50	39.1	34.8		
Brain: dilation of lateral ventricles (extreme or marked)/ hydrocephalus	0	0	1	0	1	Marked dilated lateral ventricles: 0–1% fetuses 0–5.3% litters Extremely dilated lateral ventricles: 0–0.8% fetuses 0–6.2% litters	

HCD Historical control data MCV Mean corpuscular volume ALP Alkaline phosphatase

* $p < 0.05$ ** $p < 0.01$

Maternal toxicity was evident at the top dose displaying: reductions in food consumption, body weight loss during GDs 24–29, reduced Hb, Ht and corpuscular volume, increases in platelet counts and ALP activity, and increased absolute and relative liver weights. Therefore, the NOAEL for maternal toxicity was established at 20 mg/kg bw per day. Based on embryotoxicity/fetotoxicity (small increases in early and late post-implantation loss, slightly decreased fetal weights and litter size) at the top dose, the NOAEL for fetal toxicity was also set at 20 mg/kg bw per day. No malformations or variations in the fetuses were attributable to treatment with the test substance. In the absence of any treatment-related

developmental toxicity up to the highest dose tested, the NOAEL for developmental toxicity was the top dose, 40 mg/kg bw (Hoberman, 1997).

Study 1
New Zealand White rabbits (Interfauna, UK), presumed pregnant, were assigned six to each dose group. The groups received either:

cis-metconazole,
WL13618; lots/batches 3454/078 and ST89/324: purity/content 96.9% *cis* isomer,

or (–)*cis*-metconazole,
WL161053; lots/batches 3454/081 and ST89/323: purity/content 91% ± 1% (–)*cis* isomer,

or *trans*-metconazole,
WL153996; lot/batch 88-08 ST89/312; purity/content: 97.0% *trans* isomer.

All test materials were administered in 1% methylcellulose by gavage from GD 7 to GD 19 inclusive. The dose levels selected were 10, 28 and 80 mg/kg bw per day for *cis* and (–)*cis* materials, and 10, 20 and 40 mg/kg bw per day for the *trans* material. A single control group was treated with the vehicle. On day 29 of pregnancy, the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses examined for gross changes. The study is considered to provide additional information.

Maternal data
cis-Metconazole
At 80 mg/kg bw per day all animals had cold ears (days 7–11), occasional signs of anorexia and there was reduced or altered faecal output in 4/6 animals during the treatment period. Mean food consumption was reduced during the initial treatment period (days 7–10), however recovery occurred during the remaining dosing period and, following cessation of treatment, values were either greater than or similar to controls. There was a noticeable loss of mean body weight (days 7–9). Although there was some overall recovery, a further marked loss of body weight occurred on GDs 24–29, probably due to four dams aborting in this period; mean body weights at termination were lower than predose values. At 28 mg/kg bw per day all animals had cold ears (days 7–9). A slight initial body weight loss was observed over days 7–9. At 10 mg/kg bw per day two dams had anorexia, abnormal faeces and clinical signs similar to those observed at higher doses.

(–)cis-Metconazole
At 80 mg/kg bw per day 5/6 animals had cold ears (days 7–11), anorexia and there was reduced or altered faecal output in 4/6 animals during the dosing period. There was a reduction in mean food consumption noted during the initial treatment period (days 7–8) with recovery to higher consumption by the end of the study. For dams with live young there was a slight loss of body weight (days 7–9); recovery occurred thereafter with gains essentially similar to controls. At 28 mg/kg bw per day cold ears were noted in 5/6 animals (onset on days 7–8 for four days). Although food consumption showed a marginal reduction on days 7–8, both predose values and those subsequent to day 8 were consistently lower than for controls. There was no apparent effect on food consumption, body weight or body weight gain in the 10 mg/kg per day group.

trans-Metconazole
No consistent or clear signs of reaction to treatment were observed. There were no effects on food consumption, body weight, body weight gain or any of the litter parameters at any dose of the *trans* isomer.

Litter data
cis-Metconazole
At 80 mg/kg bw per day, 4/5 pregnant animals aborted. Two abortions occurred on day 28, the other two were presumed to have occurred between days 24 and 29 due to the marked loss of body weight observed for this period (210 g and 350 g). The combination of reduction in the number of implants and an increase in embryo/fetal death and post-implantation loss (late deaths and abortions) at this

exposure levels resulted in only one dam with a live litter. For the one dam with live young on day 29, a significant reduction in number of live young, litter weight and mean fetal weight was noted. At 28 mg/kg bw per day, there was an apparent increase in post-implantation loss, which was reflected in a lower mean litter size. Mean fetal weights were similar to controls. A higher value might have been expected due to the lower litter size but litter weight was also lower than the respective control value. At 10 mg/kg bw per day, litter values were superior to controls for nearly all parameters.

The incidence of probable malformed fetuses was 2/33 (2/4 litters), 0/40 (0/4 litters), 3/41 (2/6 litters) and 0/3 (only one litter) at 0, 10, 28 and 80 mg/kg bw per day, respectively. One control fetus had microphthalmia and one fetus had great vessel malformations. In the 28 mg/kg per day dose group, one fetus had a domed cranium (suspected hydrocephalus), one fetus had missing hind paws and digits and one fetus had a retroesophageal subclavian artery.

(–)cis-Metconazole

At 80 mg/kg bw per day one dam totally resorbed her litter and at 28 mg/kg bw per day one rabbit aborted. Because the incidence was the same in each group and because one control dam also lost her litter, the losses at 28 and 80 mg/kg bw per day could not be conclusively related to treatment. At 80 mg/kg bw per day a reduction in the number of implantations and an increase in embryo/fetal death and post-implantation loss (late embryonic deaths) was observed. This resulted in a reduced number of live young. Although mean fetal weight was similar to controls, the low litter size resulted in a reduced litter weight. The incidence of probable malformations in the (–)*cis* isomer groups was 2/33 (2/4 litters), 1/56 (1/6 litters), 2/32 (2/4 litters) and 2/9 (2/3 litters) at 0, 10, 28 and 80 mg/kg bw per day, respectively. One control fetus had microphthalmia and one fetus had great vessel malformations. At 10 mg/kg per day, one fetus had retroesophageal subclavian artery. At 28 mg/kg per day one fetus showed retroesophageal subclavian artery, and the other fetus showed spina bifida and malrotated hindlimbs. At 80 mg/kg per day, one fetus had great vessel malformations and one fetus had a misshapen cranium with enlarged fontanelles. In terms of craniofacial malformations, there were defects seen in onlytwo fetuses in the 80 mg/kg per day dose group compared with one in the control group (see the *cis* isomer study above).

trans-Metconazole

There were no incidences of total litter loss at any dose; all pregnant dams had live young on day 29. Litter data at all doses showed no relationship to treatment with values similar or greater than controls. The incidence of malformations in the *trans* isomer groups was 2/33 (2/4 litters), 0/49 (0/5 litters), 3/39 (3/5 litters) and 0/55 (0/6 litters) at 0, 10, 20 and 40 mg/kg bw per day, respectively. Compared to controls (see above *cis* isomer study) there was no increase in the incidence of malformations with treatment. Three fetuses at 20 mg/kg bw per day had retroesophageal subclavian artery.

Table 46. Key findings of developmental toxicity study in rabbit

Dose	**Control**	***cis* compound**			***(–)cis* compound**			***trans* compound**		
(mg/kg bw per day)	**0**	**10**	**28**	**80**	**10**	**28**	**80**	**10**	**20**	**40**
Number of dams	6	6	6	6	6	6	6	6	6	6
				Maternal data						
Deaths	1	0	0	0	0	0	0	0	0	0
Number not pregnant	0	2	0	1	0	1	2	1	1	0
Number of abortion	0	0	0	**4**	0	1	0	0	0	0
Number with total resorptions	1[§]	0	0	0	0	0	1	0	0	0
Number with live young at day 29	4	4	6	1	6	4	3	5	5	6
Feed intake (% of control)										
days 7–8	100	94	94	**58**[#]	122	80	**64**	110	123	103
days 7–19	100	98	110	**81**	122	85	98	116	127	109

Dose	Control	*cis* compound			*(–)cis* compound			*trans* compound		
(mg/kg bw per day)	0	10	28	80	10	28	80	10	20	40
Body weight gain (g)										
days 7–9	−5	**−8**	**−32**	**−110**	17	−3	**−46**	22	−24	−19
days 7–20	163	85	225	32	272	130	164	272	214	236
Litter data										
Fetal deaths	2	0.8	**3**	**7**	0.8	**2**	**5.3**	1	0.4	1
Post-implantation loss (%)	17.8	6.7	**32.6**	**91.4**	7.7	**19.5**	**61.8**	9.9	4.9	10.1
Litter size	8.3	10	**6.8**	**0.6** [3.0]#	9.3	8	**3**	9.8	7.8	9.2
Litter weight (g)	340.1	439.4	**302.3**	- [119.3]#	416.9	**362.2**	**120.9**	430.7	335.6	366.5
Fetal weight (g)	42.8	44.2	42	- [39.8]#	45	45.8	42.6	44.1	43.8	40.2
Fetuses examined (litters examined)	33 (4)	40 (4)	41 (6)	3 (1)	56 (6)	32 (4)	9 (3)	49 (5)	39 (5)	55 (6)
Fetuses malformed (litters involved)	2 (2)	0	3 (2)	0	1 (1)	2 (2)	2 (2)	0	3 (3)	0

Values calculated for the 80 mg/kg bw per day *cis*-metconazole group include all animals that where pregnant; [values in square brackets refer to the one dam with live young at termination]

§ Dam died on day 26 after marked blood loss (vaginal bleeding) on day 25; uterus had one abortion site and 10 dead fetuses

At the dose rates used in this comparative study, the *cis* and (-)*cis* components of metconazole have been shown to be toxic to the dams and to possess embryotoxic potential, whereas, there were no clear treatment-related effects with the *trans* material.

cis-Metconazole

NOAEL(maternal): 10 mg/kg bw per day based on lowered body weight gain at 28 mg/kg bw per day
NOAEL(fetal): 10 mg/kg bw per day based on increased post-implantation loss and reduced litter size at 28 mg/kg bw per day

(–)cis-Metconazole

NOAEL(maternal): 10 mg/kg bw per day based on reduced food consumption and lowered body weight gain at 28 mg/kg bw per day
NOAEL(fetal): 28 mg/kg bw per day based on increased embryo/fetal deaths and post-implantation loss, and reduced litter weight at 80 mg/kg bw per day.

trans-Metconazole

NOAEL(maternal): 40 mg/kg bw per day;
NOAEL(fetal): 40 mg/kg bw per day (the highest dose tested).

The small number of of animals (1–6 litters with live pups) and the restricted investigations performed in this study, necessarily limited the confidence with which conclusions could be drawn from the above data (Masters, 1990).

Study 2. Preliminary study

In a preliminary study, six presumed-pregnant New Zealand White rabbits (Interfauna, UK) per dose received *cis*/*trans*-metconazole (WL148271; purity 95.3%; *cis*:*trans* 83.7:16.3; lot/batch 89-01, Ref. ST 89/088) in 1% methylcellulose by gavage at dose levels of 0, 10, 30 and 90 mg/kg bw per day from GD 7 to GD 19. Dosing volume was 2 mL/kg bw. On day 29 of pregnancy, the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses examined for gross changes. In a pilot study comprising three pairs of nonpregnant females, marked toxicity (anorexia, cold ears and marked body weight loss) was observed at 100 and

400 mg/kg bw per day, whereas at 25 mg/kg bw per day the findings were equivocal, with one animal showing an adverse response, whilst the other was unaffected.

Maternal data
Considerable maternal toxicity was observed at 90 mg/kg bw per day. At this top dose clinical signs of a generally nonspecific nature associated with initial inappetence and marked body weight loss were noted. Five of six pregnancies were terminated by total resorption (4) or abortion (1). There was reduced food intake throughout treatment, although this recovered and was higher than controls after treatment ended. At 30 mg/kg bw per day, minimal effects on maternal parameters (transient inappetence and weight loss at days 7–9) were observed. At 10 mg/kg bw per day, marginal decreases compared to control values in respect of maternal food consumption (days 7–10) and weight gains (days 7–11) were noted.

Litter data
At 90 mg/kg bw per day the single litter with live young was severely compromised in terms of high incidence of in utero death and post-implantation loss; the number of live young (three surviving fetuses), litter weight, and fetal weight were all reduced. At 30 mg/kg bw per day a possible increased incidence of post-implantation loss and slightly lower mean fetal weight were noted. There was also a possible increase in post-implantation loss in the 10 mg/kg bw per day group. At 10 mg/kg bw per day, there was also a possible association between treatment and the incidence of embryofetal deaths and fetal defects (3/48 fetuses in 2/6 litters). However, the small number of animals in each group limits the power of the study to detect such effects.

In this preliminary study the incidence of malformed fetuses was 1/51, 3/48, 6/49 and 1/3 fetuses in the control, 10, 30, and 90 mg/kg bw per day groups respectively. One fetus in the control group had forelimb flexure. In the 10 mg/kg bw per day group two fetuses had fused parietal and frontal bones, and one fetus showed forelimb flexure. In the 30 mg/kg bw per day group one fetus had short tail, two fetuses had great vessel malformations, two fetuses had alterations in the frontal bones and one fetus had a missing forelimb. Due to the variety of defects with no clear dose–response relationship, and since no skeletal evaluations were done, and the number of litters in each group was small, no conclusion can be drawn from these data.

Study 2. Main study
In the main study, 16 or 17 presumed-pregnant New Zealand White rabbits (Interfauna, UK) per dose received *cis*/*trans*-metconazole in 1% methylcellulose by gavage at dose levels of 0, 4, 10, 25 and 62.5 mg/kg bw per day from GD 7 to day GD 19. Dosing volume was 2 mL/kg bw. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined and fetuses fixed prior to examination for skeletal and visceral abnormalities.

Maternal data
No treatment-related mortalities were observed. At 62.5 mg/kg bw per day, there were three abortions (days 28/29) and total resorption of two litters. The abortion at 10 mg/kg bw per day (days 19/21) was considered unlikely to be related to treatment. The majority of the top-dose animals responded to the start of treatment by a period of anorexia/reduced or altered faecal output. This was mainly confined to the first week of dosing although it persisted for longer in some animals. Cold ears were occasionally noted at this dose. Mean food intake at 62.5 mg/kg bw per day was markedly reduced on days 7–8 of pregnancy. However, steady recovery occurred during the remaining dosing period, and after cessation of treatment values were superior to control. There was also an initial slight decrease in food consumption at 25 mg/kg bw per day. At the top dose, mean body weight gain on days 7–9 was lowered significantly. This recovered over the remainder of the study, although further loss of body weight was observed over GDs 24–29 due to three dams aborting and two others totally resorbing their litters (all late embryonic deaths). viewing mean values for dams with live young, body weight recovery was not maintained after cessation of treatment, due in part, to a high number of late resorptions (with concomitant low litter size and weight) and a clear difference from control was evident at termination. For dams with live young at 25, 10 and 4 mg/kg bw per day, although there appeared to be a slight dose-related body weight loss during days 7–9, gains thereafter were similar or greater than for control. There were a number of nonpregnant females in the 0, 4, 10 and 25 mg/kg bw per day groups, reducing the number of females with live young to 12, 15, 11, and 11 respectively. No treatment-related macroscopic changes were noticed in maternal animals at necropsy.

Litter data
At 62.5 mg/kg bw per day, there was a statistically significant increase in late embryonic deaths which resulted in a markedly increased post-implantation loss and reduced mean litter size. At 25 and 10 mg/kg bw per day, values for late embryonic death were slightly higher than controls and this was reflected in the relatively higher post-implantation loss and slightly lower mean litter size. At 62.5 mg/kg bw per day, there was a marked (and highly significant) reduction in mean litter weight. This was not only due to the lower mean litter size but was also a reflection of a concomitantly lower mean fetal weight. At the other doses, litter weights were slightly lower than controls and the findings were mainly related to the decreased litter sizes since fetal weights were unaffected. Values for post-implantation loss, late resorptions, and live litter size were far outside the historical control range at the top dose, indicating sever maternal toxicity.

The incidence of malformed fetuses was 2/113 (2/12 litters), 9/124 (7/15 litters), 2/88 (2/11 litters), 9/88 (7/11 litters) and 8/42 (4/10 litters) in control, 4, 10, 25 and 62.5 mg/kg bw per day respectively. There were a number of severe malformations reported in the study and one or more of these were seen in all dose groups, including controls. One control fetus was observed with forelimb flexure and mal-rotated hindlimb and one fetus with spina bifida. The incidence of any specific malformation at 4 mg/kg bw per day was not increased (except for a single incidence of hydrocephalus/cebocephaly). Thus, the finding at the lowest dose was considered irrelevant. The increased incidence of fetal malformations was within the test facility's historical control data up to and including the dose level of 10 mg/kg bw per day. Specifically, at 4 mg/kg bw per day one fetus had forelimb flexure, transposition of the great vessels, retroesophageal aorta and subclavian artery, duplicated inferior vena cava and interventricular septal defect. Two further fetuses showed microphthalmia, one fetus had retroesophageal subclavian artery, two fetuses showed dilated ascending aorta/arch (one with marked narrowing of the pulmonary trunk), one fetus had cranial schisis with microphthalmia, cleft lip and palate, great vessel malformations, and interventricular septal defect, one fetus had cebocephaly, anophthalmia, hydrocephalus, and malformed facial bones, and one fetus had lumbar scoliosis due to vertebral malformations. At 10 mg/kg bw per day one fetus showed forelimb flexure and a second malformed fetus had transposition of the great vessels, interventricular septal defect, small right ventricle and retroesophageal subclavian artery. Four fetuses with hydrocephalus were identified in the 25 mg/kg bw per day group in addition to two fetuses with peromelia (one with limb oligodactyly), one fetus had retroesophageal subclavian artery and cystic testes, one fetus had reduced stomach and small intestine and apparent absence of large intestine, and one fetus showed cebocephaly, facial bone malformations, umbilical hernia and absent kidney and ureter. At the top dose four fetuses had retroesophageal subclavian artery (one also with forelimb flexure), one fetus had dilated ascending aorta/aortic arch, narrow pulmonary trunk, and left ventricle, one fetus showed sacral scoliosis due to vertebral malformations, brachyury and caudal vertebral malformations, one fetus showed brachyury and caudal vertebral malformations, and another fetus had microphthalmia, reduced pinna, malformed facial bones, scoliosis and associated vertebral malformations, amelia and peromelia. The presence of four hydrocephalies at 25 mg/kg bw per day was considered treatment-related. The presence of a single incidence of hydrocephalus at 4 mg/kg bw per day was considered to be an isolated finding without a dose–response relationship and in addition the incidence was covered by the historical control data provided. The incidence of visceral anomalies was essentially comparable in all groups. At 62.5 mg/kg bw per day, the percentage of skeletal variations was significantly higher than in controls and other test groups; this was principally due to the incidence of cranial anomalies and cervical ribs. However, this conclusion may be questionable since only about 30% of the fetuses survived at the top dose. Although at 25 mg/kg bw per day the percentage of skeletal anomalies was similar to controls it was noted that the incidences of sutural bones and cervical ribs were also higher than for controls, with no instances of either at 10 or 4 mg/kg bw per day. Slight intergroup variations in mean incidence of fetuses with extra (13) ribs or variant sternebrae did not suggest any adverse effect of treatment.

Key findings for this main developmental toxicity study in rabbits are shown on the following page in Table 47.

Table 47. Key findings of developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	0	4	10	25	62.5	HCD
Maternal data						
Dams with live young/ dams mated	12/16	15/16	11/17	11/16	10/16	
Mortality	0	0	2§	0	0	
Abortions	1	0	1	0	3	
Nonpregnant	3	1	3	5	1	
Dams with total resorptions	0	0	0	0	2	
Food consumption (% of control)						
GDs 7–8	-	-	-	−19%	−60%	
GDs 7–19	-	-	-	-	−27%	
Body weight gain (g)						
GDs 7–9	3	−10	−15	−37	**−142****	
GDs 7–20	120	169	90	181	103	
GDs 7–29	302	391	275	382	**125***	
Litter observations						
Litter mean corpora lutea	11.3	10.3	11.1	10.6	11.4	
Implantations	9.6	8.9	9.3	9.5	9.1	
Pre-implantation loss (%)	8	13.7	14.2	11.2	19.5	
Post-implantation loss (%)	15.5	7.5	21.4	15	**73.8*****	3.6–17.5%, mean 10.8
Early resorptions	0.3	0.3	0.3	0.3	0.4	0.2–1.1, mean 0.5
Late resorptions	0.5	0.3	1	1.2	**4.3****	0.1–1.3, mean 0.5
Live litter size	8.7	8.3	7.3	8	**2.8*****	
Fetal weight [g (% of control)]	42.8	43.5	44.6	42.9	38.4 [−10%]	
Litter weight (g) [% of control]	398.2	357.6 [−10%]	354.4 [−11%]	341.5 **[**−14%]	**156.2** **[−61%]*****	
Fetal malformations [% fetuses]	2/113 [1.8%]	9/124 [7.3%]	2/88 [2.3%]	9/88 [10.2%]	8/42 [19.0%]	0.9%–7.5%, mean 3.0%
Gross/visceral anomalies [% fetuses]	6/111 [5.4%]	7/115 [6.1%]	4/86 [4.7%]	3/79 [3.8%]	3/34 [8.8%]	
Skeletal anomalies [% fetuses]	17/111 [15.3%]	20/115 [17.4%]	7/86 [8.1%]	13/79 [16.5%]	18/34 [**52.9****]	
Hydrocephalus: fetuses affected (litters)	0	1 (1)	0	4 (4)	0	max. 1 fetus (1 litter)
Amelia/peromelia: fetuses (litters)	0	0	0	2 (2)	1	
Sutural bones: fetuses (litters)	2 (2)	0	0	5 (3)	4 (3)	
Connected jugal to maxilla: fetuses (litters)	3 (3)	9 (4)	2 (2)	2 (2)	7 (3)	
Cervical ribs	2 (1)	0	0	5 (3)	4 (3)	

HCD Historical control data of the testing facility (Interfauna, UK) from 1989–1994 (Goettel, 2015)
Mean number of live young: 6.1–10.1, mean 8.4 %. Malformed fetuses: 0.9%–7.5%, mean 3.0%.
Hydrocephalus: max. 1 fetus (1 litter)

§ One found dead, probable dosing error; one sacrificed prior to treatment

* $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

The results obtained from this main study showed marked signs of maternal toxicity (reduced food consumption and reduced body weight gain and abortions) at the top dose of 62 mg/kg bw per day. At this clearly maternally toxic dose level strong fetotoxic effects were observed, such as increased post-implantation loss (late resorptions) and associated decreased live litter size and reduced fetal/ litter weights. Developmental toxicity at this top dose was evidenced by an increased incidence of structural defects (skeletal variations). Maternal toxic effects were already observed to a smaller extent at 25 mg/kg bw per day as indicated by an initial slight decrease in food consumption and body weight gain. Thus the maternal NOAEL was 10 mg/kg bw per day. Slightly increased embryofetal toxic effects (post-implantation loss and late resorptions and reduced litter weight) were observed at 10 and 25 mg/kg bw per day, but live litter size and fetal weights were not affected. At 4 mg/kg bw per day litter weights were reduced to a similar extent as those at the next two higher doses without any effect on post-implantation loss. Also, the values for post-implantation loss, late resorptions, and live litter size were well within the historical control range at all dose levels except the highest. Thus, the fetal NOAEL was 4 mg/kg bw per day based on post-implantation loss and late resorptions.

At 25 mg/kg bw per day, developmental effects were evidenced by an increased incidence of hydrocephalus which was clearly outside historical control ranges. Since a single incidence of hydro/ cebocephaly was also observed at 4 mg/kg bw per day in this main study, the study lab concluded that at lower dose ranges the possibility of an effect at low incidence, but probably treatment-related, could not be ruled out. Nevertheless, the apparently increased incidence of the malformations and anomalies at 4 mg/ kg bw per day was not clearly associated with specific findings and these values, as well as the single incidence of hydrocephalus, are well within the range of historical control data of the test facility with the same source of animals (Interfauna). Thus, developmental NOAEL was set at 10 mg/kg bw per day in this study. However, due to the uncertainty of a possibly treatment-related developmental effect at lower doses, an additional study was conducted (see immediately below for further information/data) (Masters, et al., 1991b).

Study 2. Additional study
According to the test facility, the singular incidence of some cranial malformations at the lowest dose (4mg/kg bw per day) in the main study justified the conduct of an additional test, to better characterize potential developmental toxicity. In the additional study, 18 or 19 presumed-pregnant New Zealand White rabbits (Interfauna, UK) per dose received *cis*/*trans*-metconazole in 1% methylcellulose by gavage at dose levels of 0, 2, 4 and 10 mg/kg bw per day from GD 7 to GD 19. Doses were selected on the basis of the previous main teratogenicity study. Dosing volume was 2 mL/kg bw. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses examined for gross changes. Fetuses were subsequently examined for skeletal abnormalities.

Maternal data
There were no obvious signs of reaction to treatment or clear dose-related differences in food consumption or body weight change; values for treated groups were either similar to or greater than controls. At 10 mg/kg bw per day, dams exhibited reduced weight gain on days 15 to 20. It is unclear whether this observation was attributable to the effects of the test substance since no dose-related decrease was observed. There were three mortalities, all considered unrelated to treatment: two occurred at 10 mg/kg bw per day and were due to probable intubation errors whilst the third rabbit died prior to the start of treatment. Occasional macroscopic findings did not suggest any association with treatment.

Litter data
There were three incidences of abortions: one in the control group, another at 4 mg/kg bw per day and a third at 10 mg/kg bw per day. The incidence was the same in each group and is therefore not related to treatment. A high pre-implantation loss was observed in the control group with a subsequent smaller litter size.

The incidence of malformed fetuses was increased above control levels: 3/98, 5/116, 5/132 and 9/110 , for controls, 2, 4 and 10 mg/kg bw per day dose groups respectively. Only at 10 mg/kg bw per day was the incidence of malformed fetuses above the historical control range. Severe types of malformation were seen in control animals as well as in the treated groups. The malformations reported in the control group were: one fetus with severe craniofacial defects including rhinencephaly, microcephaly,

anophthalmia and associated skull defects, as well as aortic arch defects, interventricular septal defect, absent spleen/left adrenal and complete situs inversus; one fetus with cranioschisis and ablepharia (open eye); and one fetus with great vessel malformations and interventricular septal defect.

At 2 mg/kg bw per day: one fetus showed cranioschisis with open eye, cleft palate, great vessel malformations, umbilical hernia, forelimb flexure, hind limb talipes and brachydactyly; one fetus showed termination of the vertebral column at sacral 2 (including anury); one fetus had brachyury; and two other fetuses had great vessel malformations (one with interventricular septal defect and altered size of ventricles). In the 4 mg/kg bw per day group: two fetuses were reported to have lumbar scoliosis and associated vertebral changes; one fetus showed thoracic scoliosis and associated vertebral and rib changes; one fetus had and interventricular septal defect with a slightly dilated aortic arch, and another fetus showed diaphragmatic hernia. In the 10 mg/kg bw per day group: one fetus had a small eye and retinal dysplasia; one fetus showed lenticular degeneration; two fetuses were found with hydrocephalus, cebocephaly and associated skull defects (one with interventricular septal defect and retroesophageal subclavian artery); one fetus also showed retroesophageal subclavian artery; two fetuses had great vessel malformations and interventricular septal defects; one fetus showed umbilical hernia, cervical and thoracic scoliosis and associated axial skeletal malformations, and another fetus had forelimb flexure.

The slightly increased incidence of hydrocephalus at the top dose was just outside the reported historical control range of the test facility, and thus a treatment-related effect cannot be excluded. Although the incidence of visceral anomalies at 10 mg/kg bw per day was higher than in controls, principally due to the occurrence of corneal or lenticular opacities, no such findings have been observed in any other rabbit studies, and they were therefore considered of doubtful toxicological relevance. The percentage incidence of skeletal anomalies was unrelated to treatment. Slight intergroup variations in mean incidence of fetuses with extra (13) ribs or variants sternebrae did not suggest any adverse effect of treatment.

Table 48. Key findings of additional developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	0	2	4	10	HCD
		Maternal data			
Dams with live young/dams mated	16/18	17/19	16/18	16/19	
Mortality	0	1 (died prior to treatment)	0	2 (intubation errors)	
Abortions	1	0	1	1	
Nonpregnant	1	1	1	0	
		Litter observations			
Litter mean corpora lutea	9.4	10.8	10.4	10.4	
Implantations	7	8.8	9.4	8.3	
Pre-implantation loss (%)	26	18.5	11.6	18.8	
Post-implantation loss (%)	14.1	19.3	11.8	16.6	3.6–17.5%, mean 10.8
Early resorptions	0.4	1.2	0.3	0.8	0.2–1.1, mean 0.5
Late resorptions	0.4	0.7	0.9	0.6	0.1–1.3, mean 0.5
Live litter size	6.1	6.8	8.3	6.9	
Fetal weight (g)	47.6	45.3	44.6	44.5	
Litter weight (g)	281.9	300.5	356.7	298	

(*Table 48 continues on the following page*)

Dose level (mg/kg bw per day)	0	2	4	10	HCD
Fetal malformations [malformations as a percentage]	3/98 [4.5%]	5/116 [6.6%]	5/132 [4.0%]	9/110 [9.5%]	0.9%–7.5%, mean 3.0%
Gross/visceral anomalies [as a percentage of fetuses]	2/95 [4.2%]	9/111 [9.8%]	6/127 [4.7%]	14/101 [12.9%]	
Skeletal anomalies [as a percentage of fetuses]	10/95 [12.9%]	18/111 [15.3%]	15/127 [10.5%]	13/101 [14.0%]	
Hydrocephalus fetuses affected [litters affected]	0	0	0	2 (2)	max. 1 fetus [1 litter]

HCD Historical control data of the testing facility (Interfauna, UK) from 1989–1994 (Goettel, 2015):

- Mean number of live young: 6.1–10.1, mean 8.4 %
- Malformed fetuses: 0.9%–7.5%, mean 3.0%
- Hydrocephalus: max. 1 fetus (1 litter)

* $p < 0.05$ ** $p < 0.01$, *** $p \leq 0.001$

Based on the results of the additional study, a NOAEL for maternal and fetal toxicity of 10 mg/kg bw per day (top dose) was suggested. The NOAEL for developmental toxicity was set at 4 mg/kg bw per day due to a slightly increased incidence of hydrocephalus at the top dose (Masters et al., 1991b).

Study 3. Preliminary study

In a preliminary study eight presumed-pregnant New Zealand White rabbits (Interfauna, UK) per dose received *cis*-metconazole (94.2% *cis*; batch 12 st90/363) in 1% methylcellulose by gavage at dose levels of 0, 2, 4, 10, 25 and 40 mg/kg bw per day from GD 7 toGD 19 inclusive. Dosing volume was 2 mL/kg bw. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined, and fetuses were examined for visceral and subsequently for skeletal abnormalities.

Maternal data

There were no abortions or total litter resorptions reported for any of the dose groups. With regard to clinical signs post dosing, an increased incidence of cold ears, occurring intermittently, was observed at the top dose. At 25 mg/kg bw per day this effect was mainly observed during the first week of treatment. The 40 mg/kg bw per day dose was associated with clear maternal toxicity. There was significantly reduced food consumption during the initial treatment period, with recovery to levels higher than controls over the remainder of the study. Body weight and body weight gain were slightly reduced during the initial treatment period and generally recovered over the remainder of the study, although there was a reduced body weight gain during the last days of the study (GDs 24–29). At 25 mg/kg bw per day, there was a similar response pattern for food consumption and a slight initial body weight loss over the first three days of dosing, thereafter with an essentially similar gain compared to the control group.

Litter data

Embryo/fetal death and post-implantation loss were significantly increased, and live young and litter weights were significantly reduced at 40 mg/kg bw per day. Mean fetal weight was slightly lower than controls at this dose, but with statistical significance. At 25 mg/kg bw per day post-implantation loss appeared to be increased and live young and litter weights appeared to be reduced, but without any statistical significance. Although this was a preliminary study, fetuses in this study were examined for external, visceral and skeletal defects. The incidence of malformed fetuses was 1/71, 4/71, 2/50, 3/55, 3/47, and 3/31 (1/8, 2/7, 1/5, 3/6, 2/7 and 3/8 litters affected) in the control, 2, 4, 10, 25, and 40 mg/kg bw per day dose groups, respectively. The incidence of malformations was somewhat higher than controls at all dose levels. Fetus malformations reported in the control group consisted of one fetus with retroesophageal subclavian artery. At 2 mg/kg bw per day: one fetus had retroesophageal subclavian artery; one fetus showed great vessel malformations; two fetuses were found with forelimb flexure (one with umbilical hernia). In the 4 mg/kg bw per day group: one fetus showed retroesophageal subclavian artery; another fetus showed thoracic scoliosis and associated vertebral and rib defects. At 10 mg/kg bw per day: one fetus had hindlimb brachydactyly; one fetus showed microphthalmia; one fetus had hydrocephalus, facial bone malformations and great vessel malformation.

In the 25 mg/kg bw per day group three fetuses were reported with hydrocephalus. At the top dose of 40 mg/kg bw per day: three fetuses showed limb defects, one with peromelia, one with forelimb flexure and brachydactyly, and one with hindlimb brachydactyly. A higher incidence of fetuses with peromelia/brachydactyly, liver anomalies and skeletal changes was noted at 40 mg/kg bw per day. At 25 mg/kg bw per day there was a larger number of fetuses with hydrocephalus, which fell outside the historical control range. At 10 mg/kg bw per day one fetus had hydrocephalus (within historical control data), and one fetus from a separate litter had brachydactyly, but again the relevance of this single excursion beyond the HCR was questioned. At 25 mg/kg bw per day and above slightly higher numbers of liver variations (including abnormal lobation, subcapsular cysts, pale subcapsular areas and necrotic lobes) and skeletal variations were observed.

Table 49. Key findings of preliminary developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	**0**	**2**	**4**	**10**	**25**	**40**
			Maternal data			
Dams with live young/dams mated	8/8	7/8	5/8	6/8	7/8	8/8
Nonpregnant	0	1	3	2	1	0
Food consumption (% difference from control)						
GDs 7–8	-	-	-	-	−11%	**−29%***
GDs 7–19	-	-	-	-	−7%	−12%
Body weight gain (g)						
GDs 7–9	−20	10	1	49	−27	−72
GDs 7–19	171	228	221	337	178	230
			Litter observations			
Fetuses examined [litters examined]	71 [8]	71 [7]	50 [5]	55 [6]	47 [7]	31 [8]
Litter mean corpora lutea	11.1	12.3	11.2	11.3	9.7	11.1
Implantations	9.5	11.4	10.8	9.8	8.7	9
Pre-implantation loss (%)	14.8	7.1	3.7	10.1	10.5	18.7
Post-implantation loss (%)	6.5	10.7	8.1	6.6	21	**58.0****
Early resorptions	0.4	0.6	0.5	0.3	0.9	0.5
Late resorptions	0.3	0.7	0.4	0.3	1.1	**4.6****
Live litter size	8.9	10.1	10	9.2	6.7	**3.9****
Fetal weight (g) [% of control]	44.7	42.4	43.9	43.7	46.4	41.1 [−8%]
Litter weight (g) [% of control]	392.4	422.3	432.5	396.7	306.7 [−22%]	**155.8** **[−60%]****
Malformations [% fetuses]	1/71 [1.4%]	4/71 [5.6%]	2/50 [4.0%]	3/55 [5.5%]	3/47 [6.4%]	3/31 [9.7%]
Malformations [% litters]	1/8 [12.5%]	2/7 [28.6%]	1/5 [20%]	3/6 [50%]	2/7 [28.7%]	3/8 [37.5 %]
Gross/visceral anomalies [% fetuses]	1/70 [1.4%]	4/67 [6.0%]	4/48 [8.3%]	2/52 [3.8%]	3/44 [6.8%]	**6/28** **[21.4%]***
Skeletal anomalies [% fetuses]	11/70 [15.7%]	14/67 [20.9%]	5/48 [10.4%]	8/52 [15.4%]	13/44 [29.5%]	12/28 [42.9%]
Skeletal anomalies [% litters]	6/8 [75%]	3/7 [42.8%]	3/5 [60%]	3/6 [50%]	7/7 [100%]	5/8 [62.5 %]

(*Table 49 continues on the following page*)

Dose level (mg/kg bw per day)	0	2	4	10	25	40
Visceral and skeletal malformations						
Fetuses examined [litters examined]	70 [8]	67 [7]	48 [5]	51 [6]	44 [7]	28 [8]
Hydrocephalus	0	0	0	1 [1]	3 [2]	0
Forelimb flexures	0	2 [2]	0	0	0	1 [1]
Peromelia	0	0	0	0	0	1 [1]
Brachydactyly	0	0	0	1 [1]	0	2 [2]
Visceral anomalies						
Fetuses examined [litters examined]	70 [8]	67 [7]	48 [5]	51 [6]	44 [7]	28 [8]
Liver: Total affected	0	0	1 [1]	0	3 [2]	5 [3]
Skeletal anomalies:						
Fetuses examined [litters examined]	70 [8]	67 [7]	48 [5]	51 [6]	44 [7]	28 [8]
Sutural bones	1	2	0	1	1	1
Connected jugal to maxilla	6	4	3	0	3	6
Cervical ribs	0	9	0	0	4	3

Historical control data (Interfauna, UK) of the testing facility from 1989–1994 (Goettel, 2015):

- Post-implantation loss (%): 3.6–17.5%, mean 10.8
- Early resorptions: 0.2–1.1, mean 0.5
- Late resorptions: 0.1–1.3, mean 0.5
- Mean number of live young: 6.1–10.1, mean 8.4
- % malformed fetuses: 0.9%–7.5%, mean 3.0%
- Hydrocephalus: max. 1 fetus (1 litter)

* $p \leq 0.05$ ** $p \leq 0.01$

At 25 and 40 mg/kg bw per day after treatment with metconazole (95% *cis*) maternal toxicity was observed as well as an increased post-implantation loss (late resorptions) resulting in reduced litter size/weight. The incidence of fetuses with hydrocephalus and/or limb malformations was increased at maternally toxic doses. However, due to the relatively few litters examined and the inconsistency of the distribution pattern of malformation (the most severely malformed fetus was in the 10 mg/kg bw per day group), it is inappropriate to draw a causal relationship for these malformations. However it should be noted that hydrocephalus occurred in other rabbit studies.

Study 3. Main study

In the main study 16 presumed-pregnant New Zealand White rabbits (Interfauna, UK) per dose received *cis*-metconazole (94.2% *cis*; batch 12 st91/106) in 1% methylcellulose by gavage at dose levels of 0, 2, 4, 10 and 40 mg/kg bw per day from GD 7 to GD 19 inclusive. Dosing volume was 2 mL/kg bw. On day 29 of pregnancy, the females were sacrificed and subjected to postmortem examination. Litter parameters were determined and fetuses fixed prior to examination for skeletal or visceral abnormalities.

Maternal data

There were no abortions or total litter resorptions reported for any of the dose groups. With regard to clinical signs at the top dose 7/12 pregnant animals had cold ears post dosing on at least three days during the treatment period compared to 2/14 control animals. This finding was considered to be a nonspecific sign. The 40 mg/kg bw per day dose was associated with clear maternal toxicity. There was significantly reduced food consumption during the initial treatment period, but this recovered to levels equal to the control over the remainder of the study. Food consumption at 10 mg/kg bw per day was slightly reduced from the onset of treatment, attaining statistical significance during days 11–14. Thereafter, intake at this dose was essentially similar to the control group. At 10 mg/kg bw per day and above there was an initial dose-related bodyweight loss (days 7–9) which attained statistical significance. At 40 mg/kg bw per day body weight gain did not recover by the end of the study. The lower body weight gain during GDs 24–29 was probably due to a general reduction of litter weight observed at termination. No relevant findings were observed at necropsy.

Litter data

There were no total litter losses at any dose. At the top dose embryo/fetal death and post-implantation loss were significantly increased. Number of live young, litter weights and mean fetal weights were lower than for controls at this dose, but it did not reach statistical significance. The incidence of malformed fetuses was 1/109, 3/133, 2/135, 5/139 and 9/69 (1/14, 3/16, 2/16, 4/14 and 6/12 litters affected) for control, 2, 4, 10 and 40 mg/kg bw per day groups, respectively. The incidence of malformations in the 40 mg/kg bw per day dose group was increased to above that in controls, but lacked a clear dose–response relationship at lower doses. Furthermore, only at the top dose was the percentage of malformed fetuses outside the historical control range. The detailed types of malformations observed in the fetuses were noted and are described below.

In the control group one fetus with cervical meningocoele was found. At 2 mg/kg bw per day one fetus had umbilical hernia and forelimb flexure; one fetus showed acephaly, transposition of the great vessels, malrotated heart, interventricular septal defect, retroesophageal subclavian artery, split sternum, spina bifida, forelimb flexure, oligodactyly and brachydactyly; another fetus showed interventricular septal defect, split sternum with umbilical hernia, forelimb flexure, oligodactyly, absent humerus and radius, and reduced scapula. In the 4 mg/kg bw per day group: one fetus was reported with brachyury and one fetus with great vessel malformations and altered size of ventricles. At 10 mg/kg bw per day one fetus was found with brachydactyly; two fetuses with forelimb flexure; one fetus with hydranencephaly with domed cranium; and one fetus with absent gonads was found. At the top dose of 40 mg/kg bw per day three fetuses had hydrocephalus, one with partially fused parietals, one with misshapen frontals and parietals; one fetus showed absent spleen; two fetuses had thoracic scoliosis and associated vertebral and rib malformations; two fetuses showed retroesophageal subclavian artery; and one fetus had forelimb flexure.

The types of defect seen at 2 mg/kg bw per day were some of the most severe findings in this study as evidenced by two fetuses with multiple severe malformations. Affected malformed fetuses in the 40 mg/kg bw per day group had individual malformations. Thus, the severity of defects did not seem to follow a dose-related pattern in this main study. Of note were the incidences of hydrocephalus at 40 mg/kg bw per day and shortened/absent limbs/digits at 2 and 10 mg/kg bw per day, but the latter finding obviously lacked a dose–effect relationship. There was also an increased proportion of fetuses with both visceral and skeletal anomalies at 40 mg/kg bw per day, mainly due to abnormal liver lobulation and various non-specific skeletal anomalies. The observed liver abnormalities at the intermediate doses were of questionable relevance in the absence of any dose-related appearance. However, the incidence of fused liver lobes at the top dose was not discounted as it was also present in the preliminary study. Although liver morphology is quite variable, the effect of treatment was considered pertinent. The slight intergroup variations in mean incidence of fetuses with extra (13) ribs or variant sternebrae did not suggest any adverse effect of treatment.

Table 50. Key findings of main developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	0	2	4	10	40
Maternal data					
Dams with live young/dams mated	14/16	16/16	16/16	14/16	12/16
Nonpregnant	2	0	0	2	4
Food consumption [% difference from controls]					
GDs 7–8	163	158	153	151 [−7%]	**93 [−43%]****
GDs11–14	164	164	156	**143 [−13%]****	**143 [−13%]****
GDs 24–28	132	138	123	**117**	**126**
Body weight (g) [% difference from controls]					
GD 9	3824	3832	3742	**3746 [−2%]****	**3641 [−5%]****
GD11	3846	3855	3763	**3778 [−2%]***	**3707 [−4%]****
GD 29	4198	4283	4156	4165	**4026 [−4%]***
Body weight gain (g)					
GDs 7–9	43	14	29	−15	−116
GDs 7–20	211	247	226	174	117
GDs 24–29	416	464	442	404	269

Dose level (mg/kg bw per day)	0	2	4	10	40
Litter observations					
Fetuses examined [litters examined]	109 [14]	133 [16]	135 [16]	139 [14]	69 [12]
Litter mean corpora lutea	10.3	11.6	11.8	12.6	11.8
Implantations	8.7	9.5	9.4	11.1	9.8
Pre-implantation loss [%]	15.3	16.4	19.8	10.5	15.6
Post-implantation loss [%]	9.9	11	10.7	10.3	**40.7****
Early resorptions	0.4	0.6	0.6	0.4	0.4
Late resorptions	0.5	0.6	0.4	0.8	**3.7****
Live litter size	7.8	8.3	8.4	9.9	5.8
Fetal weight (g) [% difference from controls]	45.7	45	44.7	42.0 [−8%]	42.1 [−8%]
Litter weight (g) [% difference from controls]	349.7	368.4	368.5	415.8	237.6 [−32%]
Malformations [% fetuses]	1/109 [0.9%]	3/133 [2.3%]	2/135 [1.5%]	5/139 [3.6%]	**9/69** **[13.0%]****
Malformations [% litters]	1/14 [7.1%]	3/16 [18.8 %]	2/16 [12.5 %]	4/14 [28.6 %]	6/12 [50 %]
Gross/visceral anomalies [% fetuses]	8/108 [7.4%]	15/130 [11.5%]	12/133 [9.0%]	8/134 [6.0%]	12/60 [20.0%]
Skeletal anomalies [% fetuses]	23/108 [21.3%]	20/129 [15.4%	18/133 [13.5%]	23/134 [17.2%]	21/60 [35.0%]
Skeletal anomalies [% litters]	10/14 [71.4%]	11/16 [68.8 %]	9/16 [56.3%]	10/14 [71.4 %]	8/12 [66.7 %]
***Visceral and skeletal malformations; fetuses* [*litters involved*]**					
Hydrocephaly	0	0	0	0	3 [3]
Hydranencephaly	0	0	0	1 [1]	0
Forelimb flexures	0	3 [3]	0	2 [2]	1 [1]
Oligodactyly	0	2 [2]	0	0	0
Brachydactyly	0	1 [1]	0	1 [1]	0
Visceral anomalies					
Fetuses examined [litters examined]	108 [14]	130 [16]	133 [16]	134 [14]	60 [12]
Liver: total affected fetuses [litters]	2 [2]	3 [3]	3 [2]	2 [2]	7 [5]
***Skeletal anomalies; fetuses* [*litters involved*]**					
Fetuses examined [litters examined]	108 [14]	130 [16]	133 [16]	134 [14]	60 [12]
Sutural bones	2 [2]	2 [2]	6 [4]	3 [3]	3 [2]
Connected jugal to maxilla	4 [4]	12 [7]	5 [3]	11 [4]	7 [4]
Irregular ossification cranial bones	1 [1]	0	1 [1]	0	5 [5]
Cervical ribs	7 [4]	1 [1]	1 [1]	3 [2]	0

Historical control data (Interfauna, UK) of the testing facility from 1989–1994 (Goettel, 2015):

- Post-implantation loss (%): 3.6–17.5%, mean 10.8
- Early resorptions: 0.2–1.1, mean 0.5
- Late resorptions: 0.1–1.3, mean 0.5
- Mean number of live young: 6.1–10.1, mean 8.4
- % malformed fetuses: 0.9%–7.5%, mean 3.0%
- Hydrocephalus: max. 1 fetus (1 litter)

* $p \leq 0.05$ ** $p \leq 0.01$

Maternal toxicity (decreased food consumption, body weight and body weight gain) was evidenced as dose-related at 10 mg/kg bw per day and above after administration of metconazole (95% *cis*) to pregnant rabbits during GDs 7–19, leading to a maternal toxicity NOAEL of 4 mg/kg bw per day. Fetal toxicity was evidenced by a significant increase in post-implantation loss (late resorptions) and an associated decreased litter size, reduced litter and fetal weight at the top dose. The fetal toxicity NOAEL was therefore 10 mg/kg bw per day. The developmental NOAEL was 10 mg/kg bw per day due to an increased incidence of hydrocephalus at the top dose (Masters et al., 1992a).

According to the test facility, the conduct of the present study was justified because incidences of brachydactily/oligodactily had been observed in the previous developmental study at 2 mg/kg bw per day (but not at higher doses).

Study 4

New Zealand White rabbits (Interfauna, UK), presumed pregnant, were allocated to groups of 18or 19. Groups received *cis*-metconazole (95.5% *cis*; batch 12 [st 91/016]) in 1% methylcellulose by gavage at dose levels of 0, 0.5, 1, 2, 10 and 40 mg/kg bw per day from GD 7 to GD 19 inclusive. Dosing volume was 2 × 2 mL/kg bw, separated as two equal doses. On day 29 of pregnancy the females were sacrificed and subjected to postmortem examination. Litter parameters were determined and fetuses fixed prior to examination for skeletal and visceral abnormalities. The study is considered to provide additional information.

Maternal data

There were no relevant findings on mortality or abortions. No treatment-associated clinical signs were observed. At the top dose of 40 mg/kg bw per day there was a marked and significant reducetion in food consumption during the initial treatment period, which recovered to levels greater than for controls by the end of the study. Following initiation of treatment at 40 mg/kg bw per day, there was a body weight loss to day 9 which affected all animals and attained statistical significance. Thereafter, good recovery occurred and weight gain throughout the remaining period of treatment was generally greater than that of the controls. Between days 24 and 29, body weight gain was again slightly reduced at this dose, however this was probably due to the general reduction of litter weight observed at termination. Necropsy revealed no findings related to treatment.

Litter data

Embryo/fetal death and post-implantation loss were increased and litter weight and mean fetal weight significantly decreased at 40 mg/kg bw per day. The number of live young was lower than for controls at this dose, but without statistical significance. The incidence of malformed fetuses was 7/152, 5/160, 1/156, 3/139, 9/165 and 18/126 (7/18, 5/17, 1/17, 3/17, 6/18 and 11/16 litters affected) for the control, 0.5, 1, 2, 10 and 40 mg/kg bw per day groups, respectively. The control incidence of malformed fetuses was high so that only the 40 mg/kg bw per day dose group was clearly increased above control levels. Percentages of malformed fetuses for all but the top dose were within the historical control range. The types of defects reported for the individual fetuses were as follows.

In the control group: two fetuses were found with retroesophageal subclavian artery; two fetuses with great vessel malformations and interventricular septal defects; one fetus with interventricular septal defect and lumbar scoliosis and associated vertebral malformation; one fetus with hydrocephalus and cebocephaly with associated facial bone malformations, and one fetus with misshapen naso-frontal region of the cranium, cleft lip and palate. In the 0.5 mg/kg bw per day group: two fetuses had great vessel malformations, one with an interventricular septal defect; two other fetuses showed retroesophageal subclavian artery; and one fetus had spina bifida and malrotated hindlimbs. At 1 mg/kg bw per day one fetus with hydrocephalus and cebocephaly with associated facial bone malformations was reported. At 2 mg/kg bw per day two fetuses showed great vessel malformations, one with interventricular septal defect, brachyury, and rib malformations; and another fetus had forelimb flexure. At 10 mg/kg bw per day five fetuses were reported with great vessel malformations, two with interventricular septal defect, one with retroesophageal subclavian artery and interventricular septal defect and altered size of ventricles and malrotated heart, and one with interventricular septal defect and forelimb flexure; one fetus showed multiple malformations including termination of the vertebral column at T6, umbilical hernia, absent adrenal, absent gonads, and malrotated hindlimbs; one fetus had an retroesophageal aortic arch; two fetuses displayed small eye and retinal dysplasia, one with

hydrocephalus. At the top dose of 40 mg/kg bw per day two fetuses were found with limb defects, one with ectrodactyly, one with brachydactyly; two fetuses showed retroesophageal subclavian artery; one fetus had multiple malformations including acephaly, cervical scoliosis with associated skeletal defects, great vessel malformations, gastroschisis, partially split sternum, and forelimb flexure; one fetus had hydrocephalus; two fetuses showed umbilical hernia; three other fetuses had forelimb flexure; one fetus showed small pinna; one fetus had vertebral and rib malformations and brachyury; two fetuses had great vessel malformations, retroesophageal subclavian artery and interventricular septal defect, one of which also had kidney defects and forelimb flexure; another fetus had spina bifida, scoliosis, kidney defects, and brachyury; one fetus showed microphthalmia and retinal dysplasia; and another fetus displayed an intestine adhered to the liver and gall bladder.

Overall, the incidence of malformed fetuses at 40 mg/kg bw per day was more than twice that seen in the control group and apart from five fetuses in separate litters with forelimb flexure, there was no consistent pattern in the type of changes observed. In this study, there were four instances of hydrocephalus, one each at 40, 10 and 1 mg/kg bw per day and one in the control group. These single cases of hydrocephalus were within the historical control data. Hydrocephalus at 1 mg/kg bw per day and the control group was associated with cebocephaly, a combination occasionally seen in historic control data. However, as the incidences were the same as those within the control group, a relationship to treatment in the context of this study was not conclusive. A further two fetuses showed marked digit reductions, both at 40 mg/kg bw per day; similar observations have been made previously at this dose. There were no limb/digit reductions seen at 10 mg/kg bw per day or at either of the lower doses. Although the percentage incidence of fetuses with visceral anomalies at 40 mg/kg bw per day was slightly (non-statistically) higher than the control value, there is no clear evidence of any specific visceral change that could be considered a response to treatment. The previously reported increase in liver variations was not replicated. At 40 mg/kg bw per day and, to a lesser extent, 10 mg/kg bw per day there was a higher number of fetuses with skeletal anomalies, attaining statistical significance at the highest dose, primarily reflecting an increased incidence of connected jugal to maxilla, a change previously present, but not specifically associated with treatment.

Table 51. Key findings of developmental toxicity study in rabbit

Dose level (mg/kg bw per day)	**0**	**0.5**	**1**	**2**	**10**	**40**
Maternal data						
Dams with live young/dams mated	18/18	17/18	17/18	17/19	18/18	16/19
Nonpregnant	0	0	1	1	0	2
Food consumption (g) [% difference from controls]						
GDs 7–8	134	144	131	144	143	**96 [−29%]****
GDs 7–19	1867	1836	1763	1852	1956	**1648 [−12%]***
Body weight gain (g)						
GDs 7–9	−12	−6	4	−10	8	−109
GDs 7–20	222	165	181	216	201	141
Litter observations						
Fetuses examined [litters examined]	152 [18]	160 [17]	156 [17]	139 [17]	165 [18]	126 [16]
Litter mean corpora lutea	10.8	11.8	11.8	11.6	11.9	11.6
Implantations	9.1	10.1	10.1	10	10.3	10.1
Pre-implantation loss (%)	15.9	13.4	14.2	12.1	13	12.2
Post-implantation loss (%)	7.1	6.4	8.6	17.1	11.5	21.1
Early resorptions	0.3	0.2	0.5	1.5	0.7	0.4
Late resorptions	0.4	0.5	0.4	0.3	0.5	1.8
Live litter size	8.4	9.4	9.2	8.2	9.2	7.9

(*Table 51 continues on the following page*)

Dose level (mg/kg bw per day)	0	0.5	1	2	10	40
Fetal weight (g) [% of control]	45.7	44.1	42.9	43.9	44	**39.8 [−13%]****
Litter weight (g) [% of control]	380	412.9	388.9	356.6	399.1	**306.3 [−19%]***
malformations [% fetuses]	7/152 4.6%	5/160 3.1%	1/156 0.6%	3/139 2.2%	9/165 5.5%	18/126 14.3%
malformations [% litter]	7/18 38.9%	5/17 29.4%	1/17 5.9%	3/17 17.6%	6/18 33.3%	**11/16** **68.8%**
Gross/visceral anomalies [% fetuses]	18/145 12.40%	5/155 3.20%	11/155 7.10%	6/136 4.40%	12/156 7.70%	13/108 −12.00%
Skeletal anomalies [% fetuses]	22/145 15.2%	17/155 11.0%	21/155 13.5%	17/136 12.5%	36/156 23.1%	**36/108** **[33.3%]***
Skeletal anomalies [%litter]	10/18 55.6%	12/17 70.6%	11/17 64.7%	12/17 70.6%	14/18 77.8%	**14/16** **87.5%**
***Visceral and skeletal malformations; fetuses* [*litters involved*]**						
Hydrocephalus	1	0	1	0	1	1
cebocephaly	1	0	1	0	0	0
Forelimb flexure/malrotated hindlimb	0	1	0	1	2 [2]	5 [5]
Visceral anomalies						
Liver: total affected fetuses [litters]	4[4]	2[2]	2[2]	1	1	3[3]
***Skeletal anomalies; fetuses* [*litters involved*]**						
Sutural bones	4[2]	2[2]	5[3]	3[3]	6[5]	3[3]
Connected jugal to maxilla	7[4]	2[2]	2[2]	6[4]	11[7]	24[11]
Cervical ribs	2[2]	9[6]	4[2]	3[2]	2[2]	6[4]
Irregular ossification vertebrae	2[2]	3[3]	8[5]	2[2]	5[5]	9[6]

* $p \leq 0.05$ ** $p \leq 0.01$

Administration of 40 mg/kg bw per day metconazole (95% *cis*) to pregnant rabbits during GDs 7–19 was associated with maternal toxicity (markedly reduced food consumption and body weight gain), leading to a NOAEL for maternal toxicity of 10 mg/kg bw per day.

Fetal toxicity was observed at the maternal toxic top dose as evidenced by increased post-implantation loss (late resorptions), decreased litter and fetal weight. The NOAEL for fetal toxicity was therefore 10 mg/kg bw per day. With regard to developmental toxicity at 10 and 2 mg/kg bw per day, one fetus in each of these two groups showed a similar structural defect as that seen at 40 mg/kg bw per day in this and previous studies. However, the findings are ambiguous and a relationship to treatment is not conclusive. The apparent increase in any skeletal anomaly at 10 mg/kg bw per day was not associated with specific changes. The only consistent malformation at the highest dose level in this study, is the forelimb flexures/malrotated hindlimbs setting the NOAEL for developmental toxicity at 10 mg/kg bw per day (Masters et al., 1992b).

Study 5

A developmental study in rabbits via the dermal route (study author Stump, 2012) with *cis*/*trans* -metconazole was conducted by another metconazole-supporting company. A detailed study summary was made available to BASF (prepared by Stump, 2014) and forms part of this submission.

Groups of 25 time-mated New Zealand White rabbits (Hra: (NZW)SPF) were exposed dermally to metconazole (purity 99.7%; *cis*:*trans* 84.2%:15.5%; batch AS2122b) at doses of 30, 90, and 270 mg/kg bw per day, six hours per day from GD 6 until GD 28. Controls received the vehicle (water) alone. Termination and laparohysterectomy on GD 29 was followed by processing and detailed necropsy of dams and caesarean section as well as detailed external, visceral and skeletal examination of fetuses.

Maternal data

One of the 25 does in each of the 90 and 270 mg/kg bw per day groups aborted on GD 23 or 26, and one of 25 does in the 270 mg/kg bw per day group delivered prior to the scheduled necropsy on GD 29, all following body weight losses (up to 20.5%) with corresponding reductions in food consumption and increased incidence of decreased defaecation. The abortion and early delivery in the 270 mg/kg bw per day group were considered to be test substance-related, but secondary to the effects on body weight and food consumption noted for surviving females at this exposure level. However, because similar effects on body weight and food consumption were not observed for other females at 90 mg/kg bw per day, the single abortion at this exposure level was not considered to be test substance-related but instead to be a random occurrence. All other females survived to scheduled necropsy on GD 29. There were no test substance-related macroscopic findings noted at any exposure level. Clinical signs observed included decreased defaecation in the 270 mg/kg bw per day group, corresponding to reduced mean food consumption.

Test substance-related, adverse dermal observations, consisted of very slight to slight erythema, desquamation, and eschar for females in the 270 mg/kg bw per day group during the treatment period. In the 90 mg/kg bw per day group, test substance-related minimal dermal irritation (primarily very slight erythema) was noted during the treatment period. However, due to the limited incidence and minimal severity of these findings, they were not considered to be adverse.

No dermal observations ere noted for the 30 mg/kg bw per day group. A test substance-related mean body weight loss following the initiation of treatment (GDs 6–10) and a test substance-related lower mean body weight gain during the later portion of the treatment period (GDs 20–29) were noted in the 270 mg/kg bw per day group, resulting in a lower mean body weight gain when the entire treatment period (GDs 6–29) was evaluated. Mean food consumption in the 270 mg/kg bw per day group was lower than for the control group throughout the treatment period. Mean net body weight, net body weight change, and gravid uterine weight for the 270 mg/kg bw per day group were similar to control group values. No test substance-related effects on maternal body weights, body weight gains, net body weights, net body weight gains, gravid uterine weights or food consumption were noted for the 30 and 90 mg/kg bw per day groups.

Intrauterine growth and survival were unaffected by test substance administration at exposure levels of 30, 90, and 270 mg/kg bw per day. Mean numbers of corpora lutea and implantation sites, and the mean litter proportions of pre-implantation loss were similar across all groups.

A significantly ($p < 0.01$) lower mean male fetal weight was noted in the 270 mg/kg bw per day group (39.7 g) compared to the concurrent control group (44.0 g). However, the value was within the historical control data range (36.6–45.2 g) and no statistically significant differences were noted when mean female or combined fetal weights were compared to the concurrent control group. In addition, the mean litter proportion of post-implantation loss (primarily late resorptions) in the 270 mg/kg bw per day group (10.6% per litter) was higher than the concurrent control group value (3.6% per litter). This resulted in a corresponding lower mean litter proportion of viable fetuses (89.4% per litter) compared to the concurrent control group value (96.4% per litter). However, these differences were not statistically significant when compared to the concurrent control group, the values were within respective historical control ranges, and the mean number of viable fetuses in this group was similar to the concurrent control group. These differences could also be attributed to two females (numbers 65481 and 65385) in the 270 mg/kg bw per day group with 55.6% and 60.0% percent per litter post-implantation loss (late resorptions), respectively.

Litter data

The numbers of fetuses (litters) available for morphological evaluation were 214 (25), 177 (22), 177 (22), and 190 (23) representing the control, 30, 90, and 270 mg/kg bw per day groups respectively. Malformations were observed in 5 (4), 3 (2), 6 (4), and 6 (5) fetuses (litters) in these same respective exposure groups during external, visceral and skeletal examinations and they were considered spontaneous in origin. In the 270 mg/kg bw per day group, one fetus was noted with hydrocephalus and a domed head (increased cavitation of both lateral ventricles and the third ventricle). Carpal and/or tarsal flexure (with no apparent skeletal origin) were noted for another fetus in this group and for two fetuses from the same litter in the 90 mg/kg bw per day group; both fetuses in the 90 mg/kg bw per day

group also exhibited paw hyperflexion (with no apparent skeletal origin) and hydrocephalus (increased cavitation of both lateral ventricles and the third ventricle). In addition, bilateral microphthalmia was noted for two fetuses from a single litter in the 90 mg/kg bw per day group (not the same litter as the carpal/tarsal flexure and hydrocephalus); no skeletal origins were apparent for the microphthalmia. Because the aforementioned malformations were observed in single fetuses or litters, were not observed in an exposure-related manner, and were within the ranges observed in the historical control data, they were not considered to be related to test substance exposure. No external developmental variations were observed in this study. Visceral and skeletal developmental variations observed in the groups exposed to test substance consisted primarily of major blood vessel variation, extra papillary muscle present in the heart, retrocaval ureter, accessory spleen(s), haemorrhagic ring around the iris, absent or small gall bladder, small heart, pale and misshapen liver, 13th full rib(s), 27 presacral vertebrae, 13th rudimentary rib(s), and sternebra(e) nos. 5 and/or 6 unossified, and hyoid arch(es) bent. These findings were not considered to be test substance-related as they did not occur in a dose-related manner, were noted similarly in the concurrent control group, and/or the mean litter proportions were within the ranges of the developmental historical control data.

Intrauterine growth and survival and fetal morphology in the 30, 90, and 270 mg/kg bw per day groups were unaffected by test substance exposure.

Table 52. Key findings of developmental toxicity study (dermal route) in rabbit

Dose (mg/kg bw per day)	0	30	90	270
Maternal data				
Mean body weight change (g)				
GDs 6–10	66	88	85	**–19****
GDs 10–13	43	58	55	34
GDs 13–20	162	173	141	149
GDs 20–29	106	21	111	70
GDs 6–29	377	340	400	**261***
Mean food consumption [g/animal per day]				
GDs 6–10	168	182	178	**133****
GDs 10–13	152	172	162	128
GDs 13–20	158	173	156	**131***
GDs 20–29	124	125	131	106
GDs 6–29	146	157	151	**124***
Litter observations				
Total number of corpora lutea	242	215	218	239
[Corpora lutea/dam]	[9.7]	[9.8]	[9.9]	[10.4]
Total number of Implantations	222	182	187	211
[Implantations/dam]	[8.6]	[8.3]	[8.5]	[9.2]
Total number of litters	25	22	22	23
Total number of live fetuses	214	177	177	190
[Live fetuses/dam]	[8.6]	[8]	[8]	[8.3]
Total number of resorptions	8	4	10	21
early resorptions	7	3	8	2
late resorptions	1	1	2	19

(*Table 52 continues on the following page*)

Dose (mg/kg bw per day)	0	30	90	270
Mean fetal weight (g)	43.2	41.8	43.8	40.2
males (g)	44	42.7	44	**39.7****
females (g)	42.6	40.5	43.7	40.1
Sex ratio (% male)	49.1	52.5	47.8	47.2
Pre-implantation loss (%)	7.9	15.3	12.9	11.7
Post-implantation loss (%)	3.6	2.3	5.9	10.6
***External examinations; fetuses* [*litters involved*]**				
Fetuses examined [litters examined]	214 [25]	177 [22]	177 [22]	190 [23]
Fetuses with malformations	0 [0]	0 [0]	4 [2]	2 [2]
Mean % affected per litter	0	0	2.3	1
Hydrocephalus with or without dome head§	0 [0]	0 [0]	0 [0]	1 [1]
Carpal and/or tarsal flexure§	0 [0]	0 [0]	2 [1]	1 [1]
Paw hyperflexion§	0 [0]	0 [0]	2 [1]	0 [0]
Microphthalmia and/or anophthalmia§	0 [0]	0 [0]	2 [1]	0 [0]
***Visceral examinations; fetuses* [*litters involved*]**				
Fetuses [litters] with malformations	1 [1]	0 [0]	2 [1]	2 [2]
Mean % affected per litter	0.5	0	1.1	0.8
Malpositioned kidney	0 [0]	0 [0]	0 [0]	1 [1]
Lungs, lobular agenesis	1 [1]	0 [0]	0 [0]	0 [0]
Hydrocephalus	0 [0]	0 [0]	2 [1]	0 [0]
Stenotic pulmonary trunk	0 [0]	0 [0]	0 [0]	1 [1]
Bulbous aorta	0 [0]	0 [0]	2 [1]	1 [1]
***Skeletal examinations; fetuses* [*litters involved*]**				
Fetuses [litters] with malformations	4 [3]	3 [2]	2 [2]	2 [2]
Mean % affected per litter	2	1.5	1.6	1
Vertebral anomaly with or without associated rib anomaly	0 [0]	0 [0]	1 [1]	0 [0]
Sternebrae fused	0 [0]	0 [0]	1 [1]	0 [0]
Costal cartilage animal	0 [0]	0 [0]	1 [1]	1 [1]
Rib anomaly	0 [0]	1 [1]	0 [0]	1 [1]
Vertebral centra anomaly	1 [1]	0 [0]	0 [0]	0 [0]
Bent rib(s)	1 [1]	0 [0]	0 [0]	0 [0]

§ Historical control data of rabbit studies from the laboratory:
- Hydrocephalus with or without dome head: 0 (0) – 5 (1)
- Carpal and/or tarsal flexure: Control: 0 (0) – 3 (2)
- Paw hyperflexion: 0 (0) – 2 (1)
- Microphthalmia and/or anophthalmia: 0 (0) – 2 (1)

* $p \leq 0.05$ ** $p \leq 0.01$

Of the 99 studies in the current WIL Research historical control data for rabbits, only two studies were performed via the dermal route other than this metconazole study. No malformations were observed in the 343 control group fetuses in these two studies (20 litters/study). With only two other dermal exposure studies conducted in the laboratory, dermal HCD are insufficient for comparison. Although the dermal route of exposure is used very infrequently for studies of developmental toxicity in rabbits, the route of

administration should have no effect on the incidence of malformations. Malformations that are the result of test substance administration are the result of systemic exposure to the developing embryo/fetus. This is true whether the compound is administered via oral, intravenous, dermal, subcutaneous, inhalation or any other route. If the dermal route used in the metconazole study were a confounder, we would expect other intrauterine parameters to be affected compared to the mean historical control data. The control animals in the metconazole study compare very favourably to the overall historical control data. Therefore, although there is little historical control data via the dermal route, comparison to historical data by all routes of administration is still appropriate.

As mentioned above, two fetuses from the same litter in the mid-dose group (90 mg/kg bw per day) and one fetus in the high-dose group (270 mg/kg bw per day) had hydrocephalus. One historical control data set had a mean litter proportion incidence of 1.2% for hydrocephalus (the maximum observed); however, there was only one occurrence of hydrocephalus in that study. Three other control data sets had two fetuses with hydrocephalus as was seen in the metconazole study. In addition, seven control data sets had one fetus each with hydrocephalus. A recently conducted oral gavage study demonstrated that five control group fetuses with hydrocephalus originated from one control group litter. The resulting incidence is much greater than seen in the 90 or 270 mg/kg bw per day metconazole groups. While hydrocephalus is not observed very often in control fetuses, previous control groups have been observed with a similar incidence to that observed in the present study, including one study in which fetuses from two different control group litters were affected.

Table 53. Historical control data for developmental toxicity studies in rabbit

Study Number	Number of affected fetuses	Number of affected litters	Affected litters (%)	Mean litter proportion affected
Hydrocephalus				
6	2	1	5	0.90%
9	1	1	6.3	0.90%
35	1	1	4.5	0.50%
43	1	1	5	0.60%
49	1	1	5.9	1.20%
55	2	2	9.1	1.10%
58	2	1	4.8	0.90%
84	1	1	4	0.40%
105	1	1	4.5	0.40%
115	1	1	5	0.70%
Conducted 4/2013 (gavage)	5	1	5	2.50%
Metconazole: 90 mg/kg bw per day	2	1	4.5	1.10%
Metconazole 270 mg/kg bw per day	1	1	4.3	0.50%
Carpal and/or tarsal flexure				
11	1	1	4.8	0.50%
19	3	2	9.5	1.40%
25	1	1	5.3	0.90%
28	1	1	4.3	0.50%
34	2	1	4	0.80%
38	1	1	4.5	0.50%
72	1	1	4.2	0.40%

(*Table 53 continues on the following page*)

Study Number	Number of affected fetuses	Number of affected litters	Affected litters (%)	Mean litter proportion affected
Conducted 4/2013 (gavage)	2	2	10	1.10%
Conducted 5/2013 (i.v.)	2	1	5.3	1.50%
Metconazole 90 mg/kg bw per day	2	1	4.5	1.10%
Metconazole 270 mg/kg bw per day	1	1	4.3	0.50%

i.v. Intravenous

In conclusion, 90 mg/kg bw per day was the NOAEL for maternal and fetal toxicity based on food consumption, body weight changes during gestation, post-implantation loss and decreased fetal weight. 270 mg/kg bw per day was the NOAEL for embryo/fetal developmental toxicity after dermal application of metconazole to time-mated New Zealand White rabbits (Stump, 2012; 2014).

2.6 Special studies

(a) Neurotoxicity

Rat

Preliminary study

A two-week range-finding sub-chronic neurotoxicity study in rats with *cis*/*trans*-metconazole (purity 98.99%; *cis*:*trans* 85:15; lot/batch #:9Z521) was conducted by another metconazole-supporting company. A detailed study summary was made available to BASF.

The sub-chronic neurotoxic potential of *cis*/*trans*-metconazole to Sprague Dawley Crl:CD®(SD) rats by dietary administration was assessed over a period of two weeks in order to aid selection of dietary concentrations to be used in a main four-week neurotoxicity study. Three groups each of five males and five females received *cis*/*trans*-metconazole in the diet at concentrations of 100, 540 or 3000 ppm and a similarly constituted control group received an untreated diet throughout the period. Achieved dosages over the two-week period were 11.0, 59.6 and 216.8 mg/kg bw per day for males and 10.6, 52.8 and 206.1 mg/kg bw per day for females receiving 100, 540 and 3000 ppm, respectively. During the study clinical condition, body weight, food consumption, neuro-behavioural investigations, organ weights and macro-pathology investigations were undertaken. Neuro-behavioural investigations (assessment in-the-hand and in a standard arena) were performed on all animals before treatment commenced and in week 2. In addition, sensory reactivity and grip strength observations and motor activity measurements were performed in week 2.

At 3000 ppm thin build was recorded from day 4 for all animals, and brown staining on the head was seen during the second week of treatment in two males receiving this dietary concentration. There were no treatment-related signs in animals receiving 100 or 540 ppm. No animals died prematurely. Body weight losses were seen in week 1 in all animals receiving 3000 ppm; the losses were greater in the first part of the week (days 0–3) than in the second part (days 3–7). Small weight gains were seen in these animals in week 2. Over the two-week period animals receiving 3000 ppm did not regain their initial body weight and overall losses were recorded (−9% for the males and −7% for the females). In addition, overall body weight gains were also low for males receiving 540 ppm (78% of the control value). No effect of treatment was seen on the body weight gains of animals receiving 100 ppm or of females receiving 540 ppm. Food consumption was markedly reduced compared with controls during the first week of treatment for animals receiving 3000 ppm; marginally reduced consumption was seen in males receiving 100 ppm and in males and females receiving 540 ppm. Low food intake continued during week 2 for animals receiving 3000 ppm, though the magnitude of the effect was less. Animals receiving 540 or 3000 ppm scattered more food than the controls in week 1 whilst in week 2 large amounts of scattered diet were only recorded for males receiving 3000 ppm. This indicated that there was an initial palatability problem with the treated diets at the higher concentrations. The amount of food eaten by females receiving 100 ppm was similar to that of the controls.

In-the-hand observations were unaffected by treatment. At arena observations, elevated gait (slight) was seen in week 2 in three males receiving 3000 ppm. This finding is occasionally reported in control animals and is thought unlikely to be related to treatment. Sensory reactivity findings and grip strength values were unaffected by treatment. Motor activity scores showed considerable intragroup and intergroup variation during week 2 of treatment; the differences could not be unequivocally attributed to treatment. High and low beam scores in all treated female groups were low when compared with controls, at 6 and 12 minutes (low beam scores) and 6, 12 and 18 minutes (high beam scores). However, in the absence of any pretreatment data, and given the flat response across all groups, the small number of animals in each sex/group and the absence of a similar effect in the males, it is difficult to confidently state that there is an effect on motor activity in these animals.

Table 54. Mean motor activity (high and low beam scores) for females during week two of a preliminary, sub-chronic neurotoxicity study in rat

		Test time point (minutes)										
Level (ppm)	**Beam level**	**6**	**12**	**18**	**24**	**30**	**36**	**42**	**48**	**54**	**60**	**Total**
Control	High	57.4	39.6	25.6	9	7	17.2	6.2	4.4	4.6	2	173
100	High	**29.2***	23.4	15.2	16	5.8	4.6	8.4	3.6	0.8	2.4	109.4
540	High	**20.6****	28.6	19	15.6	2	0.6	2.4	3	3.4	5	100.2
3000	High	**19.2****	26.8	18.8	7.4	6.2	1.4	3.8	2	3.8	4.4	93.8
Control	Low	259.4	199	125	83	68.8	94	47.8	32.8	30.6	23.6	964
100	Low	164.8	120.6	107.4	123.2	54	48.8	40.8	46.4	16.8	12.6	735.4
540	Low	**141.0***	143.2	129.8	109.8	28.2	4.6	20.8	24.8	33.8	27.8	663.8
3000	Low	**137.6****	132	132	47.8	52.8	6.8	7.8	57.8	67.8	39.6	682

* $p < 0.05$ ** $p < 0.01$

Evaluation of the organ weight data revealed statistically significant changes in many of the absolute organ weights of animals which had received 3000 ppm and, to a lesser extent, in males which had received 540 ppm. These changes were attributed to the marked effect of treatment on body weight and included variations seen in brain, adrenal, epididymides, heart, lungs, testes, thymus and thyroid weights. In addition, uterus weights were low in several females treated at 3000 ppm, though statistical significance was not attained and this change was also attributed to the effect of treatment on body weight. Slightly higher kidney weights (relative to body weight) were recorded for males given 3000 ppm and relative liver and spleen weights on the same basis were high in both sexes administered 3000 ppm. The organ weights of animals given 100 or 540 ppm were considered unaffected by treatment.

Macroscopic examination at necropsy revealed treatment-related findings in the livers of animals given 3000 ppm. These findings included pale liver, lobular pattern, accentuated and enlarged liver. The thin build observed on living animals receiving 3000 ppm was confirmed at necropsy. Small prostate and seminal vesicles were recorded for 4/5 males given 3000 ppm and for 1/5 males given 540 ppm. Thin uterus was recorded in 4/5 females given 3000 ppm and this finding correlated with the low uterus and cervix weights reported for these animals (these low weights were attributed to the effect seen on body weight). The macroscopic changes seen in the male reproductive organs were also considered to be related to the reduction in body weight seen in these animals.

Key findings for this preliminary neurotoxicity study in rat are shown on the following page in Table 55.

Table 55. Key finding in neurotoxicity study

Group/sex	1/M	2/M	3/M	4/M	1/F	2/F	3/F	4/F
Level (ppm)	**0**	**100**	**540**	**3000**	**0**	**100**	**540**	**3000**
Number of animals	5	5	5	5	5	5	5	5
Mean body weight gain (g)								
Days 0–3	27	24	**19****	**−23****	9	10	7	**−17****
[SD]	[3.8]	[2.0]	**[2.5]**	**[3.7]**	[3.3]	[2.5]	[4.0]	**[1.0]**
Days 3–7	31	32	25	**−4****	12	11	13	**−2****
[SD]	[4.8]	[4.2]	[2.2]	**[2.7]**	[3.5]	[1.6]	[2.3]	**[2.2]**
Days 0–14	111	106	**87***	**−17****	35	38	39	**−11****
[SD]	[12.4]	[7.2]	**[7.1]**	**[2.1]**	[8.0]	[6.2]	[8.3]	**[4.0]**
Organ weight; absolute (g)								
Brain	1.99	1.98	1.98	**1.86***	1.8	1.8	1.84	**1.66***
Adrenals	0.047	**0.039***	**0.038****	**0.034****	0.054	0.49	0.054	**0.045****
Epididymides	0.536	0.525	**0.441****	**0.345****				
Heart	1.211	1.132	**1.037***	**0.703****	0.748	0.789	0.776	**0.619****
Lungs and bronchi	1.605	1.511	1.451	**1.055****	1.104	1.14	1.098	**0.875****
Testes	3.22	2.99	3	**2.41***				
Thymus	0.489	0.546	0.57	**0.345***	0.47	0.505	0.43	**0.315***
Thyroids (with paras)	0.013	0.014	0.012	**0.009***	–	–	–	–
Uterus and cervix	-	-	-	-	0.431	0.501	0.375	0.163
Organ weight; relative (g)								
Kidneys	0.856	0.858	0.851	**0.929***	0.938	**0.841***	0.87	0.892
Liver	4.889	4.822	4.76	**6.501***	4.162	4.068	4.718	**6.605****
Spleen	0.2549	0.2341	0.2283	**0.4006****	0.2462	0.2749	0.2428	**0.3387****
Macroscopic findings								
Liver:								
pale	0	0	0	3	0	0	0	0
lobular pattern accentuated	0	0	0	2	0	0	0	2
enlarged	0	0	0	**5****	0	0	0	**4***
Prostate: small	0	0	1	**4***	-	-	-	-
Seminal vesicles: small	0	0	1	**4***	-	-	-	-
Uterus: thin	-	-	-	-	0	0	0	**4****

SD Standard deviation

Significant when compared with control: * $p < 0.05$ ** $p < 0.01$

It was concluded that the administration of *cis*/*trans*-metconazole to rats for two weeks via the diet at concentrations of up to 3000 ppm (equivalent to 216.8 mg/kg bw per day in males and 206.1 mg/kg bw per day in females) did not result in any neurotoxicity, although at 3000 ppm the maximum tolerated dosage was exceeded. The NOAEL in this study was 100 ppm (equivalent to 11.0 mg/kg/day in males and 10.6 mg/kg/day in females). The findings from this study demonstrated that the highest dietary concentration in the main four-week study should be in the region of 540 ppm and the lowest concentration should be below 100 ppm (Cooper, 2002a, 2015a).

Main study

A four-week sub-chronic neurotoxicity study in rats with *cis*/*trans*-metconazole (purity 98.99%; *cis*:*trans* 85:15; lot/batch 9Z521) was conducted by another metconazole-supporting company. A detailed study summary was made available to BASF.

The neurotoxic potential of the test substance *cis*/*trans*-metconazole to Sprague Dawley Crl:CD® (SD) rats by dietary administration was assessed over a period of four weeks. Three groups of 10 male and 10 female rats received *cis*/*trans*-metconazole in the diet at concentrations of 50, 170 or 500 ppm for four weeks and a similarly constituted control group received an untreated diet throughout the treatment period. Achieved dosages at dietary concentrations of 50, 170 and 500 ppm were 4.84, 15.69 and 47.08 mg/kg bw per day for males and 5.10, 17.62, and 49.82 mg/kg bw per day for females receiving 50, 170 or 500 ppm, respectively. During the study clinical condition, body weight, food consumption, food conversion efficiency, neuro-behavioural investigations, brain anatomical measurements and weight, macro-pathology and neuro-histopathology investigations were undertaken. Neuro-behavioural investigations (assessment in the hand, in a standard arena, manipulations and motor activity measurements) were performed on all animals before treatment commenced and in week 4.

The appearance and behaviour of the animals at routine examinations were unaffected by treatment. One male receiving 500 ppm (no. 33) had a palpable swelling on the right lower dorsal area; in-life signs were restricted to the FOB results. The animal was limping from week 1 and had low activity scores throughout. From week 3 onwards the animal was hunched, and it had an elevated gait during week 4. The swelling affected landing foot-splay results; the week 4 score was low when compared with the pre-treatment result. Macroscopic and microscopic findings for this animal indicated that it had an abscess of the muscle adjacent to the right hip joint with minimal dermal haemorrhage and slight subcutaneous inflammation. In the absence of any similar finding in any other animals, these findings are considered incidental and not related to treatment, rather they related to the individual animal's condition involving a swollen, subcutaneous/intramuscular lesion identified as an abscess.

No animals died prematurely. When compared with the controls, low body weight gains were observed in week 1 in males receiving 170 ppm and in animals receiving 500 ppm. At the highest concentration the effect was more marked in females than in males. Subsequent body weight gain was considered unaffected by treatment. As a result of the effect in week 1, the overall weight gain of females receiving 500 ppm was lower than that of the controls. Body weight gains of animals receiving 50 ppm and of females receiving 170 ppm were considered unaffected by treatment. Animals receiving 170 or 500 ppm consumed less food than the controls in week 1; in females receiving 500 ppm this effect persisted throughout the treatment period, and consequently the total food intake of these animals was low compared to controls. Food scatter values were generally higher than the controls in treated male groups but the effect across the groups was not always dosage-related. In females the treated groups scattered more food than the controls in week 1 but thereafter scatter values were variable throughout the groups and similar to controls. When compared with the controls, food conversion efficiency was low in week 1 for males receiving 170 ppm and for animals receiving 500 ppm. It was also low in week 2 for females receiving 170 or 500 ppm. Slightly low overall food conversion efficiencies were recorded for females receiving 170 or 500 ppm.

In-the-hand observations were unaffected by treatment. Arena observations showed some inter-group variation but there were no treatment-related changes. Rearing counts during weeks 1 and 2 in treated males were lower than those for the controls but this reflected a trend that was apparent before treatment commenced and was not evident in the motor activity assessments. Consequently, this was not attributed to treatment. Manipulations and motor activity were unaffected by treatment.

Macroscopic examination of the animals killed on completion of the treatment period revealed no treatment-related findings.Absolute brain weights were unaffected by treatment. The anatomical measurements made of the brain did not indicate any treatment-related differences between the control and treated animals. There were no microscopic pathology findings considered to be related to treatment with *cis*/*trans*-metconazole.

Key findings for the main neurotoxicity study in rat are shown on the following page in Table 56.

Table 56. Key finding in neurotoxicity study

Group/sex	1/M	2/M	3/M	4/M	1/F	2/F	3/F	4/F
Level (ppm)	**0**	50	170	500	**0**	50	170	500
Number of animals	5	5	5	5	5	5	5	5
Mean body weight gain (g)								
Week 0–1	51	53	46	**41***	22	22	20	**13***
Weeks 1–4	96	90	**78***	100	54	53	48	49
Weeks 0–4	147	144	124	141	77	75	68	62
Mean food consumption and % food conversion efficiency								
Week 1 (g)	196	199	189	184	141	140	130	126
Week 1 (%)	26.1	26.7	24.2	22.1	15.9	15.9	15.3	10.2
Week 2 (g)	-	-	-	-	146	141	144	137
Week 2 (%)	-	-	-	-	16	15	12.6	12.5
Weeks 1–4 (g)	-	-	-	-	579	570	565	531
Weeks 1–4 (%)	-	-	-	-	13.3	13.2	12.0	11.7
Rearing count								
Prior to treatment	11.3	9.9	9.7	8.5	-	-	-	-
Week 1	5.9	**2.5****	**2.5****	**2.5****	-	-	-	-
Week 2	5.6	2.6	2.7	2.5	-	-	-	-

Significant when compared with control: * $p < 0.05$ ** $p < 0.01$

It was concluded that the administration of *cis*/*trans*-metconazole to Sprague Dawley rats via the diet, at concentrations up to 500 ppm (equivalent to 47.08 mg/kg bw per day in males and 49.82 mg/kg bw per day in females) produced no evidence of neurotoxicity. Non-specific toxicity was evident at 170 and 500 ppm in both sexes. The NOAEL for neurotoxicity in this study was considered to be at least 500 ppm (equivalent to at least 47.08 mg/kg bw per day in males and 49.82 mg/kg bw per day in females). The NOAEL for the general toxicity was 50 ppm (equivalent to 4.84 mg/kg bw per day in males and 5.10 mg/kg bw per day in females), based on reduced food consumption and reduction in body weight gain (Cooper, 2002b, 2015b).

(b) Immunotoxicity

An immunotoxicty study in rats with *cis*/*trans*-metconazole (purity 99.7%; 84.6 *cis*, 15.1 *trans*; batch 1362353) was conducted for non-EU authorities by another metconazole-supporting company. A detailed study summary was made available to BASF and is part of this submission.

The immunotoxic potential of *cis*/*trans*-metconazole in male Wistar rats was analysed using dietary dose levels of 0, 70, 210 and 630 ppm (corresponding to mean intake levels of 5.4, 17 and 52 mg/kg bw per day, respectively) for 28 days. The parameters used for detection of potential test substance-related alterations in the morphology of the immune system included: the determination of lymphoid organ weights (spleen and thymus) and; the analysis of the primary humoral immune response (IgM response) to sheep red blood cells (sRBCs). No clinical signs were observed throughout the study. No mortality was observed in this study. No test substance-related findings were observed. No treatment-related effects on water consumption were noted.

The mean daily test substance intake in mg/kg bw per day over the entire study period was calculated. Body weight in animals of test group 3 (630 ppm) was slightly (but not significantly) lower from day 7 until day 28, with a maximum on day 14 of −3.7% compared to control animals. Body weight for animals treated with cyclophosphamide (CP) was slightly (but also not significantly) lower from day 7 until day 28, with a maximum of −5.5% on day 28. Body weight change in animals from test group 3 (630 ppm) was lower from day 7 until day 28 (significantly lower on day 7 (by 15.9%, compared to controls) and on day 14 (by 14.7% compared to controls). Body weight change of animals treated with CP was significantly decreased from day 7 until day 28, with a maximum of −13.8% on day 21.

Six days after immunization, no changes in the sRBC IgM titres were found in male rats dosed with the test substance, whereas the sRBC titres were significantly lower in rats of test group 4 (CP, the positive controls).

The absolute mean weights of spleen and thymus of animals in test groups administered 70, 210, and 630 ppm did not show relevant differences compared to the control group. The positive control group (CP) revealed significant decreases in spleen and thymus weights, which corresponded to the expected result. The relative mean weights of spleen and thymus of animals in test groups at 70, 210 and 630 ppm did not show relevant differences compared to the control group. The positive control group (CPA) revealed significant decreases in relative spleen and thymus weights, which corresponded to the expected result.One animal from the positive control group (CP) revealed a reduced thymus size. No gross lesions were observed in any other test animals.

Table 57. Key findings for an immunotoxicity study in rat

Treatment	**Metconazole (ppm)**				**CP (mg/kg)**
Dose level	**0**	**70**	**210**	**630**	**4.5**
Food consumption per cage (g)					
Days 6 to 7	19.69	19.55	19.98	21.41	21.4
Days 13 to 14	19.77	20.09	20.95	19.79	21.11
Days 20 to 21	20.01	20.68	21.2	20.92	20.96
Days 27 to 28	20.94	21.3	23.29	22.62	19.74
Body weight (g)					
Day 0	177.68	178.82	179.46	179.93	178.19
Day 7	220.72	220.9	220.61	216.15	216.86
Day 14	257.51	257.76	259.55	248.05	248.31
Day 21	286.81	286.59	290.69	279.68	272.31
Day 28	312.32	312.98	319.01	304.43	295.21
Day 28 (% of control)	100	100.21	102.14	97.47	94.52
Body weight gain (g)					
Days 0–7	43.05	42.07	41.15	36.23**	38.68*
Days 0–14	79.84	78.94	80.09	68.12*	70.12*
Days 0–21	109.14	107.76	111.32	99.75	94.12*
Days 0–28	134.65	134.15	139.55	124.5	117.02*
Specific IgM titre (U/mL)					
Mean ± SD	2102 ± 1722	2124 ± 1248	1581 ± 707	1917 ± 867	**584** ± 230**
Median	1206	1974	1387	1952	526
Organ weights					
Terminal body weight (g) [% of control]	291 [100]	292 [100]	296 [102]	283 [97]	273 [94]
Spleen, absolute weight (g) [% of control]	0.551 [100]	0.579 [105]	0.561 [102]	0.586 [106]	0.393** [71]
Spleen, relative weight (%) [% of control]	0.19 [100]	0.198 [104]	0.189 [99]	0.207 [109]	0.144** [76]
Thymus, absolute weight (mg) [% of control]	489 [100]	440 [90]	509 [104]	459 [94]	241** [49]
Thymus, relative weight (%) [% of control]	0.168 [100]	0.15 [90]	0.171 [102]	0.162 [97]	0.088** [52]

CP Cyclophosphamide SD Standard deviation

Under the conditions of the study metconazole did not reveal any signs of immunotoxicity when administered via the diet over a period of four weeks to male Wistar rats. The NOAEL for the immunotoxicologically relevant endpoints was 630 ppm (52 mg/kg bw per day),the highest dose tested). The NOAEL for systemic toxicity was 210 ppm (17 mg/kg bw per day). The oral administration of the positive control substance cyclophosphamide (4.5 mg/kg bw per day) led to severe findings indicative of immunotoxicity. This was represented by significantly lower sRBC IgM antibody titres as well as reduced spleen and thymus weights. Thus, assay sensitivity was verified in the present immunotoxicity study (Buesen, 2010, 2014)

(c) Mechanistic studies and position paper

(i) Enzyme induction studies

Eight male rats/dose per time point (F344 Fisher) and eight female CD-1 mice/dose per time point were fed a diet of metconazole (purity 94.2%; lot/batch ST90/369) at a dose level of 0 and 300 ppm (mice) or 0 and 1000 ppm (rats) during 7 or 28 days. Achieved doses for mice at 300 ppm were 62.5 mg/kg bw per day (days 0–7) and 58.3 mg/kg bw per day (days 0–28). Achieved doses for rats at 1000 ppm were 102.7 mg/kg bw per day (days 0–7) and 86.7 mg/kg bw per day (days 0–28). As a positive control, eight male rats and mice were fed a diet of phenobarbital at a dose level of 0.05% for 28 days. After treatment, 2/8 animals were anaesthetised with barbiturate, their livers perfused with glutaraldehyde, removed and processed for electron microscopical examination . The other animals were sacrificed by cervical dislocation, and livers were processed for biochemical analysis.

No mortality was observed. No relevant clinical signs were observed. On day 7, body weights of rats were reduced compared to controls (−5%, $p < 0.05$); the difference was maintained until day 28 of treatment. In mice, treatment did not affect body weight.

Relative (r), but not absolute (a), liver weight was increased in rats on day 7 (+9%; $p < 0.01$) and on day 28 (+4%, $p < 0.05$). In mice, both absolute and relative liver weights were increased on day 7 (a +20%, $p < 0.05$%; r +22%, $p < 0.001$), and on day 28 (a +12%; r +13%, $p < 0.01$). Phenobarbital treatment resulted in a significant increase of liver weight (a +41%; r +31%).

Table 58. Key findings in mechanistic study

Histopathological findings in the liver of rats and mice treated with metconazole and phenobarbital (Incidence from six **animals treated)**

Endpoint	Rat			Mouse		
	Control	Metconazole 1000 ppm	Phenobarbital 0.05%	Control	Metconazole 300 ppm	Phenobarbital 0.05%
Inflammatory cell foci	1	0	1	0	2	0
Midzonal vacuolization	0	5	1	0	3	0
Diffuse vacuolization	0	0	0	0	1	0
Centrilobular hypertrophy	0	0	5	0	1	6

Biochemical analysis of liver microsomes from rats and mice treated with metconazole and phenobarbital (% of control)

End-point	Rat			Mouse		
	Metconazole		Phenobarbital	Metconazole		Phenobarbital
	day 7	day 28	day 28	day 7	day 28	day 28
Protein content	117*	139***	146***	114*	124***	128**
CYP450 content	154***	140***	197***	152***	139***	146***
EMND (CYP3A)	149***	141***	172***	139***	159***	213***
ECOD (CYP2B)	173***	178***	439***	156**	143***	277***
EROD (CYP1A1)	114*	-	300***	-	-	184***
LA-11-H	-	-	189***	-	140**	165***
LA-12-H	-	-	130*	-	-	-

Statistical evaluation: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Metconazole (*cis*), administered over 7 days or 28 days in rats and mice induced some CYP450-dependent isoenzymes in a pattern similar, but not identical to, phenobarbital. In contrast to phenobarbital the compound induced some midzonal vacuolation in both rats and mice, and unlike phenobarbital, the test compound did not induce centrilobular hypertrophy. This was in line with the findings in the full 28-day feeding study, where this effect was observed at a 10-times higher dose, where the administration of 1000 ppm of the *cis*/*trans* isomer over 28 days induced such an effect predominantly in female rats (Worrell, 1991).

Metconazole (purity 98.53%; *cis*:*trans* 82.68%:15.85%; batch 9Z521) was administered via the diet to groups of 18 female Wistar rats at dose levels of 30, 300, and 1000 ppm for 3,7, or 14 days; average active substance intakes (averaged over week 1 and week 2) were: 0, 4.5, 47.6 and 151 mg/kg bw per day. At the end of the treatment, the animals were killed and subjected to blood sampling, necropsy, measurement of liver weight, hepatocellular proliferation, histopathology, and reactive oxygen species (ROS) production in the liver (oxidative stress markers). Interim kills of six animals per group were performed after three and seven days of treatment and subjected to necropsy, measurements of organ weights, cell proliferation, and hepatic drug-metabolizing enzymes (this last at 7 days only).

Statistically significant changes were observed in clinical chemical parameters at mid and top doses. These changes were considered to be treatment-related as similar effects have been observed in previous repeated-dose toxicity studies. In the 30 ppm group, there were no significant changes in any parameters. Dose-dependent increased absolute and relative liver weights were observed at 300 ppm and 1000 ppm. In the 30 ppm group, no abnormalities were observed in the liver weights for animals at either scheduled sacrifice. Slight to moderate diffuse hepatocellular hypertrophy and slight hepatocellular vacuolation (demonstrated to be lipid droplets by oil red O stain) was noted at the mid and top doses. In the 30 ppm group no treatment-related histological changes were observed.

In the 300 and 1000 ppm group ethoxycoumarin *O*-dealkylase (ECOD: CYP1A1, 1A2, 2B) activity, and pentoxyresorufin *O*-dealkylase (PROD: CYP2B) activity were significantly increased when compared to controls. With the 30 ppm group, there was no significant change in any parameter. Contents of cytochrome P-450 isoenzymes were significantly increased compared to controls at mid and top dose, especially with a marked elevation in CYP2B (11.5-fold compared to controls) at the top dose. In the 30 ppm group there was no significant change in any cytochrome P-450 isoenzyme content. Increased proliferating cell nuclear antigen labelling index (PCNA LI), a marker for cell proliferation activity, was significantly increased at 1000 ppm compared to control values, after three days (850%), seven days (600%), and to a lesser extent after 14 days (300%, not significant). These transient effects were evaluated as typical effects for a non-genotoxic mitogenic hepatocarcinogen (similar to phenobarbital), which enhances cell proliferation at the beginning of the treatment but this returns to normal thereafter although treatment may be continued. No significantly increased PCNA indices were seen in the liver at 30 ppm dietary concentration.

In 1000 and 300 ppm groups, lactoperoxidase (LPO) was significantly increased when compared to controls, but there were no significant differences in 8-oxo-2′-deoxyguanosine (8-oxo-dG) between the treated and control groups. In the 30 ppm group there were no significant changes in any parameter. Thus, metconazole is considered to lead to increased ROS levels, which are generated secondarily during metabolic microsomal enzyme induction, that is not sufficient to also induce oxidative DNA damage.

Table 59. Key finding in mechanistic study with female rats

Dose (ppm)		**0**	**30**	**300**	**1000**
Clinical chemistry					
Aspartate transaminase, AST		100	115	115	**194****
Alanine transaminase, ALT		100	129	129	**254****
Total cholesterol		100	102	74	**44****
Total bilirubin		100	111	**78****	**67****
Liver weight ± SD [***as % of control***]					
Absolute (g)	3 days	1.36 ± 0.18	1.36 ± 0.31 [100%]	1.61 ± 0.15 [118%]	2.07 ± 0.15** [152%]
	7 days	1.51 ± 0.06	1.43 ± 0.19 [95%]	1.68 ± 0.22 [111%]	2.04 ± 0.21** [135%]
	14 days	1.44 ± 0.14	1.46 ± 0.11 [101%]	1.57 ± 0.18 [100%]	2.07 ± 0.55 [144%]
Relative to body weight (%)	3 days	4.55 ± 0.33	4.61 ± 0.65 [101%]	5.11 ± 0.18 [112%]	6.75 ± 0.54** [100%]
	7 days	4.65 ± 0.16	4.52 ± 0.38 [97%]	5.31 ± 0.63 [114%]	6.79 ± 0.59** [146%]
	14 days	4.27 ± 0.36	4.52 ± 0.43 [106%]	4.99 ± 0.27 [117%]	6.67 ± 0.78** [156%]
Liver macro/hystopathological finding					
Liver enlargement	3 days	0/6	0/6	0/6	6/6
	7 days	0/6	0/6	0/6	6/6
	14 days	0/6	0/6	0/6	5/6
Hepatocellular hypertrophy	14 days	0/6	0/6	6/6	6/6
Hepatocellular vacuolation	14 days	0/6	0/6	2/6	5/6
Hepatic microsomal CYP content, enzyme activities and markers					
CYP content		100	117	**207***	**307***
ECOD		100	115	**206***	**311****
PROD		100	136	**379****	**448****
CYP P-450 isozyme content	CYP1A	100	99	222	**451****
	CYP2B	100	162	**396***	**1149****
	CYP3A	100	82	**251****	**395****
PCNA LI in the liver	3 days	100	100	**150**	**850****
	7 days	100	100	**200**	**600****
	14 days	100	{400} §	100	300
LPO (nmol/g tissue)		100	145	**261****	**231***
8-oxo-dG (ng/mg DNA)		100	94	91	92

§ Increase caused by two outliers

Statistically significant modification: * $p \leq 0.05$ ** $p \leq 0.01$

Based on the results described above, it has been suggested that metconazole is a hepatic drug-metabolizing enzyme inducer primarily via CYP2B, similar to phenobarbital and has a potential to enhance transient cell proliferation (mitogenic activity) like a known non-genotoxic mitogenic hepatocarcinogen.

The occurrence of hepatocellular tumours observed in female mice at concentrations of 300 ppm and 1000 ppm in a long-term oral carcinogenicity study is considered to be via a phenobarbital-like mechanism. In the present study, the NOAEL of the test substance was 30 ppm (4.49 mg/kg bw per day), based on the threshold of its effects on hepatic microsomal enzyme induction, cell proliferation, and ROS production (oxidative stress). (Harada, 2004)

(ii) QSAR-binding affinity

As with many other pesticides and chemicals, metconazole is part of the US ToxCast program. Several assays react to varying doses of metconazole, however no conclusive picture has emerged. The publications discussed below relate to CYP-binding affinity and estrogen receptor signaling response.

Azole compounds, such as metconazole, are known to be metabolized by CYP isozymes, including CYP3A4 and CYP2B6. Binding affinity of azoles can be measured by UV spectral analysis, as the nitrogen atom in the azole ring induces a typical UV spectral change with a characteristic absorption at 430 nm (maximum) and 400 nm (minimum). In this study the binding affinities of metconazole and other azoles were determined in rat liver CYP2B and CYP3A4 using spectral analysis followed by further QSAR (quantitative structure–activity relationship) analysis using hydrophobicity and binding affinity as parameters. Binding affinities observed with CYP2B and CYP3A using metconazole did not differ significantly. The pIC_{50}s and pK_D were 6.13 and 5.98 for CYP2B and 6.02 and 6.00 for CYP3A4, respectively. Good correlation with the bilinear model was observed between the binding affinities and the partition coefficient (log *P*). The model suggested that the optimum log *P* values of the azole compounds were nearly the same for these two CYPs. The sequence homology of amino acid residues around the substrate recognition site is significantly high between CYP2B and CYP3A. It was reported that the size of the binding pocket in CYP2B and CYP3A are not very different. These observations explain why the optimum log *P* values in the correlation equations for CYP2B and CYP3A are nearly the same (Itokawa, 2006).

In the previous study from Itokawa (2006) it was shown that the binding affinity of 18 azole compounds for rat CYP2B and CYP3A was nicely expressed by the bilinear model of log *P*. In this study the same azole compounds were examined as to their inhibitory effect on the substrates for human CYP2B6 and CYP3A4. Enzyme inhibition assays using 7-ethoxy-4-trifluoromethylcoumarin (EFC) and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as substrates for CYP2B6 and CYP3A4, respectively, were performed using high-throughput kits. The inhibitory concentration pIC_{50} was 4.87 for CYP2B and 5.8 for CYP3A4. The inhibitory activity determined was analysed as to the molecular properties of the azole compounds. A close correlation was found with the bilinear model of log *P*. These results suggested that the molecular hydrophobicity of the azole compounds plays a major role in inhibition as well as in binding. For the binding, the highest occupied molecular orbit (HOMO) was significant as an additional descriptor in the correlation equations, whereas the existence of a hydroxyl group was significant for inhibition (Itokawa, 2007).

(iii) QSAR-genotoxicity

For metconazole, the potential for presence of structural alerts was evaluated with different QSAR models.Models used were the OASIS TIMES and VEGA (CAESAR, ISS and SarPy).

Results showed that the assessment was in domain negative for Ames and in vivo micronucleus test (MNT), indicating no genotoxic potential (MultiCASE, 2015a, b, c, d, 2017, 2018a, b; Anonymous, 2018; Van Cott & Kemeny, 2018a).

A summary of genotoxicity studies on metconazole is shown on the following page in Table 60.

Table 60. Metconazole QSAR assessment

Active Substance	QSAR tools applied	Structural alerts for genotoxicity	Reliability/ Total domain	Evaluation
cis isomer: Reg. No. 4079468 *trans* isomer: Reg. No. 4079654	OA	Ames parent: negative	Low/Out of domain	Not genotoxic
		Ames metabolites: negative		
		CA parent: negative	Low/Out of domain	
		CA metabolites: 2/16 positive		
	VE	CAESAR: not mutagenic	Moderate/Could be out of domain	
		ISS: not mutagenic	Low/Out of domain	
		SarPy: not mutagenic	Moderate/Could be out of domain	
	CU	Ames: negative	High/In domain	
		In vivo MNT: known negative	High/In domain	
	ToxTree	In vivo MNT: known negative	High	

MNT Micronucleus test

(iv) Investigation of hormonal activity

cis/*trans*-Metconazole (purity 98%; *cis*/*trans* 82.7:17.3; batch 14955000), *cis*-metconazole (purity 100%; *cis*/*trans* 97.2:2.8; batch 3) and *trans*-metconazole (purity 100%; *cis*/*trans* 0.8:99.2; batch 1) were tested in vitro for their effect on human and rat aromatase activities (CYP 19). Human/rat CYP 19 supersomes (aromatase + reductase) were exposed to the test, positive and negative control substances at concentrations ranging from 10^{-4} to 10^{-13} M as well as to the solvent DMSO. Enzyme activity was determined fluorometrically using dibenzylfluorescein (DBF) as a model substrate. Resulting activity values were fitted using the 4-parameter regression model to yield a sigmoidal inhibition curve, and IC_{50} values were calculated.

The assay was conducted in a 96-well microplate utilizing the recombinant human or rat aromatase and the fluorometric artificial substrate DBF. In addition to the control substances recommended in the guideline there were included further additional positive controls: 4-hydroxy androstenedione (ASDN), fenarimol, econazole nitrate and letrozole. Similarly, negative controls: atrazine and bis(2-ethylhexyl) phthalate. These controls have been carried out in every test run, in parallel with the test compounds using a plate set up.

Metconazole technical (*cis*/*trans* mixture) shows aromatase inhibition on the human enzyme with an IC_{50} of 0.721 µM and does not differ significantly from the inhibition response of the pure *cis* isomer (IC_{50} = 0.569 µM). The *trans* isomer shows about 3.4 to 4.3-times less human aromatase inhibition compared to the *cis*/*trans* mixture or the *cis* isomer, having an IC_{50} of 2.47 µM. *cis*/*trans*-Metconazole has a comparable effect on human aromatase activity as the weakest positive control fenarimol and compared to the other azole substances tested within this study the three test substances are thousands-fold less potent than letrozole or econazole. In relation to the positive substances analysed within this study, *cis*/*trans*-metconazole has a comparable effect on rat aromatase activity to the weakest positive control fenarimol, and compared to other azole substances tested within this study the three test substances are hundreads-fold less potent than letrozole or econazole.

All three test compounds analysed within this study revealed stronger inhibition of rat aromatase than of human enzyme.

Key results for inhibition studies on rat and human aromatase are shownon the following page in Table 61.

Table 61. Aromatase IC_{50} data for isomers of metconazole

Test item	Human aromatase IC_{50} (M)		Rat aromatase IC_{50} (M)	
	mean	SE	mean	SE
Test substances				
Metconazole, *cis*	5.69×10^{-7}	6.64×10^{-8}	2.23×10^{-7}	2.54×10^{-8}
Metconazole, *cis*/*trans*	7.21×10^{-7}	1.18×10^{-7}	1.57×10^{-7}	2.28×10^{-8}
Metconazole, *trans*	2.47×10^{-6}	3.07×10^{-7}	5.79×10^{-7}	1.21×10^{-7}
Positive controls				
Letrozole	9.02×10^{-10}	8.03×10^{-11}	1.53×10^{-9}	1.03×10^{-10}
Econazole	2.30×10^{-9}	2.39×10^{-10}	1.60×10^{-9}	1.52×10^{-10}
4-Hydroxy androstenedione, ASDN	1.38×10^{-8}	2.22×10^{-8}	3.57×10^{-8}	3.48×10^{-9}
Fenarimol	1.26×10^{-6}	1.74×10^{-7}	1.79×10^{-7}	2.15×10^{-8}

SE Standard error

Under experimental conditions chosen, *cis*/*trans*-metconazole, *cis*-metconazole and *trans*-metconazole inhibited rat and human aromatase activities. The resulting human aromatase IC_{50} values were 0.721 µM, 0.569 µM and 2.47 µM for *cis*/*trans*-, *cis*- and *trans*-metconazole respectively. The resulting rat aromatase IC_{50} values were 0.157 µM, 0.223 µM and 0.579 µM for *cis*/*trans*-, *cis*- and *trans*-metconazole respectively. Aromatase inhibition was more pronounced for the rat enzyme and was 4.6-, 2.6- and 4.3-fold lower for the human enzyme by *cis*/*trans*-, *cis*- and *trans*-metconazole respectively (Mentzel, 2015).

A mechanistic one-generation study measuring serum steroid hormone concentrations and hepatic drug-metabolizing enzyme levels during late gestation in rats following exposure to metconazole KNF-474m (*cis*/*trans*) was conducted by another metconazole-supporting company.

Groups of 24 female Crj:CD(SD)[IGS] rats were given diets containing metconazole (purity 98.99%; *cis*:*trans* 83.13:15.86; lot/batch 9Z521) at concentrations of 0, 30, 150, or 750 ppm (corresponding to 0 1.82, 8.89, and 43.0 mg/kg bw per day) for three weeks and mated with normal untreated males of the same strain. Treatment of females with metconazole was continued during gestation. It has been reported in rats that towards the end of the gestation period increased serum estradiol concentrations and an increased concentration ratio of 17β-estradiol to progesterone (E:P ratio) are required to trigger parturition due to a decrease in progesterone concentrations with regression of corpora lutea from GD 19 onward. Therefore these females were euthanized on GDs 19 or 21, and their serum sex steroid hormone concentrations and hepatic drug-metabolizing enzyme levels were measured. On GD 19 or 21 blood samples were collected from all females in the afternoon by decapitation. For each dose group, serums from eight females, in the ascending order of the animal number, were analysed for 17β-estradiol and progesterone concentrations using radioimmunoassay. The E:P ratio was calculated for each female. The frozen livers of the females selected for serum hormone measurement were homogenized and centrifuged to form microsome pellets and cytochrome P-450 isozymes, CYP1A1, CYP2B1, CYP3A2, and CYP4A1 were determined using Western blot assays. The chromophoric band corresponding to the isozyme was quantified for its density using a GS-700 Imaging Densitometer (Nippon Bio-Rad Laboratories KK). CYP isozyme content in total microsomal protein (pmol/mg protein) was calculated by the linear regression of the calibration curve derived from the standard molar solution of the isozyme.

There were no deaths among females, up to 750 ppm, the highest dose tested and no treatment-related signs of toxicity up to 750 ppm.

In the 750 ppm group, mean body weights and body weight gains of females were consistently and significantly lower than those in the control group throughout the study period. Reductions in mean body weight gain of 31.5% and 17.5% were recorded during the pre-mating and gestation periods, respectively.

Mean food consumption of females in the 750 ppm group was significantly lower compared with the control group at treatment week 1 and consistently lower during the gestation period.

Mating and fertility were unaffected by treatment.

In the 750 ppm group, mean numbers of corpora lutea, implantations and live fetuses were significantly lower while mean percent resorption and fetal deaths were significantly higher than the corresponding controls.

Table 62. Key findings of one-generation mechanistic study in rat

Parameter		Dose level (ppm)			
		0	**30**	**150**	**750**
Number of animals per group		24	24	24	24
Mortality		0	0	0	0
Clinical signs		No treatment-related effects			
Body weight (g)					
Pre-mating:	week 0	228	228	228	228
	week 1	246	248	247	**234****
	week 2	260	264	262	**246****
	week 3	264	271	266	**252***
Gestation:	day 0	274	280	277	**260****
	day 7	309	314	311	**284****
	day 14	343	348	346	**313****
	day 19	404	413	408	**357****
	day 21	430	432	432	**393****
Food consumption (g/animal/day)					
Pre-mating:	week 1	15.8	16.7	16.2	**13.9****
	week 2	16.5	**17.6***	19.9	15.77
	week 3	15.6	**16.7***	15.8	15
Gestation:	days 0–7	19.7	20	19.8	**16.2****
	day 14	20.9	21.6	21.1	**18.3****
	day 19	21.9	22.4	21.2	**19.3***
	day 21	21.1	21.6	21	**19.3***
Reproductive outcome					
Mating index (%)		100	100	91.7	95.8
Fertility index (%)		95.8	100	100	100
Number of pregnant animals		23	24	22	23
Number of corpora lutea		16.7	16.3	17.3	**15.0***
Number of implantations		15.3	15	15.6	**13.9****
Number of live fetuses		14.6	14.3	15	**12.4****
Resorptions and fetal deaths (%)		4.3	5.1	4.5	**10.8****

Statistical significance: * $p \leq 0.05$ ** $p \leq 0.01$

At 750 ppm the absolute and relative (+30%) liver weights on GD 21 were significantly higher compared with the control group. The relative ovary weight on GD 19 in this group was significantly higher (+14%), compared with the control group.

There were no macroscopic findings attributable to treatment with metconazole. No significant differences were noted in the histology of corpora lutea and follicles in the ovary between the control group and any of the treated groups. The mean number of apoptotic bodies per corpus luteum in the 750 ppm group was comparable with the control group. However, the ratio of the number of corpora

lutea with PCNA-positive lutein cells to the total number of corpora lutea in the 750 ppm group was significantly higher, compared with the control group. The mean number of proliferating cell nuclear antigen-positive (PCNA-positive) lutein cells per corpus luteum in the 750 ppm group was also slightly higher than in the control.

In the 750 ppm group, serum 17β-estradiol concentrations were lower than controls on both GDs 19 and 21, and a statistically significant difference was noted in the values on GD 21. The serum progesterone concentration in this group was slightly higher than the control on GD 21, but not statistically significantly and with large standard deviations. Thus, the changes in hormone concentrations were due to decreased estradiol levels and resulted in significantly decreased E:P ratios at the top dose on both GDs 19 and 21 when compared with the control. Serum 17β-estradiol, progesterone concentrations and E:P ratios on GDs 19 and 21 were unaffected by treatment in the 30 and 150 ppm groups. A statistically significant decrease in the 17β-estradiol concentration on GD 21 in the 30 ppm group was considered incidental because no significant difference was noted between the value in the 150 ppm group and that in the control group. The E:P ratio is a calculated value based on two measured hormone parameters subject to the added variations of the two separate parameters, estradiol and progesterone. Thus, only the results based on the individual parameters, (estradiol and progesterone) should be considered as the relevant parameters. Estradiol in the 30 ppm group at GD 21 was also significantly decreased, however not dose-dependently. Thus, only the reduced estradiol levels at 750 ppm are considered to be a toxicologically relevant effect, along with accompanying parental effects (reduced body weight gain) and fetotoxic effects at this top dose of 750 ppm.

The effect on difficult delivery observed with the top-dose females were discussed and thought likely to be associated with aromatase inhibition, and the decrease in estradiol at the end of gestation would also fit with this mode of action. Furthermore, hormone changes could also indicate a physiological response secondary to other reprotoxic parameters such as reduced food consumption, number of live fetuses and fetal deaths (Lederman & Rosso, 2004,). Accordingly, higher progesterone and lower estradiol levels were observed during the end of gestation compared to the control animals.

Hepatic microsomal protein contents on GD 19 were significantly increased in the 150 and 750 ppm groups when compared with the control. Cytochrome P-450 content was significantly increased in the 750 ppm group on GDs 19 and 21 and in the 150 ppm group on GD 21. No CYP1A1 content was detected in any of the groups, including the control group, on either GD 19 or 21. Levels of CYP4A1 were increased significantly in treated groups, but the size of the increases was only less than twice that of the corresponding controls. CYP2B1 protein levels were significantly elevated in treated groups, with a slight but relevant increase only at the top dose on GDs 19 and 21 (1.9-fold and 2.2-fold). By contrast, CYP3A2 content in the 30 and 150 ppm groups was comparable to the control group, while in the 750 ppm group amounts were consistently and remarkably higher than in controls on both GDs 19 and 21 (respectively 12- and 8-fold increases over the control).

Table 63. Key finding in mechanistic one-generation study

	Gestation day 19				**Gestation day 21**			
Dose level (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals per group	11	12	11	11	12	12	11	12
Terminal observations								
Body weight at necropsy (g)	404	413	408	**357****	430	432	432	**393****
Absolute organ weight (mg)								
Liver	14 142	14 213	14 109	13 152	11 367	12 101	11 376	**13 470****
Ovaries §	61.1	61.4	62.9	61.2	60.7	58.5	61.1	60.8
Relative organ weight (% of body weight)								
Liver	3.5	3.44	3.46	3.69	2.64	2.8	2.64	3.43**
Ovaries §	0.015	0.015	0.016	**0.0172****	0.014	0.014	0.014	0.016

	Gestation day 19				Gestation day 21			
Dose level (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Serum steroid hormone concentration								
Number of animals examined	7	8	8	8	8	8	8	8
17β-estradiol (pg/mL)	22.3	24.1	23.8	16.2	23.9	**16.8***	20.9	**11.4****
[± SD]	[4.8]	[7.0]	[8.0]	[7.2]	[8.1]	[3.2]	[5.4]	[4.2]
Progesterone (pg/mL)	51.3	61.3	61.4	62.9	10.7	10	11.6	14.9
[± SD]	[7.8]	[10.6]	[10.1]	[14.9]	[2.4]	[3.4]	[2.5]	[10.1]
E:P ratio	0.441	0.396	0.402	**0.267***	2.352	1.913	1.86	**1.092****
Drug-metabolizing enzyme content								
Number of animals examined	8	8	8	8	8	8	8	8
Microsomal protein (mg/g liver)	45	49	**58****	**60****	31	32	33	33
CYP content (nmol/mg protein)	0.26	0.26	0.33	**0.46****	0.48	0.51	**0.64***	**0.76****
CYP isozymes (pmol/mg protein)								
CYP1A1	0	0	0	0	0	0	0	0
CYP2B1	6.2	**8.9****	**9.0****	**11.5****	11.9	15.5	14.4	**25.7****
CYP3A2	3.2	2.6	6.7	**39.4****	8.8	7.1	14.3	**72.7****
CYP4A1	5.2	4.6	**8.1****	**9.1****	10.6	10.8	**15.2****	12.7
Ratio of number of corpora lutea with PCNA-positive lutein cells to total number of corpora lutea [as a %]					23/49 [47%]	-	-	**28/35**** **[**80%]
Number of PCNA-positive lutein cells per corpus luteum					1.43	-	-	3.17

§ Mean weight of both sides

* $p < 0.05$ ** $p < 0.01$

The dosages used in this study were the same as those used in the previously conducted reproductive toxicity study with *cis*/*trans*-metconazole in rats. In that main reproductive toxicity study, adverse effects on reproduction such as prolonged estrus cycle length, duration of gestation and maternal deaths during delivery, as well as general systemic toxic effects (decreased body weight gains and food consumption and increased relative liver and ovary weights) were noted in F0 females at 750 ppm. These effects were also observed in F1 females at the top dose. It was concluded that 150 ppm was the NOAEL in that reproductive toxicity study. In the current supplemental study, parental toxicity was demonstrated at 750 ppm by decreased mean body weight gain during both the premating and gestation periods. The other apparently treatment-related effect was the decrease in E:P ratios on GDs 19 and 21, mainly due to decreased estradiol levels. Progesterone concentrations in this 750 ppm group tended to be slightly higher (not statistically significant) than those of the control group on GDs 21, with large standard deviations. It was suggested that corpora lutea may not have entered the stage of functional regression yet and may thus have still been active for progesterone production during late pregnancy, which was possibly in line with the finding of an increased ratio (higher than in the controls) of corpora lutea containing PCNA-positive lutein cells. However, the large variations in measured serum progesterone levels make it difficult to draw a final conclusion.

The delayed onset of parturition and difficult delivery might be associated with the decrease in the E:P ratio on GDs 19 and 21 observed in this study. It was hypothesized by the study's authors that the decrease in estradiol levels at the highest dose tested might have been due to an excessive acceleration of 17β-estradiol metabolism due to a noteworthy increase in hepatic CYP contents involved in liver estradiol metabolism.

Based on these results, a dose level of 150 ppm was considered to be the NOAEL for female rats which demonstrated no adverse effects due to increases in their E:P ratio, although nonadverse changes (increases in hepatic microsomal protein and cytochrome P-450) were observed at this dietary concentration, compared to the controls (Teramoto, 2006b; Takahashi, 2015b).

(v) US ToxCast

As with many other pesticides and chemicals, metconazole is part of the US ToxCast program. Several assays react to varying doses of metconazole, however, no conclusive picture has emerged. The following publications related to estrogen receptor signaling response are discussed.

Thousands of environmental chemicals are subject to regulatory review for their potential as endocrine disruptors (ED). In vitro high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for ED-related whole-animal tests. In this study, 1814 chemicals including pesticide active and inert ingredients, industrial chemicals, food additives, and pharmaceuticals were evaluated in a panel of 13 in vitro HTS assays. The panel of in vitro assays interrogated multiple end-points related to estrogen receptor (ER) signaling, namely binding, agonist, antagonist, and cell growth responses. The results from the in vitro assays were used to create an ER Interaction Score. Of the ca 1800 chemicals evaluated in the study; 82% did not display indications of interacting with the ER signaling pathway and would be low priorities for additional ER testing. Metconazole was one of these, and registered zero for the binding and growth group assessments (Rotroff et al., 2014).

Metconazole was tested in 18 assays for estrogenic and anti-estrogenic activity (E activity)); nine assays for androgenic and anti-androgenic activity (A activity); three assays for thyroid and anti-thyroid hormone activity (T activity); and 10 assays for steroidogenesis alteration (S activity).

Key findings for metconazole from ToxCast are shown below in Tables 64–68.

Table 64. Estrogenicity/anti-estrogenicity

Biological process indicator			**AC_{50} [µM]**			
[Estrogen (E) or anti-estrogen (Anti-E) signal detection]	**Assay ID**	**Assay end-point name**	**17β-Estradiol**	**Genistein**	**Tamoxifen**	**Metconazole**
Binding to estrogen receptor [E/Anti-E]	A1	NVS_NR_bER (bovine)	0.000243	0.0367	0.087	Inactive
	A2	NVS_NR_hER (human)	0.00003	0.0127	0.052	Inactive
	A3	NVS_NR_mERa (mouse)	0.00164	0.103	0.1	**8.04**
Receptor dimerization [E/Anti-E]	A4	OT_ER_ERaERa_0480	0.0251	3.08	0.47	Inactive
	A5	OT_ER_ERaERa_1440	0.0238	2.6	0.9	Inactive
	A6	OT_ER_ERaERb_0480	0.0162	0.791	0.34	Inactive
	A7	OT_ER_ERaERb_1440	0.0193	0.471	0.34	**19.4**
	A8	OT_ER_ERbERb_0480	0.00861	0.0819	0.5	**11.7**
	A9	OT_ER_ERbERb_1440	0.00771	0.0968	0.11	**18.9**
DNA binding of estrogen receptor complex [E/Anti-E]	A10	OT_ERa_EREGFP_0120	0.000031	1.27	0.23	Inactive
	A11	OT_ERa_EREGFP_0480	0.000179	1.27	0.2	Inactive
RNA transcription [E]	**A12**	ATG_ERa_Trans_up	1.07	0.0942	Inactive	**6.95**
	A13	ATG_ERE_Cis_up	0.0009	0.137	Inactive	**6.95**
Protein production [E]	A14	Tox21_ERa_BLA _Agonist_ratio	0.000121	9	72	Inactive
	A15	Tox21_ERa_LUC_BG1 _Agonist	0.000077	2.55	Inactive	Inactive

Biological process indicator			AC_{50} [µM]			
[Estrogen (E) or anti-estrogen (Anti-E) signal detection]	**Assay ID**	**Assay end-point name**	**17β-Estradiol**	**Genistein**	**Tamoxifen**	**Metconazole**
Cell proliferation [E]	A16	ACEA_T47D_80 hr _Positive	0.024	0.056	Inactive	Inactive
Antagonist transcription suppression [Anti-E]	**A17**	Tox21_ERa_BLA _Antagonist_ratio	48.7	34.9	1.48	**68.7**
	A18	Tox21_ERa_LUC_BG1 _Antagonist	Inactive	Inactive	14	**91.9**

Table 65. Androgenicity/anti-androgenicity

Biological process indicator			AC_{50} [µM]		
[Androgen (A) or anti-androgen	**Assay ID**	**Assay end-point name**	**Testosterone proprionate**	**Hydroxy-flutamide**	**Metconazole**
Binding to androgen receptor	A1	NVS_NR_hAR (human)	0.055	0.177	Not tested
	A2	NVS_NR_cAR (chimpanzee)	0.808	0.307	8.89
	Not in AR model	NVS_NR_rAR (rat)	0.888	18	Not tested
Cofactor Recruitment	A3	OT_AR_ARSRC1_0480	0.012	9.8	57.5
	A4	OT_AR_ARSRC1_0960	0.007	12	62.5
DNA binding of estrogen receptor complex	Not in AR model	OT_AR_ARELUC _AG_1440	0.0037	3.5	Inactive[§]
RNA transcription	A5	ATG_AR_*Trans*_up	0.953	Inactive	Inactive
Protein production	A6	ox21_AR_BLA_ Agonist_ratio	0.028	Inactive	Inactive
	A7	Tox21_AR_LUC _MDAKB2_Agonist	0.000765	7	Inactive
Antagonist transcription suppression	A8	Tox21_AR_BLA _Antagonist_ratio	71	1.7	40.2
	A9	Tox21_AR_LUC _MDAKB2_Antagonist	Inactive	23	58.6[§]

[§] The result of assay A5 was flagged "Borderline inactive" and result of assay A9 was flagged "Only highest concentration above baseline, active"

Table 66. Effects on steroidogenesis

Hormone quantified	Assay ID CEETOX_H295R_ …	AC_{50} [µM]	Assay effect cut-off, (*coff*)	Effect maximum (*Top*)§	Assay results
17α-OH pregnenolone	_OHPREG_dn	0.04	0.72	0.4	Inactive
	_OHPREG_up	1000	0.72	0	Inactive
Progesterone	_PROG_dn	ND	0.98	ND	ND
	_PROG_up	ND	0.98	ND	ND
17α-OH progesterone ↓	**_OHPROG_dn**	**0.0861**	**0.9**	**1.36**	**Active**
	_OHPROG_up	1000	0.9	0	Inactive
11-deoxycorticosterone ↓	**_DOC_dn**	**0.0974**	**0.92**	**1.20***	**Active**
	_DOC_up	1000	0.92	0	Inactive
Cortisol	_CORTISOL_dn	1000	0.98	0	Inactive
	_CORTISOL_up	1000	0.98	0	Inactive
11-deoxycortisol	_11DCORT_dn	0.0715	0.58	0.377**	Inactive
	_11DCORT_up	1000	0.58	0	Inactive
Androstenedione	_ANDR_dn	0.152	0.76	0.502	Inactive
	_ANDR_up	1000	0.76	0	Inactive
Testosterone	_TESTO_dn	1000	1.04	0	Inactive
	_TESTO_up	1000	1.04	0	Inactive
Estrone	_ESTRONE_dn	1000	0.81	0	Inactive
	_ESTRONE_up	1000	0.81	0	Inactive
Estradiol	_ESTRADIOL_dn	1000	1.04	0	Inactive
	_ESTRADIOL_up	0.329	1.04	0.884***	Inactive

ND Not determined

§ log2-fold induction

Flags: * borderline active; ** multiple points above baseline, inactive; *** borderline inactive

Table 67. Effects on aromatase

ToxCast assay name	AC_{50} [µM]	Assay Effect Cut-off (coff)	Conc. at cut-off [µM] (ACC)	Effect maximum (*Top*) §	Assay results
Tox21_Aromatase_Inhibition	32.5	31.29	13.9	104	Active

§ log2-fold induction

Table 68. Effects on thyroid hormones

#	Assay end-point name	Short assay description	Endocrine activity hypothesis (Thyroid, T; anti-thyroid, Anti-T)	Metconazole
1	ATG_THRa1_Trans_up	Inducible exogenous thyroid hormone receptor α1 (THRA1) transcription factor activity in HepG2 cells (24 h).	T	Inactive
2	NVS_NR_hTRa _Antagonist	Cell-free, chemiluminescent binding assay for thyroid hormone receptor α1; (loss-of-signal activity)	Anti-T	Inactive
3	Tox21_TR_LUC_GH3 _Agonist	Inducible endogenous thyroid receptor transcription factor activity detected by a luciferase-fusion response element in rat pituitary GH3 cells in agonist mode (24 h).	T	Inactive
4	Tox21_TR_LUC_GH3 _Antagonist	Inducible endogenous thyroid receptor transcription factor activity detected by a luciferase-fusion response element in rat pituitary GH3 cells in antagonist mode (24 h).	Anti-T	**Active**

The majority of these assays were negative following exposure to metconazole. A limited number of assays indicated some activity, but on closer examination were determined to likely represent false positives due to assay interference. No consistent patterns across multiple assays for E, A, T and S effects were observed with metconazole, with a possible exception of evidence for a weak aromatase inhibition potential at high concentrations (> 14 μM). It should be noted that the steroidogenesis assays with H295R cells were tested up to concentrations of 1 μM, which is below the cytotoxicity limit determined in ToxCast. Nevertheless, the individual concentration–response curves and the overall pattern of ToxCast results for metconazole in these assays indicate a lack of evidence for an effect on steroidogenesis (Stinchcombe, 2016).

(vi) Metabolomic study

cis-Metconazole (purity 97.4% *cis*; lot/batch AC 10925-24B) or *cis*/*trans*-metconazole (purity 98.7%; *cis*:*trans* 79.8:15.5; lot/latch COD-001163) were administered daily for 28 days to groups of five male and five female Crl:Wi(Han) Wistar rats at dietary concentrations of 1500 and 5000 ppm (*cis*), and 500 and 1500 ppm (*cis*/*trans*). Groups of 10 males and 10 females received only the base diet for the same time period and served as controls. Blood samples for metabolome analysis were taken retro-orbitally on study days 7, 14, and 28 from overnight fasted animals under isoflurane anaesthesia and the obtained EDTA-plasma was covered with nitrogen and frozen at −80°C. In total, 40 plasma samples from each test treatment were used for analysis and evaluation.

MetaMap®Tox evaluation

Test substance-related changes in the metabolome were assessed by analysis of specific metabolic changes for each dose group and by comparison with the entire metabolome of reference compounds, (called "profile comparison") using Spearman and Pearson correlations. On the basis of 297 analytes, 15 significant metabolite changes can be expected on a significance level of 0.05 (the "false positive" rate). Therefore, if up to 15 significantly changed metabolites are detected, the metabolome is still considered not to be affected by the test compound.

Profile comparison with reference compounds

At 5000 ppm of *cis*-metconazole the comparison of metabolite changes was not possible, since the animals had to be sacrificed in a moribund state on day 14. At 1500 ppm, the comparison of metabolite changes did show, in males only, similarities with patterns for triazole aromatase inhibition and oxidative stress in the liver in males. Such comparison for females suggested liver effects, indirect effects on the thyroid, and diuretic effects on the kidney. Indirect effects on the thyroids were also recorded in female

animals. However, neither in subacute nor in subchronic studies with *cis*-metconazole or *cis*/*trans*-metconazole were thyroid effects observed in rats of either sex. Profile comparison of the metabolite (profile strengths 1.6 and 1.83 for males and females respectively) did show matches with those of compounds present in MetaMap®Tox and was ranked on the level of the 87th and 97th percentile of all correlations in the database for males and females respectively.

At 1500 ppm of *cis*/*trans*-metconazole, the comparison of metabolite changes against the established specific metabolite patterns present in MetaMap®Tox did show similarities with patterns for liver enzyme induction, liver toxicity and indirect effects on the thyroid (potentially due to increased excretion of thyroid hormones) in males, but did not show any similarity for females. Profile comparison of the metabolite (profile strengths 0.99 and 1.04 for males and females respectively) was ranked on the level of the 87th and 97th percentile of all correlations in the database for males and females respectively. At 500 ppm, the comparison of metabolite changes did show weak similarities with patterns for liver enzyme induction in males, but did not show any similarity for females. The total profile comparison of the metabolite (profile strength 0.81 and 0.68 for males and females respectively) was ranked on the level of the 39th and 93th percentile for males and females respectively.

Both test articles did induce effects on the plasma metabolome of rats of both sexes, however neither in the subacute nor the subchronic studies with *cis*-metconazole and *cis*/*trans*-metconazole were thyroid effects observed in rats of either sex Comparing treatment with 1500 ppm *cis*-metconazole and *cis*/*trans*-metconazole, the effects on the metabolome of *cis*-metconazole seem to be more pronounced than for *cis*/*trans*-metconazole, reflected in the higher profile strength for *cis*-metconazole (1.6 for males and 1.83 for females) compared to that of *cis*/*trans*-metconazole (0.99 for males and 1.04 for females). At 1500 ppm the metabolite profiles of *cis*-metconazole and *cis*/*trans*-metconazole showed correlation on the 97th and 87th percentiles for females and males respectively, indicating a high degree of similarity between the two forms (Kamp, 2015).

(vii) WEC study

The effects of three formulations of metconazole test article were trialled in Whole Embry Culture (WEC), an in-vitro rat embryo toxicity assay for the independent evaluation of effects on growth and differentiation according to currently accepted interpretations.

Rat embryos were obtained from mated Sprague Dawley CD rats on GD 10. Embryos were incubated in roller-bottles (five per bottle) for 48 h (60 rev./min, 38°C, 1 mL culture/embryo) in heat-inactivated, filter-sterilized supplemented rat serum.

Ten rat embryos per concentration were incubated in the presence of *cis*/*trans*-metconazole (WL148271; purity 95.3%; *cis*:*trans* 79.8:15.5; batch 89-01), *cis*-metconazole (WL136184; purity: 95.3%; *cis*:*trans* 95.2:0.1; batch 12) or *trans*-metconazole (WL153996; purity and *cis*:*trans* content not specified; batch 88.08), at concentrations of 0, 3, 10, 30 and 100 µg/mL in the culture medium of the main experiment. Parameters of growth were yolk sac diameter, crown–rump length and head length. The differentiation landmarks were: yolk sac, allantois, heart, caudal neural tube, brain, otic and optic system, branchial bars and limb-buds. The effectiveness of the metabolic activating system was tested by treatment of the embryos with cyclophosphamide (CP) at 5 µg/mL.

In the preliminary range-finding experiment, *cis*/*trans*-metconazole showed effects on growth (crown–rump and head lengths), and on differentiation (somite number and morphological score) at 100 µg/mL. Borderline effects might have been observed on differentiation at 10 µg/mL. However, the potential effects at 10 µg/mL were not reproduced in the main experiment.

In the main experiment, embryos incubated in the presence of *cis*/*trans*-metconazole exhibited significant reductions in growth (crown–rump and head length) and differentiation parameters (somite number and partially morphological score) at 30 and 100 µg/mL. These parameters were essentially similar in all dose groups of embryos, whether incubated with either the *cis* or *trans* isomer of metconazole.

Key findings of this WEC embryo toxicity assay are shown on the following page in Table 69.

Table 69. Key findings in WEC study

End-point	Metconazole isomer or mix	Dose metconazole (µg/mL) 0	3	10	30	100	Dose CP (µg/mL) 5
General embryo development							
Yolk sac diameter (mm)	*cis*/*trans*	3.94	0%	4%	−6%	−4%	**−8%**
	cis		2%	−2%	2%	2%	
	trans		−1%	−1%	1%	−3%	
Crown–rump length (mm)	*cis*/*trans*	3.62	−3%	1%	**−7%**	**−11%**	**−30%**
	cis		2%	−6%	−2%	−3%	
	trans		0%	−1%	−1%	−4%	
Head length (mm)	*cis*/*trans*	1.77	−5%	−1%	**−12%**	**−17%**	**−40%**
	cis		6%	−5%	−1%	−6%	
	trans		2%	−3%	−1%	−3%	
Somite number	*cis*/*trans*	26.1	−1%	−2%	**−10%**	**−16%**	**−43%**
	cis		0%	−2%	−2%	−2%	
	trans		−1%	0%	−3%	−1%	
Morphological score	*cis*/*trans*	38.5	−6%	1%	−1%	**−11%**	**−44%**
	cis		1%	−2%	−1%	−2%	
	trans		1%	−2%	0%	−2%	
Embryo dysmorphology							
Posterior neuropore	*cis*/*trans*	0	3	**8**	**9**	**10**	**7**
	cis		0	**9**	**10**	**9**	
	trans		1	1	2	**9**	
Otic system	*cis*/*trans*	0	0	0	**3**	**7**	**7**
	cis		0	0	**5**	**3**	
	trans		0	0	0	**4**	
Forelimb buds	*cis*/*trans*	0	1	2	0	**4**	**9**
	trans		0	2	0	**3**	
	cis		0	0	0	1	
Hindbrain	*cis*/*trans*	1	0	0	2	**4**	**5**
	cis		0	1	2	2	
	trans		0	2	1	0	
Midbrain	*cis*/*trans*	1	0	0	2	**4**	**5**
	cis		0	1	1	1	
	trans		0	2	1	0	
Branchial part	*cis*/*trans*	0	0	0	0	**2**	0
	cis		0	0	0	0	
	trans		0	0	0	0	

CP Cyclophosphamide

cis/*trans*-Metconazole showed effects on embryo development at ≥ 30 µg/mL based on retardation in growth and differentiation. Neither *cis* nor *trans* metconazole showed a comparable effect on embryo development up to 100 µg/mL. However, *cis*/*trans* and *cis*-metconazole showed a potential to cause dysmorphogeneses at 10 µg/mL and above, but *trans*-metconazole only did so at 100 µg/mL. The pattern of dysmorphogeneses of all three isomers was comparable at the highest concentration tested,

indicating that all isomers may act via the same mode of action that results in embryo toxicity. However, the potencies of the *cis*/*trans* and *cis* test articles were greater, causing dysmorphogeneses in vitro at 10 and 30 µg/mL, whereas for *trans*-metconazole this was only apparent at 100 µg/mL (Bowden, 1991).

(viii) Rat liver microsomal assay

In this in vitro study the potential of metconazole technical (*cis*/*trans*, Reg.No. 4056343) to inhibit retinoic acid metabolism via inhibition of CYP26 activity in rat liver microsomes has been investigated. The IC_{50} values of the test substance are compared with IC_{50} values of a known CYP26 inhibitor, ketoconazole (Kirby et al., 2003; Yee et al., 2005), as well as with two further test substances of the same chemical class, letrozole as a potential negative control and epoxiconazole.

In three independent experimental runs using three different lots of rat microsomes (male or pooled male/female samples) dose response curves were generated to determine the concentration at which the test compounds show a 50% inhibition capacity (mean IC_{50}).

Test substances:

Metconazole: (*cis/trans*); batch no. COD-001163

Epoxiconazole: Purity 97.5%; batch no. COD-001456

Ketoconazole: Purity 99%; batch no. 1/0

Letrozole: Purity 98.0%; batch no. 2/0

Table 70. IC_{50} values (µM) for independent experiments (runs 1–3) in rat microsomes calculated for metconazole and the other test compounds

Compound	Run1: male rat sample 1	Run2: male rat sample 2	Run3: pooled male/female sample
Metconazole	> 100	> 100	> 100
Ketoconazole	15.3	13.1	10.9
Letrozole	> 100	> 100	> 100
Epoxiconazole	54.2	41.2	87.5

Based on the results obtained and under the conditions of this current test system metconazole technical did not inhibit rat microsomal retinoic acid hydroxylase activity, resulting in an IC_{50} greater than 100 µM. The positive control, ketoconazole, showed inhibition of rat CYP26 activity and resulted in an IC_{50} in the range 10.9 µM to 15.3 µM. These first results confirm previous published evidence for CYP26 inhibition by ketoconazole in rat microsomes (Kirby et al., 2003: IC_{50} = 22 µM; Yee et al., 2005: IC_{50} = 18 µM). Compared to ketoconazole, the test compound epoxiconazole showed a weaker inhibition activity with an IC_{50} between 41.2 µM and 87.5 µM. Letrozole was confirmed as a negative control with IC_{50} values above 100 µM in each of the three independent study runs, similar to metconazole.

The test substances were assayed up to concentrations of 100 µM, since for all test compounds the solubility limit was reached at 300 µM. Each of the three experimental runs were performed on different days and with rat microsomes of different origin. Overall, between the single experimental runs comparable results were obtained regardless of the sex of rat microsomal origin used. While the first two experimental runs were conducted using microsomes from male Wistar rats, in the third experiment microsomes from pooled male and female Wistar rat samples (1:1) were used (Schiffer, 2018).

(ix) Murine CAR nuclear translocation assay in cultured primary mouse hepatocytes

Primary mouse hepatocytes in collagen-coated 24-well plates were infected with 2–3 µL per well of Tet-On EYFP-CAR adenovirus (constructed with the Adeno-X Adenoviral System 3). The cells were cultured overnight in fresh medium and then in the presence of 100 ng/mL doxycycline for another eight hours. The cells were treated overnight with test chemicals at selected concentrations. Metconazole test concentrations were based on pretest results (HepG2 viability assay applying five metconazole concentrations from 1–100 µM). The subcellular localization of CAR was visualized using a Nikon TE-2000S fluorescence microscope and subsequently quantitatively characterized with a ThermoFisher CX7 high-content imaging platform.

In wild-type mouse hepatocytes and in CAR KO mouse hepatocytes, metconazole revealed a dose-dependent increase of CAR translocation into the nucleus of the primary cells starting at 10 µM upwards. Likewise, the positive control phenobarbital showed a statistically significant increased shift of the mCAR into the nucleus. No mCAR translocation into the nucleus was observed for the vehicle control, DMSO. The current summary seems to indicate that:

- metconazole stimulated mCAR translocation in primary mouse hepatocyte cultures,
- translocation dynamics noted were of a similar order to those obtained with phenobarbital, the prototypical CAR activator (Coslo, Lee & Su, 2018).

(d) Toxicity studies on metabolites

Table 71. Summary of toxicity studies with metconazole metabolites

Metabolite	Study	Species (strain)/ Test System	Findings	References	Note
M555F011 *cis* (Reg. No. 4111112)	Acute oral LD_{50}	Rat (Sprague Dawley)	> 5000 mg/kg bw (males and females)	Bradley, 1997	Identified in fish, plant (rape, banana and wheat straw) and in the water.
	Microbial Mutagenicity Assay (Ames)	*S. typhimurium:* TA98, TA100, TA1535, TA1537 and TA1538 *E. coli:* WP2 *uvrA*	± S9: Negative (3.3–5200 µg/plate)	Woitkowiak, 2015a	
M555F021 *cis* (Reg. No. 4558878)	Microbial Mutagenicity Assay (Ames)	*S. typhimurium:* TA98, TA100, TA1535, TA1537 and TA1538 *E. coli:* WP2 *uvrA*	± S9: Negative (3.3–5300 µg/plate)	Woitkowiak, 2015b	Identified in rat (urine: not detectable; faeces: 1–6% in males and ≤ 1% in females), fish, plants (wheat straw) and in the water.
M555F030 *cis* (Reg. No. 4110625)	Microbial Mutagenicity Assay (Ames)	*S. typhimurium:* TA98, TA100, TA1535, TA1537 and TA1538 *E. coli:* WP2 *uvrA*	± S9: Negative (3.3–5000 µg/plate)	Woitkowiak, 2015c	Identified in soil and in the water.
M555F031 *cis* (Reg. No. 5968488)	Microbial Mutagenicity Assay (Ames)	*S. typhimurium:* TA98, TA100, TA1535, TA1537 and TA1538; *E. coli:* WP2 *uvrA*	± S9: Negative (10–5000 µg/plate)	Woitkowiak, 2015d	Identified in goat and hen

QSAR evaluation

For all plant metabolites the presence of potential structural alerts was evaluated with different QSAR models. Models used were the OASIS TIMES and VEGA (CAESAR, ISS and SarPy).

Results showed that the assessment was in domain negative for Ames and in vivo MNT, indicating no genotoxic potential (MultiCASE, 2015a, b, c, d, 2017, 2018a, b; Goettel, 2018).

Table 72. Metconazole metabolites QSAR asse0ssment

Specified metabolite	QSAR tools applied	Structural alerts for genotoxicity	Reliability / total domain	Evaluation
M555F011, M555F021, M555F030 and M555F031	OA	Ames parent: negative	Low/ Out of domain	**Not genotoxic**
		Ames metabolites: negative		
		CA parent: negative	Low/ Out of domain	
		CA metabolites: §		
	VE	CAESAR: not mutagenic	Moderate/Could be out of domain	
		ISS: not mutagenic	Low/ Out of domain	
		SarPy: not mutagenic	Moderate/Could be out of domain	
	CU	Ames: negative	High/In domain	
		In vivo MNT: negative	High/In domain	
	ToxTree	In vivo MNT: negative	High	

OA OASIS TIMES VE Vega

CA Chromosomal aberration MNT = micronucleus test CU Case Ultra

§ See detail for each metabolites in the text

Triazole-derived metabolites

The sponsor stated that:

> "...with regard to the triazole-derived metabolites, no toxicological assessment has been conducted, as that would be beyond the scope of the metconazole assessment and will be reconsidered based on the outcome of the on-going UK assessment…"

Below is reported a summary of the conclusion on triazole-derived metabolites from JMPR (2008).

1,2,4-Triazole; toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.2 mg/kg bw based on a NOAEL of 250 ppm, equal to 16 mg/kg per day, on the basis of testicular effects (sperm abnormalities, sperm counts) seen at 500 ppm, equal to 30.9 mg/kg bw per day, and using a safety factor of 100. At 250 ppm, reduced body weights and body weight gains were observed in F1 males; however, the Meeting noted that the reductions in body weight observed at 250 ppm were marginal (< 6%) and were seen only in one sex and in only one generation and were not seen in short-term studies with similar doses. The Meeting therefore concluded that it was not necessary to use an additional safety factor. This ADI is protective for neurotoxic effects seen at 3000 ppm, equal to 183 mg/kg bw per day, in a short-term study of toxicity/neurotoxicity in rats in which the NOAEL was 500 ppm, equal to 33 mg/kg bw per day. The Meeting considered that it was not necessary to add an additional safety factor to allow for the lack of studies of carcinogenicity because 1,2,4-triazole is unlikely to be carcinogenic at anticipated levels of exposure since it does not bioaccumulate in the body, it is non-mutagenic, and because of the absence of preneoplastic changes at high doses. The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw based on a NOAEL of 30 mg/kg bw per day, identified on the basis of alterations of the urogenital system that occurred in several fetuses at the LOAEL of 45 mg/kg bw per day, and clinical signs of neurotoxicity in the dams in a study of developmental toxicity in rabbits, and using a safety factor of 100.

Triazole alanine and triazole acetic acid; toxicological evaluation

The Meeting established a group ADI for triazole alanine and triazole acetic acid (alone or in combination) of 0–1.0 mg/kg bw based on a NOAEL of 100 mg/kg bw per day for developmental toxicity in a study of developmental toxicity in rats given triazole alanine, on the basis of delayed ossification seen in rats at the LOAEL of 300 mg/kg bw per day, and using a safety factor of 100. The Meeting concluded that it was not necessary to use an additional safety factor for the lack of studies of carcinogenicity because the compounds are unlikely to be carcinogenic at anticipated levels of exposure, do not bioaccumulate in the body, are non-mutagenic, are not chemically reactive, and no specific target-organ toxicity was identified in the available toxicological studies with doses of up to 1510 mg/kg bw per day. The Meeting concluded that it was unnecessary to establish an ARfD for triazole alanine and triazole acetic acid because no toxicity could be attributed to a single exposure in the available database, including a study of developmental toxicity in rats.

(e) Toxicity studies on impurities

Impurities of metconazole were assessed for their genotoxic properties using QSAR models. Two state-of-the-art QSAR models were used according to the EFSA data requirement for a statistical model (Case Ultra) and a rule-based expert model (DEREK Nexus). The end-points assessed were Ames mutagenicity and DNA damage in the form of structural (clastogenicity) and numerical chromosomal aberrations (aneugenicity). In line with recommendations of EFSA (see Metruccio et al. 2017), the Case Ultra standard models for chromosome aberrations were trained using respective experimental data from BASF plant protection chemistry as well as from EFSA conclusions to increase its robustness and to ensure its applicability for this purpose. DEREK was used in its most recent standard version as provided by Lhasa. In summary, with one exception DEREK predictions for all analysed impurities were negative, as no alert in the domain of the model was fired for mutagenicity or chromosome aberrations. With metabolite Reg. No. 4539595, an alert was triggered for in vitro chromosome aberration, resulting in an "Equivocal" prediction. However, there was no alert for in vivo chromosome aberration with Reg. No. 4539595, and experimental evidence also shows this molecule to be negative for genotoxicity. The Case Ultra predictions for all analysed impurities of metconazole were a mix of "Out of domain", "Inconclusive" and "Negative" in the untrained models. However, using the more reliable trained models as well as expert judgement resulted in negative predictions for all of the impurities. Based upon the QSAR analysis, the investigated impurities of metconazole are unlikely to pose a genotoxic potential (Van Cott & Kemeny, 2018b).

(f) Literature review

Literature searches were done by Information Professionals (one biologist, one chemist) of the BASF Group Information Center. The process of selecting relevant scientific, peer-reviewed open literature was done in two steps:

The first selection step for relevance based on summary records (e.g. titles, abstracts, index terms, keywords) was done by the Agro Information Professionals:

- Obviously irrelevant records were tagged as "ballast". This ballast was controlled by scientific experts in the corresponding subject areas but was not further processed.
- Summary records which appeared to be relevant and those of unclear relevance were tagged as "hit" and went to the next level of evaluation.

The second detailed assessment step was done by the scientific experts in the corresponding areas. Records tagged "hit" were further evaluated in depth.

To facilitate a comprehensible listing of the hits in the different regulatory areas (Mammalian toxicology (including Human toxicology and Operator exposure), Residues, E-fate and Ecotoxicology) an Excel file was generated for each section with three typical registers, namely:

1 "No relevant endpoint"

2 "Evaluated – not-relevant"

3 "Used for dossier"

In a first step (rapid assessment) the "Hits" were reviewed based on the information given in the title and the abstract with regard to relevance for the regulatory end-points in the respective regulatory area. Those records that were clearly judged as not assignable to any regulatory end-point were shifted into the register "no relevant end-point" with a reasoned explanation.

In a second step (detailed assessment), all remaining records were assessed in detail based on the complete report by the respective expert(s) and separated into relevant reports for further discussion, and those clearly not relevant.

Criteria to assign a record to the register "Evaluated – not relevant" were:

- those records which were not assignable to the substance of interest (for example mixtures, not about test substance or other relevant substance),
- secondary literature linking to primary literature already discussed under relevant records,
- records with limited reliability of grade 3 or 4 based on the Klimisch scoring system (see below),
- and those which were judged as not relevant due to other reasons, with a respective justification.

Criteria to assign a record to the register "Used for dossier" were:

- Records providing information about additionaI/new/unknown/potentially contradictory effects or data which might impact the hazard assessment end-points or the risk assessments parameters in addition to having a high level of reliability, grade 1 or 2 based on the Klimisch scoring system (Klimisch, Andrea & Tillmann, 1997).

Literature search details

Section: *"Toxicology Animals And Human"*

Databases:

	Main Search	*Update Search*
Toxcenter	*20140505/UP*	*20150519/UP*
Embase	*20140508/UP*	*20150518/UP*

Date span: *2005–2015*

Search Limitation: *no patents, no tscats*

Search strategy for substances:

Toxcenter: all CAS Registry numbers, common/trade names, no mixtures

Embase: all CAS Registry numbers, common/trade names, no mixtures

Metconazole+nt/ct

Table 73. Number of records after first selection step for section "Toxicology animals and human"

Database:	***Embase – Excerpta Medica Database***	***Toxcenter – Toxicology Center Database***
Provider:	*STN International*	*STN International*
Justification for choosing the source: – for STN databases referring to STN database summary sheets	*Embase covers the most important international biomedical, drug-related and clinical literature, with a particular focus on adverse drug reactions and on Evidence -based medicine.* *Sources are more than 7 600 peer-reviewed journals. All MEDLINE records are included.* *Records contain bibliographic information, controlled terms, in-depth indexing, drug trade names, abstracts and CAS Registry Numbers.*	*Toxcenter covers all aspects of occupational hazards, adverse drug reactions, environmental pollution, chemically induced diseases, food contamination, pesticides and herbicides.* *Records contain bibliographic data, abstracts, indexing terms and CAS Registry Numbers.*
Coverage of sub-databases:		*ANEUPL, BIOSIS, CAplus, Cis, CRISP, DART, EMIC, EPIDEM, ETIC, FEDRIP, HAPAB, HMTC, IPA, MEDLINE, PESTAB, PPBIB, RISKLINE, TSCATS*
Date span of the source:	*1947 – to present*	*1970 – to present*
Date of the search:	*2014-05-09*	*2014-05-09*
Date span of the search:	*2005 – 2014*	*2005 – 2014*
Date of the latest database update included in the search:	*2014-05-08*	*2014-05-05*
Total number of summary records for metconazole and its metabolites:	*9*	*16*
Total number of summary records after removing duplicates:	*8*	*16*
Total number of summary records retrieved after first selection step:	*2*	*6*

Source: Zander, Metwally & Esswein, 2015

3. Observation in humans

Report on medical surveillance on manufacturing plant personnel

All persons handling crop protection products are surveyed by regular medical examination. There are no specific parameters available for effect monitoring of metconazole. Thus the medical monitoring programme is designed as a general health check-up, with special interest in the primary target organs presumed to be relevant by analogy from animal experiments. The surveillance program includes a general physical examination including neurological status, red and white blood cell counts, liver enzymes. No adverse health effects suspected to be related to metconazole exposure have been observed.

Report on clinical cases and poisoning incidents

Two cases of eye irritation have been registered in the BASF internal clinical incident log involving employees accidently exposed to metconazole in combination with other products. It is not clear whether metconazole was the cause of these irritations.

Medical data

Neither data on exposure of the general public nor epidemiologic studies are available for BASF SE, nor are they aware of any epidemiologic studies performed by third parties. No epidemiological studies with metconazole have been conducted. No monitoring of the general population or epidemiological studies are available for metconazole. As such, no observations regarding the health effects on the general public after exposure are known to us.

4. Microbial aspects

4.1 Mechanism and type of antimicrobial action

Metconazole, a demethylation inhibitor (DMI) fungicide, binds to the haem iron part of the cytochrome P450 sterol 14α-demethylase enzyme (CYP51). This inhibits lanosterol demethylation, which disrupts the ergosterol biosynthesis pathway.

4.2 Microbiome of the human gastrointestinal tract

There is no available information.

4.3 Antimicrobial spectrum of activity

Metconazole is a broad-spectrum triazole fungicide.

4.4 Antimicrobial resistance mechanisms and genetics

There are three major molecular mechanisms in fungi pathogenic to humans that are associated with resistance to azole compounds:

1 mutations in the target gene cyp51 which could confer reduced affinity of DMIs for their binding site,

2 up-regulation of the cyp51 gene, and

3 reduced intracellular accumulation of azoles, caused by enhanced efflux.

For further information see Lupetti et al., 2002.

Comments

Biochemical aspects

The fate of both *cis*/*trans*-metconazole (*cis*:*trans*, 85:15) and *cis*-metconazole was investigated in rats.

In bile cannulated-rats administered 2 mg/kg bw of *cis*-metconazole, bile was the major route of excretion, as up to 83% of radioactivity was eliminated via bile. Smaller amounts were excreted via urine (up to 12%) and only 0.2–0.3% in faeces. When total recovery of radioactivity was calculated based on radioactive levels in bile, urine, carcass, and cage washes, it appeared that 87–96% was absorbed during the first 48 h. Metconazole is rapidly absorbed, as 50–69% of the dose was eliminated in bile six hours after dosing (Hawkins, Elston & Kane, 1991).

When rats without bile cannulation were treated with 2 mg/kg bw of *cis/trans*-^{14}C-metconazole, 59–72% was voided via the faeces, and 14–24% eliminated renally after 48 h. By 72 h, 93–96% of the dose had been excreted (Morrison & Richardson, 1990). Based upon the plasmakinetic study in rats dosed at 2 mg/kg bw and 200 mg/kg bw of *cis*/*trans*-metconazole, and since the $AUC_{0-\mu}$ values were nearly proportional to dose levels, it was estimated that the absorption rate of the test substance was dose-independent. However, the time to reach C_{max} was dose-dependent: C_{max} was reached more slowly at the single high-dose level (4 h after dosing) than at the single low-dose level (within 0.25 h) (Yamamoto, 2002).

In rats administered *cis/trans*-metconazole at 2 mg/kg bw as a single dose and as repeated doses (14 consecutive daily low doses, 2 mg/kg bw) radioactivity was widely distributed into various organs and tissues, with the highest levels of residual radioactivity found in the liver, GI tract and adrenals, showing similar amounts after 72 h or 96 h (Morrison & Richardson, 1990, 1992a). When a single high dose of 164 mg/kg bw *cis/trans*-metconazole was administered to rats, the distribution pattern was qualitatively similar (Morrison & Richardson, 1992b). The parent compound was detected in small amounts; ≤ 2% of the dose was recovered in the faeces. Based on the identified metabolites in metabolism studies, the metabolism of metconazole appears to be initiated by monohydroxylation at the benzylic methylene, methylene, methyl groups and phenyl rings (14% in urine, 21% faeces), M21 (6% in faeces), M15 (14% in urine as conjugate and 3% in faeces) and M19 (14% in urine as conjugate and 9% in faeces). Monohydroxylation at the methylene linkage resulted in the release of triazole. The other monohydroxylated metabolites were either conjugated or further oxidized to yield carboxylated metabolites such as M12 (8% in urine, 14% faeces) and M13 (1% in urine and < 5% in faeces), dihydroxy, polyhydroxy, or hydroxylcarboxy metabolites (Morrison & Richardson, 1992b; Richardson, 1991a, b).

The test substance isomer ratio, or dosing vehicle did not affect the absorption, distribution, metabolism and excretion (ADME) of metconazole.

In a comparative in vitro metabolism study using *cis/trans*-^{14}C-metconazole, no human-specific metabolites were found, and the metabolic degradation in the tested species (human, rat and rabbit) was similar (Thibaut, 2016).

Toxicological data

The acute oral LD_{50} of *cis/trans*-metconazole in rats was between 500 and 2000 mg/kg bw (Gamer & Leibold, 2005a). The oral LD_{50} for *cis*-metconazole in rats was 1312 mg/kg bw (Gardner, 1991). In mice, the LD_{50} for *cis/trans*-metconazole was 410 mg/kg bw (Gardner, 1990a). The acute dermal LD_{50} of *cis/trans*-metconazole was > 2000 mg/kg bw in rats and rabbits (Gardner, 1990a; Gamer & Leibold, 2005b) and the acute inhalation LC_{50} was > 5.2 mg/L in rats (Gamer & Leibold, 2005c). Metconazole is neither a skin nor eye irritant in rabbits nor a skin sensitizer in guinea pigs when examined by the maximization and Buehler tests (Gardner, 1990b, 1991; Remmele & Leibold, 2005a,b).

Short-term toxicity of metconazole was evaluated for both the isomer mix (the particular isomer ratios ranged from *cis:trans* 77:18, to *cis:trans*, 84:17; nominally 80:15) and the *cis* isomer in subacute studies (rat, dog) and subchronic studies (rat, mouse, dog). Similar effects were observed in rat, mouse and dog. These consisted of a decrease in food consumption and body weight, clinical chemistry changes indicating a hepatotoxic effect associated with increase in liver weight and liver histopathological findings.

In a 90-day study, mice were treated with metconazole (*cis*:*trans*, 84:16) at 0, 30, 300, and 3000→2000 ppm (3000 on days 1–7 reduced to 2000 on days 7–90). This was equal to 0, 4.6, 50.5, and 341 mg/kg bw per day for males, 0, 6.4, 60.7, and 438 mg/kg bw per day for females. The NOAEL was 30 ppm (equal to 4.6 mg/kg bw per day) based on increased liver and spleen weights with corresponding macroscopic and microscopic changes corroborated by clinical chemistry findings observed at 300 ppm (equal to 50.5 mg/kg bw per day) (Clay, 1991).

In a 28-day study on metconazole (*cis*:*trans*, 83:17) rats were fed with diets containing 0, 30, 100, 1000, and 3000 ppm metconazole (equal to 0, 2.7, 9.1, 90.5, 261 mg/kg bw per day for males, 0, 3.1, 10.1, 97.0, 287 mg/kg bw per day for females). The NOAEL was 100 ppm (equal to 9.1 mg/kg bw per day) based on reduced body weight and food consumption as well as liver weight increase with corresponding macroscopic and microscopic changes (liver pallor/enlargement and hepatocellular vacuolation/hypertrophy) corroborated by clinical chemistry findings (enhanced aspartate aminotransferase, alanine aminotransferase reduced cholesterol and triglycerides) observed at 1000 ppm (equal to 90.5 mg/kg bw per day (Esdaile, 1990).

In a 28-day study on *cis*-metconazole, rats were fed with diets containing 0, 30, 100, 300, 1000, and 10 000 ppm metconazole (equal to 0, 2.7, 9.2, 27.3, 89.3, 721 mg/kg bw per day for males, 0, 3.0, 9.5, 29.8, 101, 784 mg/kg bw per day for females). The NOAEL was 300 ppm (equal to 27.3 mg/kg bw per day) based on reduced body weight and food consumption as well as increased liver weight with corresponding macroscopic (pallor of the liver) and microscopic (hepatocellular vacuolation) changes observed at 1000 ppm (equal to 89.3 mg/kg bw per day) (Esdaile, 1991a).

In a 90-day study rats were fed with diets containing metconazole (*cis*:*trans*, 76.5:18) at 0, 30, 100, 300, 1000, and 3000 ppm (equal to 0, 1.9, 6.4, 19.2, 64.3, 193 mg/kg bw per day for males, 0, 2.1, 7.2, 22.1, 71.4 and 208 mg/kg bw per day for females). The NOAEL was 100 ppm (equal to 6.4 mg/kg bw per day) based on hepatocellular fatty vacuolation observed at 300 ppm (equal to 19.2 mg/kg bw per day) (Esdaile, 1991b).

In a 90-day study with *cis*-metconazole, rats were fed with diets containing 0, 50, 150, 450, 1350, and 4050 ppm (equal to 0, 3.2, 9.7, 28.8, 88.6, 265 mg/kg bw per day for males, 0, 3.7, 11.0, 33.0, 96.8, and 267 mg/kg bw per day for females). The NOAEL was 450 ppm (equal to 28.8 mg/kg bw per day) based on reduced body weight and food consumption as well as increased liver and spleen weights with correlating hepatic macroscopic and microscopic changes This was corroborated by clinical chemistry findings observed at 1350 ppm (equal to 88.6 mg/kg bw per day) (Fokkema, 1992).

In a 90-day study, dogs were fed with diets containing 0, 60, 600, and 6000 ppm metconazole (*cis*:*trans*, 79.8:15.5; equal to 0, 2.5, 24.4, and 225 mg/kg bw per day for males, 0, 2.6, 24.3, 207 mg/kg bw per day for females). The NOAEL was 60 ppm (equal to 2.6 mg/kg bw per day) based on decreased food consumption and body weight gain observed in females at 600 ppm (equal to 24.3 mg/kg bw per day (Pickersgill, 1991c).

In a one-year study dogs were fed with diets containing 0, 30, 300, 1000, and 3000 ppm metconazole (*cis*:*trans*, 79.8:15.5; equal to 0, 1.1, 12.0, 38.5, and 110 mg/kg bw per day for males, 0, 1.1, 10, 36.5, and 114 mg/kg bw per day for females), the NOAEL was 300 ppm (equal to 10 mg/kg bw per day) based on significantly increased alkaline phosphatase activity in both sexes and decreased body weight gain in males observed at 1000 ppm (equivalent to 36.5 mg/kg bw per day) (Clay, 1992a).

The overall dog NOAEL for metconazole (*cis*:*trans*, 79.8:15.5) was 300 ppm (equal to 10 mg/kg bw per day) based on decreased food consumption and body weight gain at 600 ppm (equal to 24.3 mg/kg bw per day).

In a 22-month carcinogenicity study in mice, metconazole (*cis*:*trans*, 79.8:15.5) was administered at dietary concentrations of 0, 30, 300, and 1000 ppm (equal to 0, 4.4, 43.6, and 145 mg/kg bw per day in males, 0, 5.2, 53.0, and 179 mg/kg bw per day in females). The NOAEL was 30 ppm (equal to 4.4 mg/kg bw per day) based on liver (hypertrophic and hyperplastic events) and spleen (atrophy) at 300 ppm (equal to 43.6 mg/kg bw per day). The presence of liver adenoma at medium and high doses and of liver carcinoma at the high dose in female mice was considered to be treatment-related and led to the NOAEL for carcinogenicity of 30 ppm (4.4 mg/kg bw per day) (Clay, 1992b).

In a two-year chronic toxicity study in the rat, metconazole (*cis*:*trans*, 79.8:15.5) was

administered at dietary concentrations of 0, 10, 100, 300, and 1000 ppm (equal to 0, 0.4, 4.3, 13.1, 43.9 mg/kg bw per day in males, 0, 0.5, 5.3, 16.0, and 53.8 mg/kg bw per day in females). The NOAEL was 100 ppm (equal to 4.3 mg/kg bw per day) based on increased liver and spleen weight, as well as hepatocellular hypertrophy and adrenal cortical vacuolation at 300 ppm (equal to 13.1 mg/kg bw per day) (Taupin, 1992a).

In a carcinogenicity study in the same rat strain with metconazole (*cis*:*trans*, 79.8:15.5) at similar concentrations of 0, 100, 300, and 1000 ppm (equal to 0, 4.6, 13.8, 46.5 mg/kg bw per day in males, 0, 5.5, 16.6, 56.2 mg/kg bw per day in females), the NOAEL for chronic toxicity was 100 ppm (equal to 4.6 mg/kg bw per day) based on effects in the liver (pigment deposit and centrilobular hypertrophy) and the adrenals (cortical vacuolation) at 300 ppm (equal to 13.8 mg/kg bw per day). No treatment-related effects on the types or incidences of neoplasia were observed at any concentration; the NOAEL for carcinogenicity was 1000 ppm (equal to 46.5 mg/kg bw per day), the highest dose tested (Taupin, 1992b).

In a mechanistic study (28-day, rats and mice) conducted to investigate the mode of action (MOA) for mouse liver tumours, a number of biochemical parameters were assessed after exposure to 300 ppm of *cis*-metconazole or 0.05% phenobarbital.

It was demonstrated that both metconazole and phenobarbital significantly induced CYP3A, CYP2B and CYP4A (Worrell, 1991).

In a mechanistic study, mice were administered metconazole (*cis*:*trans*: 83:16) via the diet for 14 days. Hepatic cytochrome P450 (CYP), enzyme induction, production of reactive oxygen species (ROS), and transient cell proliferation were observed at 1000 ppm in a similar way to phenobarbital, a known hepatocarcinogen which is mitogenic, but not genotoxic. The NOAEL for the effects was 30 ppm (Harada, 2004).

No in vitro investigation is available on expression of Cyp2b *trans*cription levels in mouse and human hepatocytes, but in a preliminary report on a murine constitutive androstane receptor (mCAR) nuclear *trans*location assay in cultured primary mouse hepatocytes, metconazole seemed to stimulate mCAR *trans*location in primary mouse hepatocyte cultures with *trans*location dynamics similar to those obtained with phenobarbital, which is the prototypical CAR activator (Coslo, Lee & Su, 2018).

These data, although not exhaustive, are consistent with a CAR-mediated MOA, a MOA that is not relevant to humans.

The Meeting concluded that metconazole is carcinogenic in female mice, but not in rats or male mice.

Metconazole has been tested for genotoxicity in a battery of studies in vitro and in vivo. Some limitations were noted regarding these in vitro and in vivo assays, nevertheless the meeting concluded that metconazole is unlikely to be genotoxic.

As metconazole is unlikely to be genotoxic and the liver tumours in female mice occur with an MOA not relevant to the human, the Meeting concluded that metconazole is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study, rats were administered *cis*-metconazole in the diet at levels that were adjusted at intervals to maintain calculated chemical intakes of 0, 2, 8, 32 or 48 mg/kg bw per day throughout two generations. The NOAEL for parental toxicity was 8 mg/kg bw per day based on decreased body weights and ovarian weight changes at 32 mg/kg bw per day in the F1 generation. The NOAEL for reproductive toxicity was 8 mg/kg bw per day based on increased gestation length and decreased post-implantation survival in the F2 generation at 32 mg/kg bw per day. The offspring NOAEL was 8 mg/kg bw per day, based on decreased body weight gain of F1 pups until weaning at 32 mg/kg bw per day (Willoughby, 1992a).

In another two-generation study, rats were fed dietary concentrations of 0, 30, 150 or 750 ppm of metconazole (*cis*:*trans*, 83:16; equal to 0, 1.3, 6.3, 32.9 mg/kg bw per day for males, 0, 1.3, 6.4, 33.5 mg/kg bw per day for females). The parental NOAEL was 150 ppm (equal to 6.3 mg/kg bw per day), based on increased mortality, decreased body weight gain and increased liver weights associated with hepatocyte fatty changes at 750 ppm (equal to 32.9 mg/kg bw per day). The NOAEL for reproduction was 150 ppm (equal to 6.4 mg/kg bw per day) based on increased gestation length

and decreased gestation index in F1 at 750 ppm (equal to 33.5 mg/kg bw per day). The NOAEL for offspring was 150 ppm (equal to 6.3 mg/kg bw per day) based on decreased live litters born (F1, F2), decreased viability index (F2) and decreased body weight of F2 generation at 750 ppm (equal to 32.9 mg/kg bw per day) (Teramoto, 2006a).

A mechanistic one-generation study in rats investigated the mechanism by which slightly prolonged duration of gestation and dystocia had occurred when metconazole was administered in generational studies. Metconazole (*cis*:*trans*, 83:16) was administered at concentrations of 0, 30, 150, or 750 ppm (equal to 0, 1.82, 8.89, and 43.0 mg/kg bw per day). In this supplementary study a significant lack of increase in the 17β-estradiol to progesterone (E:P) ratio was observed on GDs 19 and 21 at the high dose of 750 ppm. Decreases in the E:P ratio at the top dose were mainly attributed to decreased serum 17β-estradiol concentrations on GDs 19 and 21. The delayed onset of parturition and difficult delivery might be associated with the decrease in the E:P ratio on GDs 19 and 21 observed in this study. Based on these results, the dose level of 150 ppm was considered to be the NOAEL for female rats which demonstrated no adverse effects to increases in the E:P ratio (Teramoto, 2006b).

Developmental toxicity studies have been conducted in rats and rabbits with both *cis/trans* mixture and the *cis* isomer of metconazole (treatment during GD 6–15). The toxicity of both compounds towards dams and fetuses was similar.

Metconazole (*cis*:*trans*, 84:16), was administered to rats by gavage on GD 6–15 at dose levels of 0, 12, 30 or 75 mg/kg bw per day. The NOAEL for maternal toxicity was 12 mg/kg bw per day, based on reductions in body weight gain at 30 mg/kg bw per day. The NOAEL for developmental toxicity was 12 mg/kg bw per day based on increases in skeletal ossification variations at 30 mg/kg bw per day; two cases of hydrocephalus where observed at the top dose of 75 mg/kg bw per day Masters et al., 1991a).

In a second rat developmental study, metconazole (*cis*:*trans*, 84:16) was given at doses of 0, 1, 4, 16 and 64 mg/kg bw per day. The maternal NOAEL was 16 mg/kg bw per day, based on body weight loss and lower food intake at 64 mg/kg bw per day. The embryo/fetal NOAEL was 16 mg/kg bw per day, based on post implantation loss, reduced live litter size, increased placental weight, reduction in mean fetal weight and increased incidence of minor fetal variations at 64 mg/kg bw per day (Fulcher, 2002).

In a rat developmental study with *cis*-metconazole the compound was administered by gavage at dose levels of 0, 6, 24 and 60 mg/kg bw per day from GD 6 to GD 15. The NOAEL for maternal toxicity was 24 mg/kg bw per day based on increased water consumption, decreased food consumption, and decreased body weight gain at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 24 mg/kg bw per day based on increased placental weight, decreased fetal weight and decreased litter size/viability, and increased post implantation loss (early and late resorptions) at 60 mg/kg bw per day (Willoughby, 1992c).

In a developmental toxicity study, rabbits received metconazole (*cis*:*trans*, 80:15) by gavage at dose levels of 0, 5, 10, 20 and 40 mg/kg bw per day from GD 6 to GD 28. The maternal NOAEL was 20 mg/kg bw per day based on small increases in post implantation loss (early and late), at 40 mg/kg bw per day. The embryo/fetal NOAEL was 20 mg/kg bw per day based on slightly decreased fetal weights and litter size at 40 mg/kg bw per day. No malformations or variations in the fetuses were attributable to treatment with the test substance (Hoberman, 1997).

A total of four additional main rabbit developmental toxicity studies with treatment during GDs 7–19 were conducted with *cis*-metconazole and/or *cis*/*trans*-metconazole at doses up to 62.5 mg/kg bw per day. In these studies, clear fetotoxic and developmental effects were only observed at maternally toxic dose levels. The critical finding in the rabbit was the increased occurrence of hydrocephalus. Hydrocephalus was seen in all of these four rabbit studies (including the dose range-finding studies), and although the incidence was very low or not clearly dose-related, it was seen consistently in all four studies. In one of these additional main rabbit studies, metconazole (*cis*:*trans*, 84:13) dosed at 0, 2, 4 and 10 mg/kg bw per day from GD 7 to GD 19, an increased incidence of hydrocephalus was observed, above the historical control range, at 10 mg/kg bw per day in the absence of maternal toxicity.

The overall NOAEL for embryo/fetal toxicity in rabbits was 4 mg/kg bw per day based on an increased incidence of hydrocephalus above the historical control range at 10 mg/kg bw per day in one rabbit study (Masters et al., 1991b).

The Meeting concluded that metconazole is teratogenic, producing hydrocephalus in rats and rabbits.

Metconazole did not cause neurotoxic effects in a two-week oral neurotoxicity (range finding) study in rats at concentrations of 0, 100, 540 or 3000 ppm (equal to 0, 11.0, 59.6 and 217 mg/kg bw per day for males, 0, 10.6, 52.8 and 206 mg/kg bw per day for females) and in a four-week oral main neurotoxicity study in rats at dietary concentrations of 0, 50, 170 and 500 ppm (equal to 0, 4.84, 15.7 and 47.1 mg/kg bw per day for males, 0, 5.1, 17.6, and 49.8 mg/kg bw per day for females) (Cooper, 2002a, b, 2015a, b).

The Meeting concluded that metconazole is not neurotoxic.

No immunotoxic potential was observed in a 28-day immunotoxicity study in rats using dietary dose levels of 0, 70, 210 and 630 ppm (equal to 0, 5.4, 17 and 52 mg/kg bw per day).

The Meeting concluded that metconazole is not immunotoxic.

In a recombinant aromatase assay, *cis/trans*-metconazole, *cis*-metconazole and *trans*-metconazole inhibited rat and human aromatase activity. The resulting human aromatase IC_{50} values were 0.721 μM, 0.569 μM and 2.47 μM for *cis/trans*-metconazole, *cis*-metconazole and *trans*-metconazole, respectively. The resulting rat aromatase IC_{50} values were 0.157 μM, 0.223 μM and 0.579 μM for *cis/trans*-metconazole, *cis*-metconazole and *trans*-metconazole, respectively

Toxicological data on metabolites and/or degradates

Metabolites *cis*-M555F001 and *cis*-M555F012 were found in rat urine at about 10% of the dose, and their toxicity is considered to be covered by the parent compound.

Metabolite *cis*-M555F011 had an acute oral LD_{50} in rats of greater than 5000 mg/kg bw, and was not mutagenic in an Ames test. Metabolites *cis*-M555F021, *cis*-M555F030 and *cis*-M555F031 were investigated by in vitro genotoxity studies (Ames) and all the tests were negative. For metabolites *cis*-M555F011, *cis*-M555F021, *cis*-M555F030 and *cis*-M555F031, the threshold of toxological concern (TTC) approach can be applied (Cramer class III) for chronic toxicity.

For metconazole and its plant metabolites, the presence for potential structural alerts was evaluated with different QSAR models. Models used were the OASIS TIMES and VEGA (CAESAR, ISS and SarPy). Results showed that the assessment was in domain negative for Ames and in vivo micronucleus tests, indicating no genotoxic potential.

Several unidentified hydroxylated metabolites were found in residue studies and were of potential relevance for the residue definition for risk assessment. The Meeting concluded that, as the addition of a hydroxylated group is unlikely to add any alerts for genotoxicity, the TTC approach can be applied (Cramer class III) for chronic toxicity.

Triazole derivative metabolites were detected in animal matrix (1,2,4-triazole) and plant matrix (triazolyl alanine and triazolyl acetic acid). A relevant complete evaluation has been conducted by JMPR in 2008, leading to specific reference values.

Human data

No specific data have been provided

The Meeting concluded that the existing database on metconazole was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.04 mg/kg bw on the basis of the embryo/fetal NOAEL of 4 mg/kg bw per day from the developmental toxicity study in the rabbit based on increased incidence of hydrocephalus at 10 mg/kg bw per day. A safety factor of 100 was applied. A margin of 250 for the upper bound of the ADI to the LOAEL for the increased incidence of hydrocephalus was observed. This ADI is supported by the NOAELs in several other studies (90-day mouse, 18-month mouse, two-year rat).

The Meeting established an ARfD for women of child-bearing age of 0.04 mg/kg bw, on the same basis as the ADI.

An ARfD is required for the general population based on the LD_{50} studies. In the absence of appropriate data, the Meeting cannot establish a population-specific ARfD. Therefore the ARfD for women of child-bearing age (0.04 mg/kg bw) is used as a conservative value.

Levels relevant to risk assessment of metconazole

Species	Study	Effect	NOAEL	LOAEL
Mixed cis/trans isomers				
Mouse	90-day[a]	Toxicity	30 ppm, equal to 4.6 mg/kg bw per day	300 ppm, equal to 50.5 mg/kg bw per day
	22-month study on toxicity and carcinogenicity[a]	Toxicity	30 ppm, equal to 4.4 mg/kg bw per day	300 ppm, equal to 43.6 mg/kg bw per day
		Carcinogenicity	30 ppm, equal to 4.4 mg/kg bw per day	300 ppm, equal to 43.6 mg/kg bw per day
Rat	90-day toxicity study[a]	Toxicity	100 ppm, equal to 6.4 mg/kg bw per day	300 ppm, equal to 19.2 mg/kg bw per day
	Two-year chronic toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 4.6 mg/kg bw per day	300 ppm, equal to 13.8 mg/kg bw per day
		Carcinogenicity	1000 ppm, equal to 46.5 mg/kg bw per day[c]	-
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	150 ppm, equal to 6.3 mg/kg bw per day	750 ppm, equal to 32.9 mg/kg bw per day
		Parental toxicity	150 ppm, equal to 6.3 mg/kg bw per day	750 ppm, equal to 32.9 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 6.4 mg/kg bw per day	750 ppm, equal to 33.5 mg/kg bw per day
	Developmental toxicity[b]	Maternal toxicity	12 mg/kg bw per day	30 mg/kg bw per day
		Embryo and fetal toxicity	12 mg/kg bw per day	30 mg/kg bw per day
Rabbit	Developmental toxicity[b]	Maternal toxicity	10 mg/kg bw per day[c]	-
		Embryo and fetal toxicity	4 mg/kg bw per day	10 mg/kg bw per day
Dog	90 day and 1 year[a,d]	Toxicity	10 mg/kg bw per day	24.3 mg/kg bw per day
Cis isomer alone				
Rat	Two-generation study of reproductive toxicity[a,d]	Reproductive toxicity	8 mg/kg bw per day	32 mg/kg bw per day
		Parental toxicity	8 mg/kg bw per day	32 mg/kg bw per day
		Offspring toxicity	8 mg/kg bw per day	32 mg/kg bw per day
Rabbit	Developmental toxicity[b]	Maternal toxicity	4 mg/kg bw per day	10 mg/kg bw per day
		Embryo and fetal toxicity	4 mg/kg bw per day	10 mg/kg bw per day

[a] Dietary administration [b] Gavage administration [c] Highest dose tested [d] Two or more studies combined

Acceptable daily intake (ADI) applies to metconazole, M1 and M12, expressed as metconazole

0–0.04 mg/kg bw

Acute reference dose (ARfD) applies to metconazole, M1 and M12, expressed as metconazole

0.04 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to metconazole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and efficient (95–97%)
Dermal absorption	Not evaluated
Distribution	Widely distributed up to 72 hours, residue radioactivity: highest tissue levels found in GI tract, liver and adrenals
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid excretion mainly in the faeces, following biliary excretion (79–83% within 48 h) for the low dose; elimination was delayed by approximately 48 h for the repeated dosing and for the high dose
Metabolism in animals	Extensively metabolized, mainly by hydroxylation of cyclopentane or benzyl rings
Toxicologically significant compounds in animals and plants	Metconazole, (sum of *cis* and *trans* isomers), M1, M12, M11, M21, M30, M31 and hydroxylated metabolites
Acute toxicity	
Mouse LD_{50} oral	410 mg/kg bw
Rat LD_{50} oral	> 500 and < 2000 mg/kg bw
Rat, rabbit LD_{50} dermal	> 2000 mg/kg bw
Rat LC_{50} inhalation	> 5.2 mg/L
Rabbit, dermal irritation	Non-irritant
Rabbit, ocular irritation	Non-irritant
Guinea pig, dermal sensitization	Not sensitizing (maximization, Buehler)
Short-term studies of toxicity	
Target/critical effect	Liver, mild hypochromic microcytic anaemia, adrenal (rat) Liver, slight microcytic anaemia at highest dose (mouse) Reduction in body weight gain, increased ALP, lens degeneration at higher dose levels (dog)
Lowest relevant oral NOAEL	4.6 mg/kg bw per day (mouse) 19.2 mg/kg bw per day (rat) 10 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEL	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver toxicity; corticomedullary adrenal pigmentation, spleen atrophy, reduced cholesterol/triglyceride level
Lowest relevant oral NOAEL	4.3 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in female mice but not in rats or male mice; mode of action not relevant for humans[a]
Genotoxicity	Unlikely to be genotoxic[a]

Reproductive toxicity	
Target/critical effect	Parental toxicity: reduced body weight and body weight gain, increased liver weights, hepatocyte fatty change Reproductive toxicity: prolonged gestation length, dystocia and associated maternal deaths, decreased gestation index, reduced post-implantation survival Offspring toxicity: decreased number of live fetuses, reduced bw gain
Lowest relevant parental NOAEL	6.3 mg/kg bw per day (with *cis*/*trans*-metconazole)
Lowest relevant offspring NOAEL	6.4 mg/kg bw per day (with *cis*/*trans*-metconazole)
Lowest relevant reproductive NOAEL	6.3 mg/kg bw per day (with *cis*/*trans*-metconazole)
Developmental toxicity	
Target/critical effect	Maternal toxicity: reduced bw gain and food consumption (rat, rabbit) Embryo/fetal toxicity: increased skeletal ossification variations, increased placental weight (rat), Increased post implantation loss, decreased live litter size, reduced fetal weight, and hydrocephalus (rat, rabbit)
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	4 mg/kg bw per day (rabbit, with *cis*/*trans*-metconazole)
Neurotoxicity	
Acute neurotoxicity NOAEL	No findings indicative of neurotoxic potential reported
Sub-chronic neurotoxicity NOAEL	47.1 mg/kg bw, highest dose tested (rat, in 2-week and 4-week neurotoxicity studies)
Developmental neurotoxicity	No data
Other toxicological data	
Mechanism studies	
Metconazole, (*cis*)	28-day mechanistic study (male rats and mice): hepatic CYP induction (rat, mouse): pattern similar, but not identical to phenobarbital
Metconazole, (*cis*/*trans* mix)	14-day mechanistic study in female mice: CYP2B induction (protein and enzyme activity) and transient hepatocellular proliferation after 3 and 7 days at 300 and 1000 ppm. 28-day immunotoxicity study in rats: no immunotoxin potential Mechanistic one-generation study: extended gestation length and dystocia may be associated with decrease of estradiol:progesterone ratio during late gestation, mainly due to decreases in serum estradiol
Metconazole: *cis*/*trans*, *cis*, and *trans*	Recombinant aromatase assay on rat and human enzymes: inhibition of aromatase activity (rat greater than human; *cis*/*trans* and *cis* similar, and greater than *trans* alone.)
Metabolite data	
Oral LD_{50}	*cis*-M555F011: > 5000 mg/kg bw (rat)
Genotoxicity	*cis*-M555F011, *cis*M555F021, *cis*M555F030, *cis*M555F031: No genotoxic concern (Ames test and QSAR)

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

Metconazole

	Value	Study	Safety factor
ADI	0–0.04 mg/kg bw[a]	Rabbit developmental	100
ARfD	0.04 mg/kg bw[a]	Rabbit developmental	100

1,2,4-Triazole[b]

	Value	Study	Safety factor
ADI	0–0.2 mg/kg bw	Two-generation	100
ARfD	0.3 mg/kg bw	Rabbit developmental	100

Triazole alanine and triazole acetic acid[b]

	Value	Study	Safety factor
ADI	0–1 mg/kg bw	Rat developmental	100
ARfD	Unnecessary		

[a] Applies to metconazole, M1 and M12, expressed as metconazole

[b] Triazole fungicide metabolites from JMPR 2008, pp 437–490

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PYFLUBUMIDE

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Explanation

Pyflubumide is the ISO-approved common name for 3′-isobutyl-*N*-isobutyryl-1,3,5-trimethyl-4′-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]-1*H*-pyrazole-4-carboxanilide (IUPAC), with the Chemical Abstracts Service number 926914-55-8. It is a novel acaricide and, so far, the only one with a carboxanilide structure. Pyflubumide provides control mainly of *Tetranychus* and *Panonychus* species including such that have developed resistance to conventional acaricides. Its highly specific pesticidal mode of action is by an inhibition of the mitochondrial complex II in the respiratory chain caused by *N*-deisobutylated pyflubumide, known as “pyflubumide-NH form”, to which pyflubumide is rapidly metabolized in the arachnid’s body (van Leeuwen et al., 2015; Furuya et al., 2017).

Pyflubumide has not been evaluated before by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). Most critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current Japanese or Organisation for Economic Cooperation and Development (OECD) test guidelines. A few non-GLP pharmacological and mechanistic studies were also of good quality and reported in detail. Since pyflubumide is a new compound, published information is very scarce.

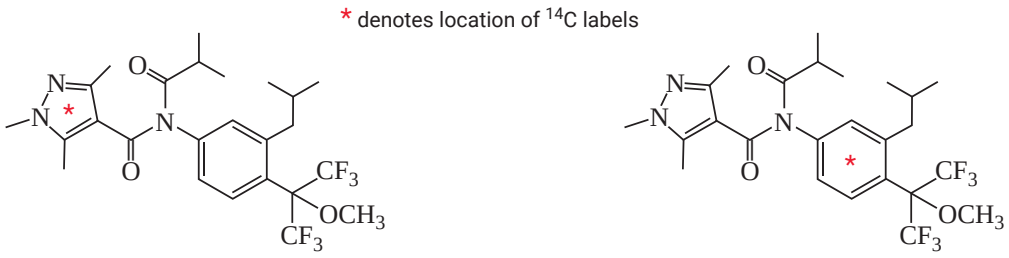
Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution, metabolism and excretion (ADME), as well as toxicokinetics, of pyflubumide have been investigated in Fischer rats by the oral route. For this purpose, the molecule was radiolabelled either on its pyrazole or its phenyl ring (see Fig. 1) and applied as a single oral dose in amounts of 1 or 100 mg/kg body weight (bw). In addition, a study with bile-fistulated rats was conducted to determine biliary excretion and thus allow an estimate of oral absorption. However, no experiment with repeated administration of pyflubumide is available, leaving some uncertainty, particularly with respect to the potential for bioaccumulation, since body fat was one of the tissues with highest residues in single dose studies. In addition, limited evidence of retention in fat was obtained in the 28-day feeding study in rats (Horiuchi, 2007). Lipophilicity of the compound is also suggested by a high octanol/water partition coefficient ($\log P_{O/W}$) of 5.34 (Furuya et al., 2017) and proven by significant excretion in the milk by nursing rats (Murata, 2012, see sub-sections 1.2 and 2.6(c)).

Figure 1. Structure of pyflubumide and position of radiolabels



[Pyrazole-3(5)-^{14}C]pyflubumide

[Phenyl-U-^{14}C]pyflubumide

(a) Oral route

Single-dose experiments with ^{14}C-(pyrazole ring labelled) pyflubumide

For all experiments, the test material (radiolabelled at the 3′- and 5′-positions in the pyrazole ring, radiochemical purity > 98%) was suspended in an aqueous vehicle of 0.1% Tween 80 in 0.5% sodium carboxymethyl cellulose (CMC). The dose levels in this study of 1 or 100 mg/kg bw had been chosen to reflect a "non-toxic" and a "slightly toxic" dose. The test item was applied by single oral gavage at a volume of 5 mL/kg bw to male and female Fischer (F344/DuCrlCrlj) rats. In total, eight groups of four animals per sex were employed. An overview on the study design is given in Table 1.

Table 1. Summary of the different groups and treatment regimens in the single dose ADME study with pyrazole-3,5-^{14}C radiolabelled pyflubumide

Endpoint under investigation	Single dose (mg/kg bw)	Number and sex of animals	Termination post-dosing (hours)	Samples
Pharmacokinetics	1	4 M, 4 F	168	Blood at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h post dosing
Pharmacokinetics	100	4 M, 4 F	168	
Mass balance, excretion and tissue distribution	1	4 M, 4 F	168	Expired air for 24 h; Urine and faeces at 24, 48, 72, 96, 120, 144, and 168 h; Organs/tissues at termination after 168 h
	100	4 M, 4 F	168	
Tissue distribution (interim investigations)	1	4 M, 4 F	6	Organs/tissues
	1	4 M, 4 F	24	
	100	4 M, 4 F	9	
	100	4 M, 4 F	24	

In males and females receiving the low dose of 1 mg/kg bw, the highest blood or plasma concentrations were measured 3–6 hours after dosing (Table 2). In high-dose males and females, maximum blood and plasma concentrations were reached after 12 h. Mean half-lives for excretion were consistently a little more than 24 hours when the blood was considered and much shorter when determined in plasma; this suggested a certain affinity to red blood cells (RBCs). All these parameters, including the area under the concentration–time curve (AUC), showed a remarkable similarity between males and females.

Table 2. Selected pharmacokinetic and elimination parameters (mean values) for pyrazole-3,5-^{14}C pyflubumide in male and female rats

Parameter	Low dose (1 mg/kg bw)		High dose (100 mg/kg bw)	
	Males	Females	Males	Females
T_{max} (h), blood/plasma	6/6	6/3	12/12	12/12
C_{max} (µg eq/g), blood/plasma	0.197/0.238	0.209/0.264	9. 1/10.6	8.8/10.0
$T_{½}$ (days), blood/plasma	1.07/0.61	1.06/0.59	1.30/0.71	1.31/0.81
AUC (µg equiv./h per g), blood/plasma	4.242/4.555	4.682/4.932	321.4/312.5	337. 1/320.7
Excretion in urine/faeces (% of dose) over 24 hours	2.79/74.62	3.74/75.35	1.58/65.45	2.13/64.20
Excretion in urine/faeces (% of dose) over 168 hours	3.29/92.47	4.35/94.98	2.21/92.84	2.77/89.68

The predominant excretion route was the faeces, the urine playing only a minor role (confirmed also by very little radioactivity in the cage wash) and no evidence of exhalation of the test substance was observed. The oral absorption rate could not be estimated from this data since biliary excretion was not determined and intravenous application for a possible comparison with the oral route was also not performed. Based on the biotransformation rate (see below) and a comparison of pharmacokinetic parameters, however, it seems reasonable to assume that absorption of the high dose (as a percentage) was a bit lower than that of the low dose. Again, there were no obvious differences between the sexes. Total recovery of radioactivity at 168 h was nearly complete at the low dose level and clearly above 90% in the animals receiving the high dose.

In both sexes, at 6 h (low dose) or 9 h (high dose), the highest levels of radioactivity were detected, in the liver, the adrenals, the gastrointestinal tract (GIT) and its contents, in fat and in the kidneys. Some radioactivity was also measured in bone marrow, indicating exposure of this highly perfused organ. With

regard to blood, erythrocytes contained more radioactivity than the plasma, suggesting some affinitiy of pyflubumide or its metabolites to RBCs. At termination after 168 h, all residues had markedly declined. Radioactive residues were still most abundant in liver and fat, followed by adrenals (females only), kidneys, and bone marrow (Yoshizane, 2010a).

Single dose experiments with ^{14}C-(phenyl ring labelled) pyflubumide

For all experiments, the test material (radiochemical purity > 98%) was suspended in an aqueous vehicle of 0.1% Tween 80 in 0.5% sodium CMC. Again, a low dose of 1 mg/kg bw or a high dose of 100 mg/kg bw was applied by single oral gavage at a volume of 5 mL/kg bw to male and female Fischer (F344/DuCrlCrlj) rats. An overview of the study design is shown in Table 3. Excretion, distribution and organ/tissue residues were investigated in low-dose males only since there were no differences in the pharmacokinetic parameters between the sexes and doses, and since urinary and faecal excretion was similar in quantity to the study with the pyrazole ring-labelled test substance. Since a complete study with another radiolabel is available for pyflubumide (Yoshizane, 2010a, see above), this is not considered a data gap or of concern but the actual study is regarded as supplementary only, providing additional information.

Table 3. Summary of the different groups and treatment regimens in the single-dose ADME study with phenyl-U-^{14}C radiolabelled pyflubumide

Endpoint under investigation	Single dose (mg/kg bw)	Number and sex of animals	Termination post-dosing (hours)	Samples
Pharmacokinetics	1	4 m / 4 f	168	Blood at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h post-dosing
Pharmacokinetics	100	4 m / 4 f	168	
Mass balance, excretion and tissue distribution	1	4 m	168	Expired air for 24 h;Urine and faeces at 24, 48, 72, 96, 120, 144, and 168 h; Organs/tissues at termination after 168 h
Tissue distribution (interim investigations)	1 1	4 m 4 m	6 24	Organs/tissues

In males and females receiving the low dose of 1 mg/kg bw, the highest blood or plasma concentrations were measured six hours after dosing (Table 4). In high-dose males and females, maximum blood and plasma concentrations were reached after 9–12 hours. Mean half-lives suggested a slightly faster elimination from blood than for the pyrazole-radiolabelled test substance (Yoshizane, 2010a). Again, there was not much difference in pharmacokinetic parameters between the sexes, although, as with the phenyl label, maximum blood and plasma concentrations were consistently higher in females.

The predominant excretion route of the phenyl label was via the faeces with the urine playing only a minor role and no evidence of exhalation of the absorbed radioactivity was observed; this was much as was found with the pyrazole label. Over the first 24 h following dosing, faecal and urinary elimination of the phenyl-labelled test material by low-dose males, in sum, was a bit lower than with the pyrazole label (66% compared with 77%) but total recovery at termination was of the same magnitude. Again, the oral absorption rate could not be calculated since biliary excretion was not determined in this study and intravenous application was not performed. Based on a comparison of pharmacokinetic parameters, however, it seems reasonable to assume that absorption of the high dose (as a percentage) was lower than that with the low dose.

Table 4. Selected pharmacokinetic and elimination parameters (mean values) for phenyl-U-^{14}C pyflubumide in rats

Parameter	Low dose (1 mg/kg bw)		High dose (100 mg/kg bw)	
	Males	**Females**	**Males**	**Females**
T_{max} (h), blood/plasma	6 / 6	6 / 6	9 / 12	12 / 12
C_{max} (µg equiv,/g), blood/plasma	0.193 / 0.254	0.219 / 0.297	9.5 / 11.1	12.3 / 14.5
$T_{½}$ (days), blood/plasma	0.61 / 0.55	0.59 / 0.54	0.74 / 0.65	0.69 / 0.60
AUC (µg equiv./h per g), blood/plasma	3.850 / 4.699	4.099 / 5.123	270.7 / 305.9	334.9 / 372.3
Excretion in urine/faeces (% of dose) over 24 hours	2.32 / 63.74	Not determined	Not determined	Not determined
Excretion in urine/faeces (% of dose) over 168 hours	3.31 / 91.95	Not determined	Not determined	Not determined

At six hours, the highest levels of radioactivity in the tissues and organs of low-dose males were detected in the liver, in fat, GIT, adrenals, kidneys, and lungs. Some radioactivity was also measured in bone marrow indicating exposure of this highly perfused organ. At 24 h, radioactivity in all organs and tissues had markedly declined but distribution was still the same. At termination after 168 h, very little radioactivity was still retained in the organs and tissues, with the highest relative values measured in liver and fat (Yoshizane, 2010b).

Study on biliary elimination

An additional study was performed in surgically bile-cannulated male Fischer (F344/DuCrlCrlj) rats to determine the amount of biliary excretion of ^{14}C-(pyrazole ring-labelled) pyflubumide and to investigate the metabolites in bile. The test material (radiolabelled at the 3′- and 5′- positions in the pyrazole ring, radiochemical purity > 98%) was suspended in an aqueous vehicle of 0.1% Tween 80 in 0.5% sodium CMC and applied as a single dose of 1 mg/kg bw by oral gavage to four animals. Immediately after dosing, the male rats were connected to free-moving sampling devices and transferred to glass metabolic cages to collect bile, urine, and faeces for 72 h. At 72 h after dosing, the animals were killed to obtain the GIT and its contents, liver and carcass. Radioactivity was measured in bile and excreta, in GIT contents and the carcass.

Mean cumulative excretion in the bile over 72 h accounted for 43% of which nearly three-quarters (31%) had been already excreted within the first 24 hours post-dosing. Total urinary excretion amounted to 5.9%. If the low amount of radioactivity in cage wash (ca 0.3 %) and the total carcass residues (2.8% at termination) are added to the biliary and urinary excretion, an oral absorption rate of 52% can be estimated for pyflubumide when applied at the low dose level of 1 mg/kg bw. Mean cumulative excretion via the faeces was ca 40%, representing the unabsorbed portion of the administered dose. Less than 1% of radioactivity was found in the GIT contents. A total recovery of ca 93% is considered acceptable for this type of study (Yoshizane, 2010c).

(b) Dermal route

No data were available for pyflubumide.

1.2 Biotransformation

Extensive metabolism was observed in the study with ^{14}C-(pyrazole ring-labelled) pyflubumide (Yoshizane, 2010a, see above). At the low dose level of 1 mg/kg bw, eight metabolites could be identified in the urine of males, along with only traces of the parent compound. In faeces, eight metabolites and the parent were found in much higher amounts than in urine. Two of the identified metabolites were unique to urine and two were unique to faeces, whereas the others were detected in both matrices. In females, the qualitative pattern was virtually the same with some quantitative differences compared to males. At the high dose level of 100 mg/kg bw, unchanged parent compound was found at a notably high percentage of around 40% in the faeces of males and females, but not in their urine, suggesting nearly complete biotransformation of the absorbed portion of the dose. Again, eight metabolites were identified in the urine of male rats and nine in females. In faeces, eight metabolites could be detected in both males and females (see Table 5). A similar number of plasma metabolites were found in males and females at T_{max}, that is 6 h (with the pyrazole label and in females only) or 3 h respectively, following dosing.

Table 5. Excretion of identified metabolites (% of dose) after single oral administration of low or high doses of ^{14}C-(pyrazole ring-labelled) pyflubumide (NNI-0711) to rats

Metabolite, number (as in Fig. 2) and designation (as in Annex 1)	Males				Females			
	1 mg/kg bw		100 mg/kg bw		1 mg/kg bw		100 mg/kg bw	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Pyflubumide (parent), 1	0.02	11.16	ND	42.63	ND	11.24	ND	38.35
NH- form *(N*-deisobutylated pyflubumide), NNI-0711-NH*, 2 (= B)	ND	11.23	ND	17.35	ND	10.86	ND	18.90
NNI-0711-NH-1-H-RfOH, 5 (= F)	0.05	4.28	0.03	1.04	1.66	13.49	0.37	2.09
NNI-0711-NH-1-H-5-CH_2OH-RfOH, 6 (= G)	ND	1.95	ND	0.37	N.d.	4.31	ND	0.35
NNI-0711-acid, 7 (= H)	0.04	ND	0.04	ND	0.05	ND	0.04	ND
NNI-0711-acid-1-H, 8 (= I)	0.06	ND	0.04	ND	0.05	ND	0.04	ND
NNI-0711-acid-5-CH_2OH, 9 (= J)	ND	ND	ND	ND	ND	ND	0.02	ND
NNI-0711-NH-1-H-3′-(2-OH)-RfOH, 11 (= O)	0.09	5.56	0-08	5.65	0.10	5.25	0.27	7.84
NNI-0711-NH-1-H-5-CH_2OH-3′-(2-OH)-RfOH, 12 (= P)	0.05	4.04	0.03	2.50	0.03	2.61	0.03	2.41
NNI-0711-NH-1-H-3′-(2-COOH)-RfOH, 13 (= Q)	0.30	8.21	0.09	2.74	0.20	2.48	0.11	1.50
NNI-0711-NH-1-H-3′-(3-OH)-RfOH, 14 (= R)	0.11	5.13	0.03	0.93	0.33	6.60	0.5	1.09
NNI-0711-NH-5- CH_2OH-3′-(2-COOH)-RfOH, 15 (= S)	0.13	1.41	0.06	0.76	0.07	0.65	0.05	0.41

ND Not detected or quantified

* The metabolite exhibiting main acaricidal activity in target pests

Source: Yoshizane, 2010a, (amended)

The main metabolic pathways comprised deacylation of the nitrogen atom, followed by hydroxylation and demethylation. Cleavage of the molecular backbone of pyflubumide was very limited. The proposed metabolic pathway is shown in Fig. 2.

In the study with ^{14}C-(phenyl ring-labelled) pyflubumide, metabolism was confirmed to be extensive in low-dose males but was not investigated in female rats or at the high dose level. In urine, five metabolites could be identified whereas the parent compound was not detected. In faeces, eight

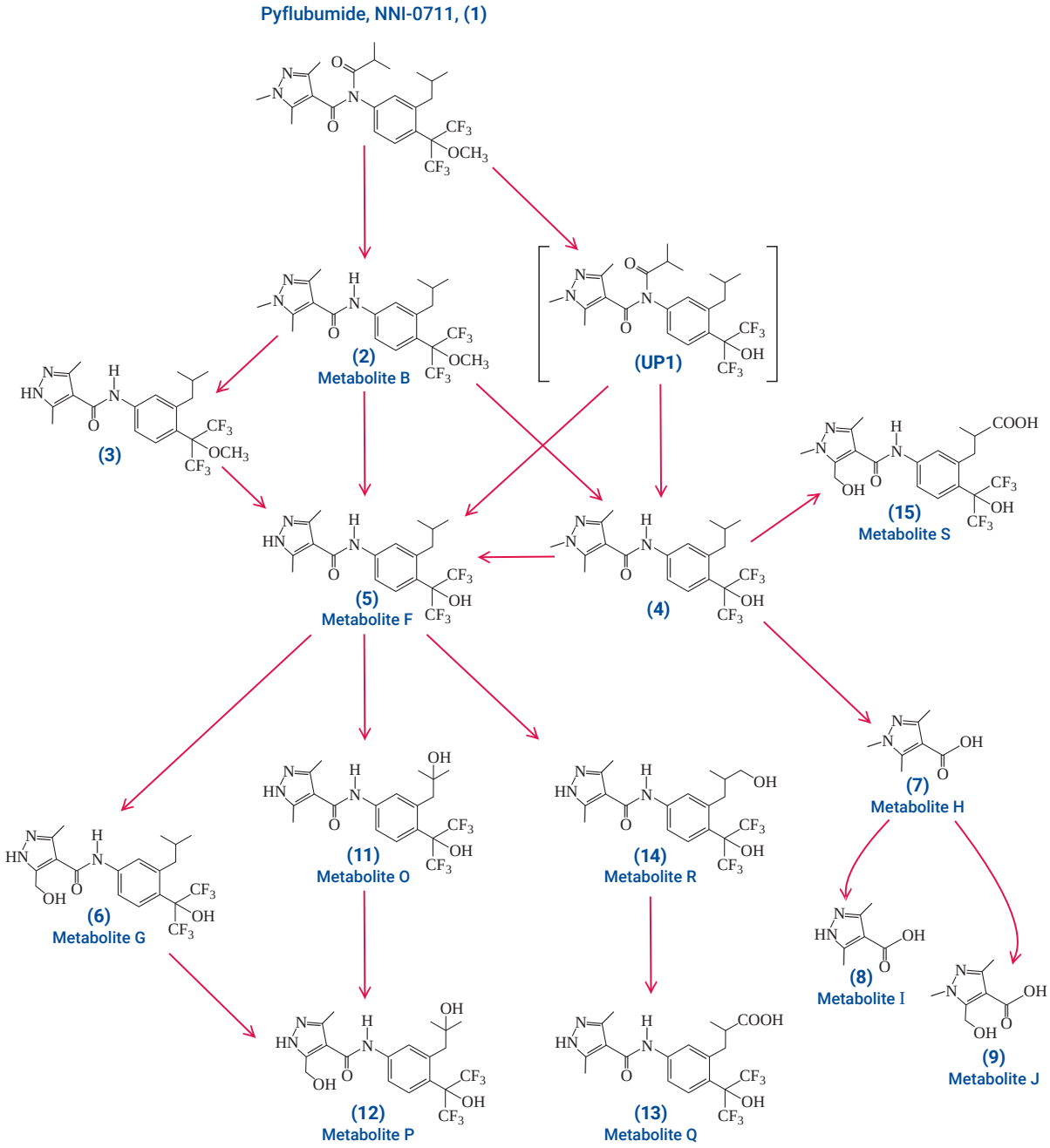
metabolites were identified, along with a small amount of the parent. At the time of T_{max}, (6 hours after dosing) nine metabolites in total were identified in plasma. The urinary, faecal and plasma metabolites were virtually the same as were found in the study with the pyrazole label. In an additional examination, 10 metabolites were found in the GIT contents, of which six were identical to those in plasma. This finding suggests a certain contribution by the gut microflora to the metabolism of pyflubumide. The most abundant substances in the gut contents were the (apparently unabsorbed) parent compound and its NH-form, (the *N*-deisobutylated pyflubumide, Metabolite B), which is thought to exhibit acaricidal activity in the target organisms (Furuya et al., 2017).

In the study of bile-cannulated rats, 12 metabolites were identified in the bile of which six could not be detected either in urine or faeces. Four biotransformation products were found in all three matrices, while two were found in bile and urine but not in faeces. In contrast, unchanged parent and its NH- form which occurred with the highest percentages of applied dose (7.7 and 12.1%, respectively) were found only in faeces. However, it must be acknowledged that the metabolites accounting for more than half of the total material excreted via the bile, could not be chemically identified.

Based on all available information, the main metabolites (exceeding 10% of administered dose in either excreta or plasma in the absorption, distribution, metabolism and excretion studies) were pyflubumide-NH (Metabolite B), pyflubumide-NH-1-H-RfOH (Metabolite F) and pyflubumide-NH-1-H-3'-(3-OH)-RfOH (Metabolite R), along with the parent compound (see also the list of metabolites in Annex 1) (Yoshizane, 2010a; Yoshizane, 2010b; Yoshizane, 2010c).

In rat milk, four major and four minor metabolites could be identified, along with their parent. The highest absolute amounts of the major metabolites were detected at 9 h following single oral administration to nursing rats of pyflubumide at a dose of 100 mg/kg bw. At this time point, the parent compound accounted for 9% of the radioactivity in milk, whereas the NH- form (Metabolite B) was the most abundant at 58%. A similar ratio between the parent compound and this metabolite had been detected after administration of 1 mg/kg bw. At the high dose level of 100 mg/kg bw, pyflubumide-NH-1-H (Metabolite C) accounted for 13%, pyflubumide-NH-RfOH (Metabolite D) for 7% and pyflubumide-NH-1-H-RfOH (Metabolite F) for 9%. The four minor metabolites G, O, P, and R (see Table 5) were found only in traces 24 h following application of the high dose. For more detailed description of the experiment, see sub-section 2.6(c) (Murata, 2012).

Figure 2. Presumed metabolic pathway of pyflubumide (obtained from sponsor)



2. Toxicological studies

2.1 Acute toxicity

A summary of acute toxicity, irritation and skin sensitisation studies is given in Table 6.

Table 6. Overview of acute studies with pyflubumide (inc. skin and eye irritation and skin sensitization)

Study type	Species	Result	Reference
Acute oral	Rat	LD_{50} > 2000 mg/kg bw (females)	Tsukushi, 2010a
Acute dermal	Rat	LD_{50} > 2000 mg/kg bw	Tsukushi, 2010b
Acute inhalation	Rat	LC_{50} > 5.23 mg/L air (4 h nose-only exposure)	Fukuyama, 2010
Skin irritation	Rabbit	Non irritant	Munechika, 2010a
Eye irritation	Rabbit	Non irritant	Munechika, 2010b
Skin sensitization (local lymph node assay)	Mouse	Negative	Munechika, 2011

(a) Lethal doses

An oral, a dermal and an inhalation study in rats are available and reveal that pyflubumide is of low acute toxicity via all three routes.

In an oral study which was performed according to OECD TG 423 (acute toxic class method), pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered by gavage to fasted female Fischer (F344/DuCrlCrlj) rats. The test item had been suspended in 0.5% CMC sodium salt in a 0.2% solution of Tween 80. For each experimental step, three animals were employed resulting in 12 rats on study in total. Two groups were given a dose of 300 mg/kg bw with a two day interval between. A further group received a single dose of 2000 mg/kg bw, five days after the initial groups, and yet another group 2000 mg/kg ten days after the initial groups. The animals were observed for mortality, clinical signs of toxicity and body weight effects for 14 days after dosing before they were killed and subjected to gross examination for evidence of organ or tissue damage.

There were no unscheduled deaths, nor clinical or behavioural signs of toxicity noted. All animals gained weight over the course of the study. Gross findings at termination were confined to diaphragmatic nodules on the liver in two rats receiving the low dose and in one high-dose rat. Because of this distribution, these nodules were considered to have occurred by chance and were not attributed to treatment. The oral LD_{50} in female rats was greater than 2000 mg/kg bw (Tsukushi, 2010a).

The low acute oral toxicity of pyflubumide was confirmed in a more recent acute neurotoxicity study in another rat strain, Sprague Dawley rats, in which no mortality was observed up to the top dose level of 2000 mg/kg bw (Barnett, 2018b, see section 2.6 (a) for further details). The only possibly treatment-related effects were a lower body weight gain in males and a reduced body temperature on the day of dosing in both sexes. Both findings were noted from the lowest tested dose of 500 mg/kg bw onwards but a clear dose response was lacking. In addition, a single-dose oral study in which several pharmacological parameters were investigated in rats and partly in mice also revealed a low acute toxicity, since most end-points were not altered up to doses as high as 2000 mg/kg bw (Tsuchiyama, 2012b, see section 2.6 (c) for further details).

In a limit test via the dermal route, a single dose of 2000 mg/kg bw (pyflubumide Lot no. 9HZ0013P, purity 95.8%) was applied to the shaved, intact skin of five male and five female Sprague Dawley rats. A control group of the same size was treated with the vehicle, 0.5% CMC sodium salt in 0.2% solution of Tween 80. The application site covered approximately 10% of each animal's body surface area and was semi-occluded for 24 hours after which the remaining test substance was removed with absorbent cotton moistened with warm water. The rats were observed for 14 days following application for mortality, clinical signs including skin lesions and body weight effects. At termination, they were killed and examined for gross pathological changes.

No mortalities were observed. All animals survived until scheduled termination. There were no systemic clinical signs and no signs of skin irritation. All rats, including those in the vehicle control

group, lost some weight (consistently less than 5%) on the day of application but gained body weight thereafter. This observation was attributed to the application method but not considered an adverse effect of the test substance. As in the oral study, the only gross finding was a diaphragmatic nodule on the liver which was observed in one vehicle control male. According to the study author, such nodules are inherent to rats of this strain and source. The dermal LD_{50} of pyflubumide in rats was >2000 mg/kg bw (Tsukushi, 2010b).

In an acute inhalation study, groups of five young male and female Sprague Dawley rats were exposed, snout only, for a 4 h period to pyflubumide dust (Lot no. 9HZ0013P, purity 95.8%). For successful generation of the dust, it was necessary to add 20% (w/w) of white carbon before the mixture could be finely pulverized. A vehicle control group of the same size (i.e., consisting of five males and females) was exposed to white carbon dust only. The nominal concentration of pyflubumide in this limit test was 5 mg/L. The actual mean (gravimetric) concentration was 5.23 mg/L, with a mass median aerodynamic diameter of 2.7 µm and a geometric standard deviation of 1.96. Animals were observed for mortality, clinical signs of toxicity and body weight effects for 14 days after treatment. At scheduled termination, rats were killed and examined for gross pathological changes.

No mortalities were observed. The only clinical sign was abnormal respiratory sound in three males and two females in the group exposed to pyflubumide. This finding was apparent for up to 4 h after the end of treatment and was not noted in the vehicle group. On the day of exposure, all animals, including the control group, lost some weight (less than or ca 10%) but gained body weight again afterwards. No test substance-related gross lesions were observed at necropsy. Accordingly, the inhalation LC_{50} of pyflubumide in rats was >5.23 mg/L (Fukuyama, 2010).

(b) Dermal irritation

Pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was applied as a single dermal dose of 500 mg (moistened with 0.7 mL of distilled water) to the shaved intact skin of three young adult Japanese White (Kbl:JW) rabbits (all males). The rabbits were exposed to the test substance under semi-occlusive conditions for 4 h after which it was removed and the treated 2.5 × 2.5 cm area gently cleaned. Test sites were visually observed for signs of dermal irritation at 1, 24, 48, and 72 h after test substance removal. The rabbits were also monitored for the possible occurrence of clinical signs and were weighed prior to application of the test substance and at study termination.

No signs of dermal irritation (such as erythema or oedema) were noted in any animal at any time point of reading during the study. There were no abnormal clinical signs and body weight was not affected by treatment since all animals slightly gained weight. Pyflubumide was not irritating to the skin in this study (Munechika, 2010a).

(c) Ocular irritation

A single dose of 59 mg of pulverized pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was instilled into the left eye of six young adult Japanese White (Kbl:JW) rabbits (all males). About 30 seconds later, the eyes of three of these animals were washed using warm distilled water whereas the other three rabbits remained unwashed. The right eye of each animal served as an individual control. The cornea, iris and conjunctiva were visually inspected under room light 1, 24, 48, and 72 hours after instillation and the findings scored according to the Draize criteria. For each animal, an individual ocular irritation index was determined. For the two small groups ("washed" and "non-washed"), a mean ocular irritation index (MOI) for the whole study duration was calculated, as well as an acute ocular irritation index, which was based on the maximum MOI at any reading. The rabbits were also monitored for the possible occurrence of clinical signs and were weighed prior to application of the test substance and at study termination.

The cornea and iris were not affected in any animal. The only finding was a slight congestion of the conjunctiva (redness score 1) as observed in one animal from the "washed" as well as in one rabbit from the "non-washed" group. In both cases, this reaction was noted one hour following application but had disappeared within 24 hours. No chemosis nor discharge were observed in any animal under study. There were no abnormal clinical signs nor was body weight affected by treatment since all animals very slightly gained weight. Based on these observations, pyflubumide may be considered non-irritating to the eyes since the only effect was transient, very minor and confined to only two out of six animals (Munechika, 2010b).

(d) Dermal sensitization

In a local lymph node assay according to OECD TG 429, 35 female CBA/JNCrlJ strain mice were assigned to seven groups of five animals each. Three groups were exposed to concentrations of 10, 25 or 50% (w/v) pyflubumide (Lot no. 9HZ0013P, purity 95.8%). Three other groups were treated with concentrations of 5, 10, or 25 % (v/v) of the positive control substance α-hexylcinnamaldehyde (HCA). Both compounds were dissolved in a 4:1 mixture of acetone and olive oil. The seventh group was the vehicle control group. The test or control mixtures were applied to the dorsal skin of the ears at a volume of 25 µL for three consecutive days. Five days after the first treatment, all animals were intravenously injected with ^{3}H-methyl thymidine (^{3}HTdR). Five hours later, they were killed and incorporation of the radiolabelled thymidine was measured in cells which had been prepared from drained auricular lymph nodes by means of liquid scintillation. For each group, the mean number of disintegrations per minute (dpm) for each animal and the stimulation index (SI) were calculated.

Based on SI of 1.1, 1.3 and 1.3 for the three groups which were treated with pyflubumide, it can be concluded that incorporation of ^{3}HTdR was not increased due to pyflubumide. In contrast, the positive control substance gave the expected increases in SI of 2.6 (5%), 3.9 (10%) or even 11.9 (25%). Accordingly, pyflubumide proved negative in the local lymph node assay and is not a skin sensitizer (Munechika, 2011).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

In a subacute feeding study, pyflubumide (Lot no. 7HZ0006N, purity 98.4%) was administered to male and female Crlj:CD1(ICR) mice (five animals per sex and dose level) for 28 consecutive days at dietary concentrations of 0, 20, 200, and 2000 ppm. The mean daily intakes for males were 0, 3.0, 32, and 297 mg/kg bw. The mean daily intakes calculated for females were 0, 4.1, 40, and 397 mg/kg bw. A further group receiving 10 000 ppm (mean daily dose not reported) was prematurely terminated on day six (females) or day seven (males) and excluded from further evaluation since this dose was clearly in excess of the maximum tolerated dose (MTD).

During the administration period, the animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded weekly. In addition, the mice were weighed on day three of the exposure period. At scheduled termination, blood samples were taken to determine erythrocyte parameters, platelet and white blood cells count before the animals were killed and necropsied. In addition to gross examination, organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and thymus were taken and selected organs subjected to histopathology. Livers in all groups were examined microscopically (except the 10 000 ppm group) whereas histopathological examination of adrenals, femur, heart, kidneys, pituitary, spleen, thymus and thyroid was confined to the control group and the group receiving 2000 ppm.

Effects at dose levels up to 2000 ppm which could be administered over the entire study period must be clearly distinguished from those observed in the animals receiving the excessive dose of 10 000 ppm. In the latter group, food intake was markedly reduced and a significant body weight loss of more than 10% already observed over the first three days of treatment. Most animals (four males and three females) displayed severe clinical signs of toxicity from day 4 (females) and day 5 (males) of the administration period onwards. These signs comprised decreases or even complete loss of spontaneous movements, hypothermia and smudge of fur around genitals and anus. All the four males and three females displaying these signs were eventually found dead on days 5–7 (males) or 4–6 (females).

In contrast, at the lower dose levels up to 2000 ppm, there were no premature deaths and no clinical signs of toxicity were noted at any dietary concentration in males or females. Furthermore, there were no adverse test substance-related effects, in any group, on body weight, body weight gain, food consumption, or food efficiency. In fact, body weight gain appeared even higher in all treated female groups compared to the controls, and the differences were occasionally statistically significant in the groups receiving 200 or 2000 ppm.

Haematological examination after 28 days of continuous substance administration did not reveal any effects in male mice. In females, however, mean cell volume of the erythrocytes and mean cell haemoglobin were significantly reduced at 2000 ppm (by ca 5%) providing evidence of anaemia.

At necropsy, no lesions were found that could be related to treatment. However, a significant and dose-related increase in mean liver weights was observed in females at 200 ppm (+40% for absolute and +30% for relative organ weight) and 2000 ppm (+50% for absolute and +42% for relative organ weight). The same effect in males was confined to the high dose of 2000 ppm and was a bit less pronounced (+33% for absolute and +28% for relative organ weight). These increases were accompanied by histopathological liver findings. In both sexes, centrilobular hypertrophy was seen in the livers of all animals receiving 200 or 2000 ppm, severity increasing with dose. In addition, vacuolar degeneration was observed in three males and three females from the 2000 ppm group, whereas a similar effect in one male and one female at 200 ppm was of the lowest degree and, thus somewhat equivocal. No effects were noted on organs other than the liver.

On balance, the lowest dose of 20 ppm (equal to a daily intake of 3 mg/kg bw for males and 4.1 mg/kg bw for females) was the NOAEL in this study, based on liver findings from 200 ppm (32/40 mg/kg bw per day) onwards. The liver was the target organ of toxicity in both sexes even though it was noted that this NOAEL was not consistent with the results obtained in the subsequent 90-day study in which liver toxicity was confined to a much higher dose of 4000 ppm (however, it is worth noting that different batches of the test substance were administered.) In addition, a weak effect on RBCs could not be excluded in females at 2000 ppm (396 mg/kg bw per day). Excessive toxicity was observed at 10 000 ppm (Inagaki, 2009).

Study 2

In a 13-week feeding study, pyflubumide (Lot no. 8HZ0008P, purity 96.9%) was administered to male and female Crlj:CD1 (ICR) mice (10 per sex and dose) at concentrations of 0, 40, 400, and 4000 ppm. The mean daily intakes by male mice were 0, 5.3, 51, and 505 mg/kg bw. Calculation of the mean daily intakes for female mice revealed 0, 6.4, 64, and 596 mg/kg bw for the different groups. The animals were observed daily for mortality and clinical signs. Ophthalmoscopy, however, was not performed. Body weight and food consumption were recorded weekly. Food efficiency and mean daily substance intake were calculated. At scheduled termination after 13 weeks of treatment, blood samples were taken from all animals to determine erythrocyte parameters, reticulocyte, platelet, and total and differential white blood cell counts. Except for haemoglobin, all these parameters were measured by means of laser flow cytometry. Clinical chemistry comprised the most relevant parameters but it must be noted that blood coagulation was not investigated and urine not analysed at all. The mice were killed, gross examination performed and a representative selection of organs and tissues taken. Weights of adrenals, brain, heart, kidneys, liver with gall bladder, ovaries, spleen, thymus, testes and epididymis were determined in all animals. Full histopathology of these and a wide selection of other organs and tissues was performed on all animals from the control and high dose groups. In addition, livers and all gross lesions were microscopically examined in all mice, along with thyroids and adrenals in low- and mid-dose females.

There were no unscheduled deaths at any concentration and no clinical signs of toxicity were observed in males or females. Body weight, body weight gain and food efficiency were not affected in any group. In high-dose males and females, lower food consumption (reduction of less than 10%) was observed during the first week of treatment but normalized thereafter.

A number of statistically significant alterations in haematological parameters were noted in high-dose females as well as in mid- and high-dose males (Table 7). An increase in mean corpuscular haemoglobin concentrations (as seen in males from 400 ppm) is, in contrast to its reduction, not regarded as adverse. There was also a 50% decrease in eosinophils and a 15% increase in platelet count in high-dose females. These isolated changes are not considered to be of concern.

Changes in clinical chemistry parameters (Table 7) were confined to top-dose animals and clearly indicated some liver toxicity. The increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in plasma, together with the skewed ratio between protein fractions in males, suggest liver damage. The higher triglyceride levels in males and females might point to a possible impact of pyflubumide on lipid metabolism, whereas reduced cholesterol was of no toxicological relevance. Raised bilirubin levels might reflect the weak effect on RBCs. The increase in creatinine in high-dose males was only slight and not accompanied by any evidence of kidney damage.

Table 7. Selected haematological and clinical chemistry findings in the 13-week feeding study in mice at termination (mean values for males/females, n = 10 per sex and dose)

Dose level (ppm)	0	40	400	4000
Haematology				
Haematocrit (%)	43.1 / 43.9	43.8 / 43.3	42.0 / 43.2	41.4 / 40.3**
Haemoglobin (g/dL)	12.9 / 13.4	13.3 / 13.2	12.9 / 13.3	12.7 / 12.5**
Mean cell volume (fL)	52.5 / 52.6	52.5 / 51.8	50.4** / 52.0	50.2** / 50.8*
Mean cell haemoglobin concentration (g/dL)	29.9 / 30.4	30.4 / 30.5	30.6** / 30.9	30.6* / 30.9
Clinical chemistry				
AST (U/L)	52 / 53	61 / 52	66 / 58	111** / 144**
ALT (U/L)	36 / 23	47 / 23	57 / 26	201** / 189**
Triglycerides (mg/dL)	75 / 118	57 / 77	58 / 79	126** / 271**
Cholesterol (mg/dL)	123 / 89	119 / 97	122 / 78	91** / 45**
Albumin (g/dL)	3.00 / 3.30	2.97 / 3.33	3.03 / 3.29	2.78** / 3.17
Albumin/globulin ratio	1.60 / 2.25	1.59 / 2.28	1.54 / 2.31	1.38** / 2.04
Bilirubin (mg/dL)	0.08 / 0.07	0.10 / 0.07	0.09 / 0.07	0.09 / 0.09**
Creatinine (mg/dL)	0.14 / 0.18	0.15 / 0.15	0.15 / 0.16	0.18* / 0.15

* $p < 0.05$, ** $p < 0.01$, Dunnett's test

At necropsy, enlargement of the liver became apparent in all high dose animals. In females, the bigger size of the organ was accompanied by a grossly visible accentuated lobular pattern. In line with that, absolute and relative liver weights were markedly increased in males and females receiving 4000 ppm. Compared to the control groups, mean liver weight was doubled in females and nearly 70% higher in males. In addition, absolute and relative spleen weight was significantly increased in high-dose males (+21% for absolute and +24% for relative organ weight) and in mid (+26% for absolute, +35% for relative organ weight) and high-dose females (+31% for absolute, +39% for relative orgsn weight) even though the dose–response curve was very flat for females. Other changes in organ weights comprised a higher relative weight of heart and adrenals in high-dose males but the increase over the control values was moderate (10% or less) and no such effect was apparent in females.

Histopathological examination of livers revealed the frequent occurrence of fatty changes in both sexes at the high-dose level, but their localization was different. In seven out of 10 males, there was periportal fatty degeneration whereas this finding was characterized as centrilobular in all females of this group. Single-cell necrosis was observed in seven high-dose males and five high-dose females and centrilobular hypertrophy of liver cells was present in all animals from this dose group. No notable liver histopathology was observed at 400 ppm.

Microscopic findings in organs other than the liver were confined to high-dose females. They comprised follicular cell hypertrophy of the thyroid in 5/10 animals and eosinophilic changes in the zona fasciculata of the adrenals in the same number of female mice. The higher spleen weight was not accompanied by any remarkable microscopic lesions at the top dose in neither sex: mid-dose animals were not examined.

In this guideline 90-day study in mice, the mid dose of 400 ppm (equal to 51 mg/kg bw per day in males, 64 mg/kg bw per day in females) was considered the NOAEL, based on liver toxicity in both sexes at the top dose of 4000 ppm (505 mg/kg bw per day in males, 596 mg/kg bw per day in females). In addition, at the same high dose, hypertrophy of the thyroid and eosinophilic changes to the adrenals were seen in females, and RBC parameters were reduced in males. Spleen weight was increased in top-dose males and in females from 400 ppm. This organ weight increase, in the absence of histopathological changes, was considered treatment-related but not adverse. The same holds true for the marginal decrease in mean RBC volume in mid-dose males since this was the only significant alteration in erythrocyte parameters at this dose level (Kashimoto, 2009).

Rat

Study 1

In a 28-day feeding study, pyflubumide (old developmental code CE-164059, lot no. 9, purity at least 99%) was administered to male and female Fischer (F344/DuCrlCrlj) rats (five per sex and dose) at concentrations of 0, 200, 2000, or 20 000 ppm. Due to severe clinical signs and mortality in high dose-males and females, the top-dose group was terminated after one week and the few survivors killed. The overall mean daily intakes at the remaining dose levels were 0, 17, and 137 mg/kg bw for males and 0, 17, and 140 mg/kg bw for females. In addition, a small satellite group was included to support the toxicokinetic and tissue residue investigations. It comprised one male and one female on control diets plus two males and females which received the dose of 2000 ppm but only for seven days.

During the administration period, the animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded weekly. Ophthalmoscopy took place prior to treatment and at its end. Urinalysis was conducted on day 23. Assessment for neurological functions (functional observational battery, FOB) was performed before the start of administration and on treatment days 7 and 28. The FOB comprised assessment of motor activity, assessment of visual responses and of those to touch, sound or pain and of the aerial righting reflex, as well as the determination of grip strength and of landing foot splay of hindlimb. At scheduled termination, blood samples were taken to determine erythrocyte parameters, platelet and white blood cells count and a number of clinical chemistry parameters including thyroid hormones triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) in serum. In addition to gross examination, adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, testes, thymus and thyroid were weighed. Histopathological examination was first performed on selected organs and tissues from the control group and from the animals receiving 2000 ppm. In the case of any apparent lesions, preserved samples from the low-dose animals were also examined.

Following termination, test substance concentrations were determined in serum, liver and fat in all surviving animals from the main and satellite groups. After four weeks of feeding, no pyflubumide was apparent, but some metabolites were detected in blood serum at concentrations which were proportional to the dose in males, but showed no such pattern in females. Meaningful assessment of liver residues was not possible since the only data provided was obtained from one male and one female receiving 2000 ppm for four weeks, and from one male and one female from the 20 000 ppm group which were killed in a moribund condition after six or eight days. The parent compound itself was detected in fat, along with its metabolites. When the residues in fat after 7 or 28 days of administration of 2000 ppm were compared, a tendency to increase was noted since the concentrations of the parent compound at the end of treatment were twice to nearly 10 times greater than after one week of feeding. Even though these preliminary findings suggest at least the possibility of some retention in fat, no firm conclusion may be drawn since only one male and one female were examined on day 8.

At the excessive dose level of 20 000 ppm, one male rat was found dead on day 8 whereas the other rats had to be killed for humane reasons on days 7 or 8. In females, three were found dead on days 4, 5, and 6, respectively, and the remaining rats killed as moribund on day 6. Clinical signs were noted from day 1 onwards and comprised piloerection, prone position, abnormal gait, hypothermia, smudge of fur, lacrimation, discoloration of eyeballs, diarrhoea, a decrease or loss of spontaneous movement, but in one case also hyperactivity. Neurological examination revealed lower grip strength and reduced motor activity. Food intake was reduced and the animals lost weight.

Up to the dose level of 2000 ppm, no premature deaths occurred and the only test substance-related clinical sign was thin appearance at the high-dose level. In fact, mean body weight was reduced in males from the 2000 ppm group from day 14 onwards and the difference from the control group was statistically significant ($p < 0.001$ in Dunnett's multiple comparison test). In females, the same effect was apparent at this dose level only at the end of treatment whereas the 200 ppm-group displayed a higher mean body weight from day 14 onwards. The lower body weight gain at 2000 ppm in both sexes was paralleled by lower food consumption. However, males and females fed 200 ppm tended to consume more food, at least for some periods. In line with these findings, food efficiency was severely compromised in males and females at 2000 ppm.

The FOB at day 28 revealed a reduction in forelimb and hindlimb grip strength in males receiving 2000 ppm but no effects in females. This observation could well reflect the much lower body weight.

There were no ophthalmoscopic findings that could be attributed to treatment.

Haematology revealed significant reductions in nearly all (except mean corpuscular haemoglobin) RBC parameters in males at 2000 ppm. In addition, total leucocyte count and platelets were decreased in this group. In the males receiving 200 ppm, only RBC count was decreased but there was a clear dose response for this parameter. In females, RBC count, haematocrit and haemoglobin were significantly reduced at 200 and 2000 ppm. A clear dose response was apparent. Mean erythrocyte volume was depressed in the 2000 ppm group only. As with males, total leucocyte count was also reduced in that group.

There were a number of significant alterations in clinical chemistry parameters, in particular in the 2000 ppm male rats. Some of these effects, although treatment-related were not adverse, for example, lower activity of AST or alkaline phosphatase (AP). However, an adverse effect on the liver (suggesting perhaps cholestasis) was indicated by the increased activity of γ-glutamyl transpeptidase in high-dose males, as well as by a significantly higher total cholesterol concentration. Albumin and total protein were also increased. The same findings were obtained in females fed 2000 ppm, but cholesterol was already increased in the group receiving 200 ppm. In contrast, urinalysis did not indicate any adverse findings.

Reduced absolute organ weights of adrenals, brain, heart, kidneys, spleen, thymus, and testes in high-dose male rats were most likely related to the lower body weight in this group. Relative organ weights of liver, testes, pituitary and brain were increased whereas those of kidneys, spleen, and thymus were decreased. Because of the dramatically reduced body weight (by more than 25%), organ weight data at this high dose level are difficult to interpret. At 200 ppm (where the body weight was similar to the controls), there was an increase in absolute and relative liver and heart weight. In females, the pattern was very similar.

The most prominent organ weight finding, however, was a very large increase in absolute and relative thyroid weight in both sexes receiving 2000 ppm which cannot be explained by the lower body weight. In male rats, absolute thyroid weights ranged from 82.1 to 106.8 mg in the treated group compared to a range from 17.6 to 20.1 mg in the controls. In females, the range in the group fed 2000 ppm was 63.1–82.1 mg compared to 9.3–13.4 mg in the control group. Measurement of thyroid hormones revealed a significant reduction in T3 and T4 and a very large increase in TSH in males and females receiving 2000 ppm.

Gross examination revealed a reddish region in the thymus in four out of five males at 200 and 2000 ppm. Discoloration and clear lobules of the liver were noted in all 2000 ppm males and the thyroids were enlarged in four of them. The same liver, thymus and thyroid findings were reported to have occurred in females receiving 200 and 2000 ppm.

Main histopathological findings were follicular cell hypertrophy in the thyroid of males and females at 200 and 2000 ppm. Severity increased with dose. Follicular cell hyperplasia was noted in all animals of both sexes at 2000 ppm. Liver findings in males and females comprised hepatocyte hypertrophy and cytoplasmic vacuolation but were confined to 2000 ppm rats, at which dose cellular vesicles were also noted. Further organs with lesions were, in both sexes, the kidneys and the pituitary. Small droplets and vacuolization were observed in the epithelium of the proximal renal tubules at 2000 ppm. At the same dose, hypertrophy, hyperplasia and vacuolization of basophilic cells in the pituitary were noted.

On balance, a NOAEL could not be established from this 28-day study in rats since organ weight changes (heart, liver, thyroid), histopathological lesions of the thyroid and alterations in some haematological and clinical chemistry parameters observed at the low dose of 200 ppm (16 mg/kg bw per day) already. The main target organs of toxicity of pyflubumide in the rat were the thyroid, the liver, and the blood, but additional adverse findings were seen at 2000 ppm (137/140 mg/kg bw per day) in kidney, thymus and pituitary (Horiuchi, 2007).

Study 2

In a subsequent 90-day feeding study, pyflubumide (Lot numbers 7HZ0003P, purity 97.7% and 7HZ0006N, purity 98.4%) was administered to male and female Fischer (F344/DuCrlCrlj) rats (10 per sex and dose) at dietary concentrations of 0, 20, 200, and 1200 ppm. Two additional (satellite) groups of the same size as above were kept to investigate the reversibility of adverse findings. These groups were either fed an untreated diet or 1200 ppm pyflubumide for 90 days but observed then for a four-

week recovery period during which all the animals were given substance-free diet before being killed. In the main study, the mean daily intakes for male rats were 0, 1.2, 12, and 72 mg/kg bw, respectively. The mean daily intake in the satellite group receiving 1200 ppm was virtually the same at 72 mg/kg bw. The mean daily intakes for female rats were 0, 1.4, 14, and 81 mg/kg bw, the highest dose being used for the satellite group also. The dose levels were selected on the basis of the 28-day study by Horiuchi (2007) as reported above in Study 1.

Rats were observed daily for mortality and clinical signs of toxicity. Ophthalmoscopy was conducted on all animals during the acclimatization period and on all animals from the control and high-dose groups (including satellite groups) at week 13 of treatment. Since there was no evidence of adverse findings, the ophthalmological examination was not repeated in the satellite groups at the end of the recovery period. An FOB comprising investigations of motor activity, grip strength and responses to various sensory stimuli was conducted on all animals on study at week 11. Body weight and food consumption were recorded weekly, the latter for three consecutive days per week. Food efficiency and mean daily substance intake were calculated. At scheduled termination after 13 weeks of treatment, or at the end of the recovery period, blood samples were taken from all animals to determine erythrocyte parameters, as well as reticulocyte, platelet, and total and differential white blood cell counts. In addition, prothrombin time and activated partial thromboplastin time were determined. Bone marrow samples were taken from the femur or sternum of all animals at terminal kill to measure the nucleated bone marrow cell count and to prepare bone marrow smears for cytology if necessary. Clinical chemistry at terminal kill or at the end of the recovery period comprised the most relevant parameters. Urinalysis was conducted on all animals at week 13 and then, again, in the satellite groups, at the end of the recovery period. At scheduled termination after 13 or 13 + 4 weeks, the rats were killed, gross examination conducted and a representative selection of organs and tissues taken. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, testes with epididymis, thymus, thyroid with parathyroid, and uterus were determined in all animals. Full histopathology of a wide range of organs and tissues was performed in all animals from the main study control and high-dose groups. In the low- and mid-dose groups, histopathological examination was confined to liver, thyroid, testes and epididymis and all gross lesions. In the satellite groups, liver, spleen, thyroid and bone marrow were examined under the microscope.

There were no unscheduled deaths at any concentration and no clinical (including ophthalmological) signs of toxicity were observed that could be clearly attributed to treatment in either males or females. Very few findings such as an increase in hindlimb grip strength and a transient decrease in motor activity in the high-dose satellite group became apparent during the functional observations in females, but their interpretation is difficult since they were not confirmed at the same dose level in the main study.

From the first week of treatment onwards, mean body weights were increased in mid- and high-dose female groups and in males receiving the highest dose. As compared to the control groups, significant increases by 4–8% were noted for the individual weeks and, in females, there was no difference between the dose levels at 200 and 1200 ppm. At the end of the recovery period, the effect had completely disappeared in the high-dose groups and mean body weight was the same as in the controls. It is worth noting that the satellite group males and females, on average, lost 12 or 9 g, respectively, in the first week of the recovery period, that is just after cessation of treatment. Food consumption was reduced in the first week of treatment at the top dose level in both sexes, but thereafter consumption was significantly increased in most weeks of the administration period in high-dose males (by 5–11%) and occasionally in females receiving 200 or 1200 ppm. During the recovery period, the only finding was an increased food intake in its first week in both sexes, despite the body weight losses. Food efficiency was higher in both sexes at the top dose level during the first week of treatment and tended to remain higher over the course of the study in females. As expected because of higher food intake with concomitant body weight losses, food efficiency was markedly decreased in the first week of the recovery period in both males and females but was similar to the control values thereafter.

A number of significant alterations in RBC parameters were noted in mid- and high-dose males and females (Table 8). In addition, coagulation was impaired at the top dose level in both sexes. In males only, neutrophil count was reduced at the two upper dose levels. In high-dose females, bone marrow cytology, as well as a significant increase in nucleated cells in bone marrow, suggested a regenerative response to anaemia even though these findings could also be indicative of bone marrow hyperplasia

as a direct effect of treatment on this tissue. In fact, erythroid cells (most likely this is synonymous with proerythroblasts), immature and mature erythroblasts were observed more frequently than in the control, with the difference being statistically significant. After the recovery period, the changes in RBC parameters persisted whereas the other alterations, including bone marrow changes, had disappeared or were at least alleviated.

A number of significant changes in clinical chemistry parameters were detected in males and females at the mid- and high-dose levels. Only a few of these, however, were considered adverse (Table 8). These findings comprised a marked increase in γ-glutamyltranspeptidase (GGTP) activity and in glucose concentration in high-dose females. Evidence of liver toxicity was further supported by higher cholesterol in mid- and high-dose females, with a non-significant increase in high-dose males too, increases in total protein and albumin in both sexes at the two upper dose levels and of globulin at 1200 ppm. In addition, lower triglyceride levels were observed in mid- and high-dose male rats. Even though a decrease in this parameter, in contrast to an increase, is usually considered to be of no concern, it is sometimes regarded as an early (but mostly overlooked) indicator of hepatotoxicity in the rat whereby different mechanisms might be involved (Provost, Hanton & LeNet, 2003). It was noted that the mean values for this parameter, in all groups, were extremely different between males and females. It is a common finding that triglyceride levels are higher in male rats than in females but, in this pyflubumide study the concentrations measured in females appear remarkably low. Total bilirubin was significantly decreased in high- and mid-dose males and in high-dose females. Such a decline is not considered adverse but might suggest that the observed RBC findings were not haemolytic in nature. Lower activities of some liver enzymes (ALT, AST, AP, creatine phosphokinase) in both sexes at the mid- and/or high-dose levels may be treatment-related but, again, are not regarded as adverse. Urinalysis did not reveal any abnormal findings in any group.

Table 8. Selected haematological and clinical chemistry findings at termination in the main study groups from a 13-week rat feeding study (mean values for males/females, n = 10 per sex and dose)

Dose level (ppm)	0	20	200	1200
Haematology				
Haematocrit (%)	48.1 / 45.6	48.0 / 45.4	46.8** / 44.0**	45.9** / 42.5**
Haemoglobin (g/dL)	15.8 / 15.8	15.8 / 15.7	15.5** / 15.3*	15.1** / 14.8**
Red blood cell count (10^6/μL)	9.41 / 8.47	9.39 / 8.44	9.27 / 8.38	9.09** / 8.20**
Prothrombin time (s)	20.4 / 17.1	21.6 / 16.9	22.7 / 17.7	28.1** / 17.4
Activated partial prothrombin time (s)	27.0 / 18.0	27.7 / 18.4	28.1 / 18.8	32.6** / 20.3**
Clinical chemistry				
AST (U/L)	82 / 73	78 / 68	76 / 71	67** / 65
ALT (U/L)	51 / 35	46 / 34	43* / 34	39** / 30
GGTP (U/L)	0 / 1	0 / 1	0 / 1	0 / 3**
Glucose (mg/dL)	134 / 117	130 / 120	137 / 116	136 / 124*
Triglycerides (mg/dL)	69 / 10	62 / 9	41** / 9	36** / 9
Cholesterol (mg/dL)	52 / 63	50 / 66	55 / 69*	56 / 89**
Total protein (g/dL)	6.55 / 6.13	6.55 / 6.24	6.78** / 6.60**	7.06** / 6.94**
Albumin (g/dL)	4.40 / 4.33	4.41 / 4.41	4.54** / 4.65**	4.68** / 4.81**
Globulin (mg/dL)	2.15 / 1.80	2.14 / 1.83	2.24 / 1.95**	2.38** / 2.13**

* $p < 0.05$, ** $p < 0.01$, Dunnett's test

After the recovery period, the following findings were still present in the high-dose satellite animals: higher glucose concentration in females; higher cholesterol concentration in males (whereas normalization was noted in females); higher globulin and total protein concentrations in males even though a distinct decline (when compared to the results at the end of treatment) was apparent.

The only gross pathological effect at necropsy immediately after treatment was enlargement of the thyroid in all male and female animals receiving 1200 ppm. In the high-dose satellite groups, however, this finding could no longer be confirmed. Several organ weights were increased either in the high-dose group only or at the two upper dose levels (Table 9) indicating an effect of pyflubumide on the liver, the heart, the spleen, and the thyroid. The mean weights of a number of reproductive organs in males and females were also affected but in both directions. At the low-dose level of 20 ppm, relative liver weight was already significantly increased in females. Since the absolute organ weight was comparable to the controls (in contrast to the findings at 200 and 1200 ppm) and since there were no concomitant histopathological changes in the low-dose female group, this low-dose effect is not considered adverse. The significant organ weight changes at 200 and 1200 ppm were dose-related, with the ovaries being the only exception. The high-dose effect on thyroid weight in both sexes was by far the most pronounced finding in this study.

Table 9. Mean organ weights at terminal kill in the 90-day rat feeding study (n=10 per sex and dose)

Sex, dose (ppm)	**Males**				**Females**			
	0	**20**	**200**	**1200**	**0**	**20**	**200**	**1200**
Mean absolute liver weight (g)	7.07	6.92	7.43	9.08**	3.64	3.74	4.26**	5.20**
Mean relative liver weight (%)	2.29	2.26	2.36	2.86**	2.24	2.33*	2.47**	3.05**
Mean absolute heart weight (mg)	930	929	1100**	1262**	563	575	706**	819**
Mean relative heart weight (%)	0.30	0.30	0.35**	0.40**	0.35	0.36	0.41**	0.48**
Mean absolute spleen weight (mg)	618	611	637	654*	386	381	420**	435**
Mean relative spleen weight (%)	0.20	0.20	0.20	0.21	0.24	0.24	0.24	0.25**
Mean absolute thyroid weight (mg)	18.0	18.0	19.8	32.5**	13.0	12.7	12.7	28.4**
Mean relative thyroid weight (%)	0.0058	0.0059	0.0063	0.0102**	0.0080	0.0079	0.0074	0.0166**
Mean absolute testes weight (mg)	3193	3177	3092*	3036**	-	-	-	-
Mean relative testes weight (%)	1.04	1.04	0.99*	0.96**	-	-	-	-
Mean absolute epididymis weight (mg)	968	946	920*	904**	-	-	-	-
Mean relative epididymis weight (%)	0.31	0.31	0.29	0.29**	-	-	-	-
Mean absolute ovary weight	-	-	-	-	56.2	59.5	74.9**	71.4**
Mean relative ovary weight (%)	-	-	-	-	0.034	0.037	0.044**	0.042**

* $p < 0.05$, ** $p < 0.01$, Dunnett's or Dunnett-type test

Following the recovery period, significant increases in absolute and relative organ weights of heart, liver, spleen, and thyroid were still present in both sexes even though the differences compared to the controls were, with exception of the spleen, a bit smaller than in the main study. In contrast, no organ weight changes in the male or female reproductive organs were apparent any longer. However, there were increases in absolute adrenal and kidney weight in females and in relative kidney weight in males which had not been observed before and are therefore regarded as chance findings.

In contrast to the marked organ weight changes, only a few histopathological lesions were observed. In high-dose males clear increases in diffuse liver cell hypertrophy (7/10 animals affected as compared to zero in the control group) and in diffuse follicular cell hyperplasia of the thyroid (in 10/10 males as compared to 0/10 in the control) were noted. No further microscopic changes were observed in any group. In the satellite group receiving 1200 ppm, the only finding at the end of the recovery period was variable size and irregular shape of thyroid follicles seen in all males from this group. In females, the same histopathological findings as in males were noted at the end of treatment and these were also confined to the high-dose groups where all animals were affected, compared to a zero incidence in the controls, low- and mid-dose groups. In the high-dose satellite group, thyroid follicles of various sizes and irregular in shape were observed in all females. In addition, deposition of brown pigment was noted in the spleen of seven animals, which had not been seen before.

The lowest dose of 20 ppm (equivalent to a mean daily intake of 1.2 or 1.4 mg/kg bw in males and females, respectively) was the NOAEL in this study and was mainly based on the higher heart weight at the two upper dose levels in both sexes, supported by higher liver and (absolute) spleen weights at least in female rats, even though these changes were mostly not associated with histopathological findings. In addition, treatment-related and persisting haematological findings and alterations in some clinical chemistry parameters suggestive of liver toxicity were observed from 200 ppm (equivalent to 12/mg/kg bw per day in males, 14/mg/kg bw per day in females). The thyroid was another target organ but the very pronounced effects on this organ were confined to the highest dose level, 1200 ppm. These effects, along with some weight changes in reproductive organs, might suggest endocrine disturbances but hormones were not measured to substantiate this assumption (Kuwahara, 2009).

Dog

Study1

In a 28-day range-finding study, pyflubumide (Lot no. 7HZ0005N, purity 98.5%) dietary doses of 0, 30, 300, or 3000 ppm were fed to two male and two female Beagle dogs per group. The calculated mean daily intakes at these different dose levels were 1.1, 10, and 76 mg/kg bw in males and 1.1, 11 and 80 mg/kg bw in females. A wide range of standard parameters was examined. In addition, electrocardiography (ECG) was performed, blood pressure measured and blood hormone concentrations of T_3, T_4 and TSH determined. Also, thyroid microsome samples were analysed for protein concentration and thyroid peroxidase (TPO) activity.

The only clinical sign that was attributed to treatment was tachycardia (> 160 heart beats per minute) in males and females receiving 3000 ppm on study day 8. In contrast, blood pressure and ECG parameters were not affected. Apparently, there was an effect on body weight since high-dose males and females either failed to gain weight, or even lost weight, over the treatment period. This might be due to reduced food consumption in these groups, on the other hand, similar intergroup and interindividual differences in food intake had already been observed in the pre-dosing interval and, thus, it is equivocal if a lower food intake was indeed sufficient to explain the adverse effect on body weight. Haematology, urinalysis and blood clinical chemistry did not reveal clear effects but analysis was hampered by the small number of animals. The same holds true for hormone measurements but when the values for the individual weeks were compared, it was noted that T_3 and T_4 levels, at least, tended to decrease under treatment in high-dose males and females, whereas no effect on TSH was seen. Microsomal protein in the thyroid and TPO activity were not altered.

There were no consistent changes in organ weights or gross lesions that could be attributed to pyflubumide administration. A higher relative liver weight in high-dose dogs of both sexes might be related to the slightly lower terminal body weights. Adverse histopathological findings in the heart (such as atrial oedema and haemorrhages, pigmentation or new formation of blood vessels) were seen in high-dose males and females, whereas myocardial fibrosis and epicardial infiltration were observed also in mid-dose females. Adrenal lesions (lipid depletion in the zona fasciculata) were confined to one male and both female dogs receiving 3000 ppm. On balance, the heart was identified as a target organ of pyflubumide in dogs and effects could be expected from 300 ppm onwards. The selection of the dose levels for the subsequently reported 90-day study appears appropriate (Ishikawa, 2008).

Study 2
In a 90-day feeding study, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered to groups of four male and four female Beagle dogs in dietary concentrations of 0, 40, 300, or 2500 ppm. At the beginning of treatment, the animals were around six months old. Actual mean daily intakes of 1.2, 9.1, and 77 mg/kg bw were calculated for male dogs and of 1.3, 9.5, and 75 mg/kg bw for females.

The animals were monitored daily for mortality and occurrence of clinical signs of toxicity. Along with visual inspection, dogs were subjected to palpation and auscultation. Once a week, an in-depth clinical examination was performed including measurement of respiration, heart and pulse rates and recording of body weight. Ophthalmological examination took place two weeks prior to the administration period and in week 12. Food intake was determined daily. An ECG was taken and blood pressure measured once in study weeks −1, 1, 2, 4, 8 and 13. Haematology comprised determination of erythrocyte parameters, as well as of reticulocyte, platelet, and total and differential white blood cell counts. In addition, prothrombin time and activated partial thromboplastin time were measured. Standard blood and urine clinical chemistry parameters were included. Haematological and clinical chemistry analyses were performed on blood samples taken in weeks −2, −1, 4, 8, and 13. In the same weeks, urinalysis was run, although urine was also collected in weeks 1 and 2.

At terminal kill after 13 weeks, all animals were necropsied and a representative range of organs and tissues taken, preserved and subjected to histopathological examination. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes with epididymis, thymus, thyroid, and uterus were determined.

All the dogs survived until scheduled termination. With first onset in at least one animal noted on study day 6, tachycardia of more than 180 beats per minute was frequently observed in high-dose males during the first two to three weeks of the treatment. Very rarely, other clinical signs such as vomiting or loose stool were noted; these were confined to the two upper dose levels. A similar pattern was observed in the females. Estrous haemorrhages were noted in a total of three females, one in each of the treated groups, but not in the control. No further clinical (including ophthalmological) signs were recorded. When the distribution of the few findings is taken into consideration, tachycardia was the only one that could be clearly attributed to treatment. An effect on the heart was further supported by electrocardiography which confirmed the increased heart rate and revealed a shorter QT interval in high dose males and females. This change is consistent with the tachycardia and suggests an impact mainly on the main ventricles of the heart. With ongoing duration of the study, these heart effects became weaker and disappeared, in males markedly faster than in females in which ECG changes persisted until week 8. A lower blood pressure, mainly affecting the diastolic phase, was observed in high-dose males at the beginning of the administration period. There were no effects on the heart and blood pressure at 300 ppm.

No effects on body weight or its gain were noted, either in males or in females, at any dose level. Food consumption was not affected.

In high-dose males, a lower erythrocyte volume compared to the control group was observed over the whole duration of treatment but statistical significance was achieved only at week 8. Mean corpuscular haemoglobin in that group tended to decrease over the course of the study and was significantly reduced at termination. In females, there were no significant differences in RBC parameters in any dose group.

Clinical chemistry parameters were only marginally affected by treatment at the top dose. An increase in serum triglycerides was noted in high-dose males at study termination. Calcium concentrations were reduced in the same group in weeks 8 and 13. Urinalysis did not reveal any abnormalities that could be attributed to treatment, but urine volume was significantly increased in high-dose males and females from week 8 onwards, whereas osmotic pressure was reduced in the high-dose male group only.

Significant differences in mean organ weights were confined to a higher absolute and relative heart and liver weight in the high-dose male group. The increase was also obvious when the individual organ weights were taken into consideration. In high-dose females, the mean absolute liver and heart weights were also higher but the difference to the control group was statistically significant only for the liver. In contrast, the relative organ weight of both organs was increased.

At gross examination, white discoloration of the heart was noted in two high-dose males. In one dog, in addition, the liver was enlarged. In high-dose females, the pattern of observations was very similar; heart discoloration was seen in two dogs whereas an enlarged liver was noted in three females from that group.

Histopathology revealed lesions of the heart in high-dose males comprising oedema and proliferation of the epicardium in all four animals, and brown pigmentation and formation of new blood vessels in three dogs. Myocardial fibrosis, in contrast, was present in one male only. In the liver, central hypertrophy of hepatocytes was noted in two mid-dose and all high-dose males. In high-dose females, similar heart findings were obtained with up to three animals affected. Myocardial fibrosis, oedema, neovascularization and brown pigmentation were the most common observations. With regard to the liver, central hypertrophy of hepatocytes was seen in one mid-dose and all high-dose females. Minimal thickening of afferent arterioles was observed in the kidney in one high-dose male and one high-dose female. In both sexes, lesions in other organs appeared incidental.

The mid dose of 300 ppm (equivalent to 9.1 or 9.5 mg/kg bw per day in males and females, respectively) is considered the NOAEL based on effects on the heart, liver, kidney, and blood, on clinical chemistry and urinalysis parameters. At this dose, hepatocyte hypertrophy was occasionally observed but, in the absence of liver weight increase or related clinical chemistry findings, is not considered adverse (Ishikawa, 2010).

Study 3

In a one-year study, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered to groups of four male and four female Beagle dogs in dietary concentrations of 0, 40, 300, or 2000 ppm. At the beginning of treatment, the animals were around six months old. Similar actual mean daily intakes of 1.1, 8.0, and 54 mg/kg bw were calculated for male and female dogs. The animals were monitored daily for mortality and occurrence of clinical signs of toxicity. Along with visual inspection, dogs were subjected to palpation and auscultation. Once a week, an in-depth clinical examination was performed including measurement of respiration, heart and pulse rates and recording of body weight. Ophthalmological examination took place two weeks prior to the administration period and then in weeks 25 and 51. Food intake was determined daily. Standard blood and urine clinical chemistry parameters were included. Haematological and clinical chemistry analyses were performed on blood samples taken in weeks −2, −1, 13, 26, 39, and 52. In the same weeks, urinalysis was conducted.

At terminal kill after one year, all animals were necropsied and a representative range of organs and tissues taken, preserved and subjected to histopathological examination. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes with epididymis, thymus, thyroid, and uterus were determined.

All the dogs survived until scheduled termination. At the high dose level of 2000 ppm, tachycardia of more than 180 beats per minute was observed in three male dogs and three females on many occasions between administration days 4 and 13 for males, days 4 and 27 for females. A number of clinical signs such as vomiting, soft or mucous stool, or estrous haemorrhages were noted over the course of the study but, because of their rare or transient occurrence and nearly equal distribution among the groups, theses effects were considered incidental and not attributed to treatment. Ophthalmology did not reveal new abnormalities during the administration period. Body weight and food consumption were not affected at any dose level.

Haematology revealed a lower mean RBC count and decreases in haematocrit and haemoglobin in high-dose male dogs from week 13 onwards even though statistical significance was only occasionally reached. Perhaps to compensate for this slight anaemia, reticulocyte count was significantly increased in that group in weeks 39 and 52. In contrast, an increase in platelet count was apparent in week 13 and then until termination, but never reached statistical significance. Coagulation parameters were not affected.

Effects on RBC parameters and platelets were the same for females as males. Statistically significant differences from the control group were confined to the high-dose group, except in week 39 when lower haematocrit, RBC count and haemoglobin were also observed at 300 ppm. Reticulocytes, white blood cells and coagulation parameters were not affected.

There were only very few alterations in clinical chemistry parameters considered potentially adverse, statistically significant and which could be attributed to treatment. In high-dose males an increase in cholesterol was noted, whereas albumin and calcium concentrations were reduced in that group. In high-dose females increased activity of lactate dehydrogenase was the most prominent finding.

A reduction in albumin and calcium levels was also seen but was less pronounced than in males. On balance, these findings suggest a rather weak effect due to the high dose of 2000 ppm on the liver and perhaps due to metabolic acidosis, on muscle.

Urinalysis did not reveal any abnormalities that could be attributed to treatment but urine volume was markedly higher in top-dose males at weeks 39 and 52 than in the control, low- and mid-dose groups. Since a similar increase had been observed in the 90-day study in male and female dogs receiving 2500 ppm, a treatment-related effect is likely even though the difference was not statistically significant in the actual study. In high-dose females a significant increase in urine volume was observed at weeks 13 and 26 whereas no difference was seen at study termination. At the same measurement times, osmotic pressure was reduced in this group. Water intake was not determined in dogs. Accordingly, it is not clear whether the higher urine volume might be due to polydipsia.

Significant differences in mean organ weights in male dogs were confined to a higher heart and liver weight at the high dose level. In addition, a significant increase in absolute and relative organ weights of the adrenals and the prostate were noted in that group. In high-dose females, significant increases in absolute and relative liver weight were apparent.

The only presumably treatment-related gross necropsy finding in males was enlargement of the liver in all high-dose animals. In high-dose females enlargement of the liver in three dogs was accompanied by heart changes (discoloration, hard consistency of an auricle and foci) in a single animal. A summary of histopathological findings is given in Table 10.

Table 10. Selected histopathological findings in the one-year feeding study in dogs (n = 4 per sex and dose)

Sex, dose (ppm)	Males				Females			
	0	**40**	**300**	**2000**	**0**	**40**	**300**	**2000**
Liver: central hypertrophy	0	0	0	2	0	0	0	3
Heart: fibrosis (heart muscle)	0	0	0	1	0	0	0	2
Adrenals: hyperplasia/hypertrophy of zona fasciculata	0	0	0	1	0	0	2	4
Adrenals: lipid depletion in zona fasciculata	0	0	1	2	0	0	0	0
Adrenals: thickening of zona fasciculata	0	0	1	2	0	0	0	2
Kidney: thickening of afferent arteriole	0	0	0	0	0	1	0	1
Stomach: mucosal cell infiltration	0	2	1	2	0	1	1	4

Histopathological findings were partly in line with organ weight changes. Based on dose response, it seems likely that the adrenal findings in mid-dose males and females were treatment-related. Also, the findings in heart, liver and (in females) in stomach were attributed to substance administration but were confined to the highest dose. Toxicological significance of the thickening of afferent kidney arterioles is equivocal since, in contrast to the 90-day study, this lesion was observed in females but not in males and there was no clear dose response. On the other hand, the highest dose in the one-year study was lower, thus on balance an additional (even though marginal) high-dose effect of pyflubumide on the kidneys in dogs cannot be excluded, whereas the same finding in one low-dose female in the one-year study was regarded as incidental since there was no dose response and no evidence of progression at higher doses. The resulting NOAEL of 40 ppm (equivalent to 1.1mg/kg bw per day in both males and females) is based on histopathological adrenal findings and further supported by a few haematologial findings which had occurred also at the mid dose level (Ishikawa, 2011).

(b) Dermal application

No data were available for pyflubumide.

(c) Exposure by inhalation

No data were available for pyflubumide.

2.3 Long-term studies of toxicity and carcinogenicity

The chronic toxicity and carcinogenicity of pyflubumide was investigated in long-term feeding studies in both mice (combined chronic and carcinogenicity study) and rats (one-year chronic study and two-year carcinogenicity study).

Mouse

In a carcinogenicity study, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered to male and female Crlj:CD1(ICR) mice for 18 months at nominal dietary concentrations of 0, 40, 400, and 1600 ppm. Group size was 52 animals per sex and dose. The dose levels were based on a 90-day feeding study in the same mouse strain (Kashimoto, 2009, see above). The overall mean daily intakes in male mice were 0, 4.4, 45, and 176 mg/kg bw. The mean daily intakes in female mice were 0, 4.0, 43, and 178 mg/kg bw.

All animals on study were observed daily for mortality and clinical signs of toxicity. During the first 13 weeks of treatment, they were weighed once a week, food consumption was determined on three consecutive days per week and food efficiency was calculated. Thereafter, body weights and food intake were recorded every four weeks. As usual for a carcinogenicity study, blood and urine clinical chemistry parameters were not examined. Haematology was confined to total and differential white blood cell counts in the surviving mice at scheduled termination. At terminal kill, organ weights of adrenals, brain, heart, kidneys, liver with gall bladder, ovaries, spleen, thymus, testes and epididymis were determined in ten animals per sex from each dose group. Gross examination was carried out on all animals (including those which were found dead or had to be killed for humane reasons during the study), and a broad selection of organs and tissues harvested and preserved. Full histopathology of these samples was performed only for the control and high-dose groups, but additionally and if possible, also for all animals from the low- and mid-dose groups which were killed in extremis or found dead. For low- and mid-dose mice surviving until scheduled termination, histopathological examination was confined to just the few organs in which treatment-related findings had been noted at the top dose level: the livers of both sexes, the spleen of mid- and low-dose males and the adrenals and the thyroid of mid- and low-dose females. In addition, where gross lesions were observed these too were examined.

There were no premature deaths that could be attributed to treatment. Survival was similar in all groups with the highest within-study mortality of 19/52 occurring in both control and low-dose males and 17 premature deaths among 52 low-dose females. Accordingly, no impact of the test substance on survival became apparent. Likewise, no clinical signs of toxicity were reported for any group. However, as a gross observation, an increase in the number of females with loss of fur was noted at scheduled termination from the mid-dose (see Table 11).

In males, body weight, food consumption and food efficiency were not affected by treatment up to the highest dose level. In contrast, mean body weight was reduced in the high-dose female group from week 32 onwards. In subsequent months, body weight was consistently lower than in the control group by 9–11%. Food intake was not compromised in top-dose females and was occasionally even slightly higher towards the end of the treatment period. Accordingly lower body weight cannot be attributed to lower food consumption. It is mentioned in the study report that food efficiency did not differ among the female groups but this parameter was calculated only for the first 13 weeks of the study. As in top-dose females, a lower body weight was observed despite similar food consumption, so food efficiency must have been impaired at later stages.

The limited haematological examination at the end of the study revealed a significant increase by 53% in the mean number of both neutrophils and of monocytes in high-dose males compared to the control means. Interpreting this finding is not easy, but a physiological unspecific immune reaction of the organism to a xenobiotic may be a likely explanation. If so, the effect would be treatment-related but not adverse. The same holds true for a 60% decrease in eosinophil count in high-dose females. A similar effect of the same magnitude had been observed in the 90-day study in mice receiving 4000 ppm (Kashimoto, 2009, see above), making a chance event unlikely.

Gross examination at scheduled necropsy revealed an increased number of males with masses and spots of the liver at the top dose (Table 11). Another common finding was enlargement of the spleen at all doses, but given the absence of a dose response it is unlikely this finding was related to treatment.

Thickened wall of the glandular stomach was observed in high-dose males but in the absence of any microscopic findings this was considered to have occurred by chance. The number of males displaying the physiological, age-related hypertrophy of the seminal vesicle and coagulating gland was decreased at the two upper dose levels. These findings are considered to be of no toxicological significance. Even though this finding in itself is not adverse, rather beneficial, it is indicative of some interference with endocrine regulation or activity, however hormones were not measured in any study in mice. The background to the more frequent loss of fur in females is not known but could be also be hormone-mediated. It is worth noting that it was clearly dose-related at scheduled termination with 9, 14, 18, and 21 females affected and a significant difference to the control groups at the two upper dose levels.

Table 11. Selected gross pathological findings in the 18-month feeding study in mice (males/females), based on terminal kill and premature deaths (n = 52 per sex and dose)

Dose level (ppm)	0	40	400	1600
Loss of fur	11 / 14	10 / 20	8 / 25*	9 / 23
Liver masses	19 / 7	19 / 4	21 / 3	33** / 10
Spots on liver surface	1 / 3	4 / 3	4 / 2	10** / 5
Enlarged spleen	1 / 5	3 / 14*	7* / 11	5 / 9
Glandular stomach wall thickened	0 / 0	1 / 1	4 / 1	6* / 2
Hypertrophy of seminal vesicle	19 / -	17 / -	8* / -	9* / -
Hypertrophy of coagulating gland	18 / -	17 / -	9* / -	9* / -

* $p < 0.05$, ** $p < 0.01$, Fisher's exact test

Increases in absolute and relative liver weight in both sexes at the highest dose level were attributed to treatment, as well as increases in absolute and (even though not statistically significant) relative spleen weight, at least in females of the mid- and high-dose groups; a similar effect in males was equivocal (see Table 12). A significantly higher relative organ weight of heart and kidneys in top-dose females was most likely due to the lower mean body weight in this group since absolute organ weights were similar to control means.

Table 12. Mean organ weights, with standard deviations (SD) in the 18-month feeding study in mice at terminal kill (n = 10 per sex and dose)

Sex, dose (ppm)	Males				Females			
	0	40	400	1600	0	40	400	1600
Mean absolute liver weight (g)	2.40 ± 0.60	3.19 ± 1.55	2.86 ± 1.17	4.11 ± 1.24**	2.28 ± 0.41	2.19 ± 0.45	2.51 ± 0.31	3.62 ± 0.69**
Mean relative liver weight (%)	5.17 ± 0.62	6.43 ± 2.63	6.01 ± 2.39	8.32 ± 1.96**	3.95 ± 0.64	4.33 ± 0.85	4.89 ± 1.01	7.45 ± 1.56**
Mean absolute spleen weight (mg)	126 ± 58	163 ± 93	132 ± 81	174 ± 66	155 ± 136	145 ± 57	245 ± 122*	253 ± 185*
Mean relative spleen weight (%)	0.27 ± 0.12	0.34 ± 0.22	0.28 ± 0.17	0.35 ± 0.12	0.28 ± 0.28	0.29 ± 0.14	0.49 ± 0.29	0.52 ± 0.38

* $p < 0.05$, ** $p < 0.01$, Dunnett's or Dunnett-type test

Histopathological examination revealed clearly treatment-related findings in the liver in both sexes at the high dose level, including some preneoplastic lesions. As in the 90-day study, localization of fatty changes within the liver was different between the sexes.

The spleen was identified as an additional target organ in high-dose males. The increase in extramedullary haematopoiesis could well be related to haematological changes as observed in the 90-day study in mice (Kashimoto, 2009, see above) but unfortunately RBC parameters had not been measured in this carcinogenicity study. It is remarkable that increased haematopoiesis was observed in males whereas a significant increase in spleen weight was noted in females only, and was apparently not accompanied by histopathological findings that could explain an effect on the organ weight.

The adrenals were affected in top-dose females. The increase in eosinophilic staining of the zona fasciculata of the adrenal cortex most likely suggests the presence of foci of cellular alteration in this organ. In this high-dose female group there was also a significant increase in thyroid follicular cell hypertrophy. but a smaller increment was also seen in top-dose males.

Table 13. Selected neoplastic and non-neoplastic histopathological findings in the 18-month feeding study in mice, based on terminal kill and premature deaths (number affected/number examined)

Sex, dose (ppm)	Males				Females			
	0	**40**	**400**	**1600**	**0**	**40**	**400**	**1600**
Neoplastic findings								
Liver: adenoma	10 / 52	15 / 52	16 / 52	27**/ 52	2 / 52	2 / 52	3 / 52	4 / 52
Liver: carcinoma	9 / 52	1 / 52	4 / 52	7 / 52	0 / 52	0 / 52	0 / 52	0 / 52
Lymph nodes: haemangiosarcoma	0 / 52	0 / 19	0 / 16	3 / 52§	0 / 52	1 / 18	0 /13	3 /52
Spleen: haemangiosarcoma	1 / 52	2 / 52	2 / 52	2 / 52	0 / 52	1 /19	2 /18	3/52
Non-neoplastic findings								
Liver: centrilobular fatty change	9 / 52	10 / 52	6 / 52	0** / 52	2 / 52	0 / 52	0 / 52	0 / 52
Liver: periportal fatty change	0 / 52	1 / 52	0 / 52	5* / 52	0 / 52	0 / 52	2 / 52	1 / 52
Liver: Diffuse fatty change	0 / 52	0 / 52	0 / 52	2 / 52	2 / 52	2 / 52	3 / 52	35** / 52
Liver: eosinophilic foci of cellular alteration	4 / 52	6 / 52	6 / 52	19** / 52	0 / 52	0 / 52	0 / 52	10** / 52
Liver: inflammation	7 / 52	9 / 52	6 / 52	30** / 52	1 / 52	0 / 52	0 / 52	18** / 52
Spleen: increased haematopoiesis	14 / 52	16 / 52	22 / 52	28** / 52	14 / 52	9 / 19	8 / 18	17 / 52
Adrenals: eosinophilic changes in zona fasciculata	4 / 52	4 / 19	2 / 15	7 / 52	2 / 52	4 / 52	7 / 52	14** / 52
Thyroid: follicular cell hypertrophy	4 / 52	0 / 19	1 / 15	10 / 52	6 / 52	4 / 52	8 / 52	34** / 52

* $p < 0.05$, ** $p < 0.01$, Fisher's exact test;

§ $p < 0.05$, asymptotic Cochran-Armitage test, one-sided

The NOAEL for chronic toxicity in this study was 400 ppm (equivalent to 45 and 43 mg/kg bw per day in males and females respectively), based on a lower body weight in females in the second half of the treatment period, on markedly increased liver weights in both sexes and on histopathological findings in the liver in both sexes, in adrenals (females only), spleen (males only) and thyroid (females and perhaps in males). All these effects were confined to the top dose level of 1600 ppm (equal to 176 or 178 mg/kg bw per day in males and females respectively). The higher spleen weights in females at the two upper dose levels were however dismissed as not adverse, since in contrast to the more frequent observation of haematopoiesis in high-dose males, they were not accompanied by any histopathological findings to explain the organ weight increase. Effectively, the observations in the preceding 90-day study by Kashimoto (2009) have been confirmed and the same dietary NOAEL was established.

The toxicological significance cannot be determined in considering the of loss of fur in females and a (arguably beneficial) reduction in age-related changes in male reproductive organs from the mid-dose onwards.

An oncogenic effect of pyflubumide on the liver was confined to the maximum tested dose at which a significant increase in hepatocellular adenoma in male mice was observed. No progression towards carcinoma was noted. There was no increase in liver tumours in females. No mechanistic studies have been performed to further investigate the mode of action (MOA) of these tumours, but since the liver was a common target organ of pyflubumide in the mouse, it seems likely that the adenomas fit into this pattern of hepatotoxicity.

In addition to the above, an increase of haemangiosarcoma in mesenteric lymph nodes was noted in both sexes that had not been reported in the study summary and was not subject to further considerations by the sponsor. As to be expected for such a rare tumour, Fisher's exact test failed to elucidate a statistically significant difference between the control and any dose group, but a test for trend provided a *p* value of 0.0127 for males. No positive trend was obtained in females, most likely because of the single case in the low-dose group. When the sexes were combined, however, statistical significance was achieved by the Cochran-Armitage test for trend, as well as by Fisher's exact test. The actual study incidences were compared to historical control data from the same laboratory and mouse strain which were included in the study report. In male mice of the same strain, haemangiosarcoma of the lymph nodes had been observed in only two out of 733 animals, which occurred in two out of 14 studies in the provided historical database. In females, this finding was also very rare since it was reported in only one out of 14 studies. Even though it was seen in this study in two control animals, the total incidence of 2/734 was as low as in males. In particular, since the median or the mean are more relevant than the range when it comes down to the interpretation of historical control data, the zero incidences in the actual male and female control groups were completely in line with the historical database. In contrast, the increase in haemangiosarcoma in lymph nodes in both sexes at the high dose level was clearly in excess of the historical data. No MOA has been proposed.

A concomitant increase of haemangiosarcoma of the spleen in females, by contrast, was more equivocal against the background of historical control data. The total incidence of 16/734 animals in the database suggests that haemangiosarcoma of the spleen is more common in untreated female ICR mice than is haemangiosarcoma of the lymph nodes. In fact, splenic haemangiosarcoma was observed in female control mice in seven out of 14 studies from the same laboratory, and an incidence of three cases, (that is the same as in the high-dose group from the actual study) was noted in three of them. In males there was apparently no dose response in the actual study. However, for the high dose level, it was confirmed that different males were bearing this malignant tumour in mesenteric lymph nodes and in the spleen. In females, one high-dose animal was identified with haemangiosarcoma on both sites whereas the other tumours had been found in different animals.

On balance, pyflubumide proved carcinogenic in this long-term study in mice at the high dose level of 1600 ppm (equal to 176 or 178 mg/kg bw per day in males and females, respectively). Based on the increase in haemangiosarcoma of mesenteric lymph nodes in both sexes and on the higher incidence of liver adenoma in females, the NOAEL for carcinogenicity was 400 ppm (equal to 45 and 43 mg/kg bw per day in males and females, respectively). This is the same as for chronic toxicity (Kuwahara, 2011a).

Rat

Study 1

Chronic toxicity of pyflubumide was investigated in a one-year feeding study in F344/DuCrlCrlj rats. Groups of 20 male and female animals were fed the test substance (Lot no. 9HZ0013P, purity 95.8%) at dose levels of 0, 10, 20, 120, or 600 ppm. The mean daily intakes of male rats were 0, 0.4, 0.9, 5.1, and 26 mg/kg bw, respectively. The mean daily intakes of female rats were 0, 0.5, 1.1, 6.4, and 32 mg/kg bw, respectively. The nominal dietary dose levels were the same as in the two-year carcinogenicity study (Kuwahara, 2012) reported below. Accordingly, this study can be considered the chronic toxicity part of the long-term study in this species.

The rats were observed daily for mortality and clinical signs of toxicity. Ophthalmoscopy was conducted on all animals during the acclimatization period and on all animals from the control and high-dose groups at week 52 of treatment. Since there was no evidence of adverse findings, ophthalmological examination was not repeated in the other dose groups. An FOB comprising investigations on motor

activity, grip strength and responses to various sensory stimuli was conducted on ten animals per sex and dose group in study week 49. Body weight was recorded weekly for the first three months of the study and once every four weeks thereafter. Food consumption was measured on four consecutive days per week. This was done weekly for the first three months of the study and once every four weeks thereafter. Food efficiency was calculated weekly but only until week 13. Haematological examinations were conducted on 10 animals per sex and group after 14, 26 and 52 weeks of treatment. Erythrocyte parameters, reticulocyte, platelet, and total and differential white blood cell counts were determined. In addition, prothrombin time and activated partial thromboplastin time were measured and the nucleated cell count in bone marrow determined. Blood smears were prepared but not examined. In blood samples from the same 10 animals per group, taken at the same time points, a representative selection of clinical chemistry parameters was determined. Urinalysis was conducted on 10 animals per sex and dose at weeks 13, 25, and 51. At scheduled termination after 52 weeks, the rats were killed, gross examination carried out and a representative selection of organs and tissues taken. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, testes with epididymis, thymus, thyroid, and uterus were determined. Full histopathology of a wide range of organs and tissues was performed in all animals from the control and high-dose male and female groups. In the three lower-dose groups, histopathological examination was confined to liver, lung, thyroid, urinary bladder and all gross lesions in both sexes. In addition, heart and kidneys were examined under the microscope in male rats but adrenals only in females.

One male animal from the low-intermediate dose group (20 ppm) died at week 43. A malignant tumour (schwannoma) was the most likely cause of death and, because of its isolated occurrence at a rather low dose level, this fatal outcome was considered incidental. There were no further unscheduled animal losses during the study. No clinical (including ophthalmological) signs of toxicity were observed in either males or females that could be clearly attributed to treatment. Loss of fur, which has been a common finding with pyflubumide in other studies in rats and mice, was in fact noted in two females at 120 ppm and in three females at 600 ppm, as compared to zero in the control or in the 10 and 20 ppm groups. In males, by contrast, loss of fur was seen in three animals in both the control and high dose groups. A decrease in motor activity was observed during the functional observations in high-dose males with statistical significance achieved for the first 10 recording minutes only which tended to indicate an unspecific finding of general toxicity rather than suggesting neurotoxicity.

From the first week of treatment onwards, mean body weights were increased in high-dose males by ca 5% for the whole course of the study. A less pronounced (< 5%) but still significant increase in body weight was also noted at 120 ppm from week three to week 20.

In females, a higher mean body weight was noted at the two upper dose levels from the first treatment week onwards, but was not always dose-related. Differences to the control group approached, but did not exceed, 10%. At first occasionally, but from week 20 onwards consistently, mean body weight was significantly higher, also at the low dose level of 10 ppm and for individual weeks increases were noted at 20 ppm. There was no dose response at the lower dose range but, of course, the difference between 10 and 20 ppm is not large. A higher body weight is not considered adverse from a toxicological point of view but may be considered an unwanted effect if human health is concerned. A significantly higher mean food intake was observed in both sexes at the high dose during most of the study and for the next lowest dose of 120 ppm in many individual weeks. Since the higher mean body weights were paralleled by an increase in food consumption, food efficiency was not altered.

A number of significant alterations in RBC parameters were noted in high-dose males and in mid- and high-dose females as it is typical for slight anaemia. In addition, a prolonged prothrombin time suggested impaired coagulation at the top dose level but only in males (Table 14). Most of these findings had been observed already at earlier time points during the study (and also in the 90-day study). White and differential blood cell or platelet counts did not differ among the groups.

Table 14. Selected haematological findings in the one-year feeding study in rats at termination (mean values for males/females, n = 10 per sex and dose)

Dose level (ppm)	0	10	20	120	600
Haematology					
Haematocrit (%)	45.1/45.5	45.4/45.1	44.9/45.2	44.8/44.6	43.3**/42.4**
Haemoglobin (g/dL)	14.9/15.3	15.0/15.1	14.9/15.1	14.9/15.0	14.4**/14.3**
Mean corpuscular haemoglobin (pg)	15.7/17.3	15.6/17.2	15.6/17.1	15.5/16.8**	15.4**/16.6**
Mean cell volume (fL)	47.5/51.7	47.2/51.5	47.2/51.1	46.7/50.0**	46.4**/49.5**
Prothrombin time (s)	18.1/17.2	17.7/17.1	18.2/17.3	18.6/17.1	19.5**/16.9

*$p < 0.05$, **$p < 0.01$, Dunnett's test

An increase in γ-glutamyltranspeptidase activity in high-dose females in treatment weeks 14 and 26 suggested some liver toxicity but this was not confirmed at the end of substance administration after one year. At termination, however, glucose concentration was significantly increased in high-dose females. An impact on the liver became further apparent by higher cholesterol, albumin, globulin and total protein concentrations in high-dose males at most time points of measurement, but were only occasionally observed in females. Triglycerides and total bilirubin were significantly decreased in high-dose males and females. Occasionally changes in calcium and potassium levels were observed but the only consistent effect was indicated by a significant decrease in females receiving 120 or 600 ppm at termination.

Urinalysis revealed a significant decrease in specific gravity of urine in high-dose males from the middle of the study onwards. Perhaps in line with that, urine volume was clearly increased in that group. In addition, more protein was found in the urine. A higher urinary volume was also noted in females receiving 120 and 600 ppm but this finding was confined to the examination in week 51 and was not accompanied by further changes. Therefore, and since it had not been observed at higher dose levels in the 90-day study, it is not regarded as adverse.

The only remarkable gross pathological effect at necropsy was the observation of spotted lungs in high dose males (6/20 affected). The difference to the control group was statistically significant. As revealed by subsequent histopathology, five of these animals suffered from pneumonia. Apart from this group, spots on lung surface were a rare finding in this study since they were only noted in one male receiving 120 ppm and in one female receiving 10 ppm.

A statistically significant and dose-related increase in absolute and relative heart weights was noted in both males and females at the two upper dose levels. Likewise, absolute and relative ovary weight was increased in females at 120 and 600 ppm. Mean absolute organ weights of liver, kidneys, thyroid, spleen, and adrenals were increased in both sexes at 600 ppm but were not always accompanied by concomitant increases in relative organ weights.

A summary of histopathological changes is provided in Table 15. In male rats, the findings in the heart, thyroid, kidneys and urinary bladder at the two upper dose levels or in the highest dose group were considered adverse and treatment-related. The small increase in medullary hyperplasia of the adrenals does not suggest a treatment-related effect per se, but should be assessed against the background of an increase in this lesion in the two-year study reported below. The increase in pneumonia in high-dose males was unique to this study and not observed in any other study with pyflubumide. This pathological lesion was also different from the lung findings in rat pups or young adult offspring in the multigeneration study and in a few mechanistic studies (see below). Most likely, pneumonia was not related to substance administration.

In females, hyperplasia of the bile duct was increased at the two upper dose levels confirming the liver as a target organ. The treatment-related effects on thyroid and urinary bladder were confined to the highest dose level. Even though statistical significance was not achieved, the increase in haematopoiesis in bone marrow might indicate regeneration in response to slight anaemia.

No increase in the (very few) neoplastic findings was noted in neither sex.

Table 15. Selected histopathological findings in the one-year feeding study in rats (n = 20 per sex and dose[#])

Sex, dose (ppm)	Males					Females				
	0	**10**	**20**	**120**	**600**	**0**	**10**	**20**	**120**	**600**
Liver: bile duct hyperplasia	20	20	19	20	20	4	7	4	14**	16**
Lung: pneumonia	0	0	1	0	5*	0	0	0	0	1
Heart: fibrosis (heart muscle)	6	3	7	5	13*	0	NE	NE	NE	0
Thyoid: follicular cell hyperplasia	0	0	0	0	20**	0	0	0	0	16**
Adrenals: medullary hyperplasia	0	NE	NE	NE	2	0	0	0	0	0
Bone marrow (sternum): increased heamatopoiesis	1	NE	NE	NE	0	1	NE	NE	NE	5
Bone marrow (femur): increased haematopoiesis	1	NE	NE	NE	0	4	NE	NE	NE	9
Kidney: tubular basophilic change	4	5	8	10*	16**	3	NE	NE	NE	2
Kidney: urinary casts	3	2	3	9*	16**	1	NE	NE	NE	3
Urinary bladder: mononuclear cell aggregation in lamina propria	3	5	3	3	10*	6	8	9	8	13*

NE Not examined

[#] except male 20 ppm group consisting of 19 animals due to one incidental death

* $p < 0.05$, ** $p < 0.01$, Fisher's exact test

Based on haematological effects, increased heart and ovary weights and histopathological lesions in kidneys (males) and liver (females), the low-intermediate dose of 20 ppm (equivalent to a mean daily intake of 0.9 and 1.1 mg/kg bw in males and females, respectively) was the NOAEL in this study. At the top dose level of 600 ppm, in addition, thyroid and urinary bladder were also detected as target organs of pyflubumide, whereas an effect on the lungs in males appears questionable. Taking into consideration the available information from other studies, it cannot be excluded that non-significant high-dose effects on adrenals in males and on bone marrow in females might also be treatment-related (Kuwahara, 2011b).

Study 2

In a separate carcinogenicity study, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered to male and female F344/DuCrlCrlj rats at dietary concentrations of 0, 10, 20, 120, and 600 ppm. These dose levels were based on the 90-day feeding study in the same rat strain (Kuwahara, 2009, see above). The dose groups treated for up to two years comprised 50 rats per sex. The overall mean daily intakes for male rats were 0, 0.4, 0.7, 4.5, and 23 mg/kg bw. In females, the mean daily intakes were 0, 0.5, 0.9, 5.6 and 29 mg/kg bw.

All animals on study were observed daily for mortality and clinical signs of toxicity. During the first 13 weeks of treatment, they were weighed once a week, food consumption was determined on four consecutive days per week and food efficiency was calculated. Thereafter, body weight and food intake were recorded every four weeks. Haematology was confined to total and differential white blood cell count in the surviving rats at scheduled termination. In addition, blood smears had been prepared from samples taken after 52 and 78 weeks but were not evaluated since there was no evidence of tumours of the haematopoietic system at study termination. At terminal kill, organ weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes with epididymis, thymus, thyroid with parathyroids, and uterus were determined in ten animals per sex from each dose group. All animals (including those which were found dead or had to be killed for humane reasons during the study) were subjected to gross examination and a broad selection of organs and tissues harvested and preserved. Full histopathology of these samples was performed in the control and high-dose groups only. However, in addition and if possible, the same examination was performed on all animals from the low- and mid-dose groups which were killed in extremis or found dead. In the groups receiving 10, 20, or 120 ppm, regular histopathological examination of the animals of both sexes surviving until scheduled termination comprised the adrenals,

heart, kidneys, liver and thyroid, since pathological changes had been observed at the top dose level. For the same reason, skin from the lumbo-dorsal reason was examined under the microscope in all dose groups but only the females.

There were no premature deaths that could be attributed to treatment. Survival was generally good and was not affected by treatment. Possible treatment-related clinical signs were confined to the highest dose level. In males a higher incidence of eye opacity was observed (13/50 at 600 ppm versus 4/50 in the control group and 3–7 cases in the other treatment groups). Upon terminal necropsy, discharge from the eyes was a common finding and was more frequent at the highest dose level. However, histopathology failed to reveal typical findings such as keratitis or cataracts which might explain the opacity. In females, an increase in the occurrence of skin masses (swelling) was seen, as well as a loss of fur. The differences from the control group (25 or 27 compared to 15) were statistically significant for the two upper dose levels. In particular, the lumbo-dorsal region was affected. Histopathology revealed a high incidence of perifolliculitis (see Table 16) that could explain this finding, at least partly. Set against this there were many animals (mostly in the 120 ppm group) in which the gross finding of skin masses was not accompanied by any remarkable microscopic skin findings. On balance, eye opacity and skin masses at high doses were considered to be of equivocal toxicological significance.

In high dose males, mean body weight was significantly higher (but by less than 8%) than in the control group over the most of the study. Occasional increases were also seen at 120 ppm. The same effect was apparent in females at the two upper dose levels but only up to week 60. Thereafter, body weight was similar to the controls suggesting a slight reduction in body weight gain as the study progressed. Increases in body weight were paralleled by a higher food intake. All these changes, even though relatively minor, were considered treatment-related and potentially adverse.

Haematology in the animals surviving until scheduled termination revealed a significant increase in total white blood cell count in males that was confined, however, to the top dose level. In this group, mean numbers of lymphocytes, neutrophils and monocytes were significantly increased compared to the control group but the ratio between these different leucocyte subpopulations was not skewed. When the low and intermediate dose levels were taken into consideration, there was no clear dose response as sometimes the mean values for total white cell count or the individual populations were markedly above the high-dose mean. Statistical significance was not achieved, due to a very large standard deviation, indicating strong interindividual variability. Accordingly, only significant changes at the maximum dose level were attributed to treatment even though their toxicological significance is equivocal.

A similar pattern became apparent in females. A marked increase in mean total white cell count in the high-dose group did not gain statistical significance since the SD was very large. However, as with high-dose males, there was a significant increase in neutrophil and monocyte numbers in the females receiving 600 ppm. In addition, basophil granulocytes were reduced. Again, the toxicological significance of these presumably treatment-related findings is equivocal. A further remarkable finding was a significant increase in large unstained cells in high-dose females that may have also contributed to the high mean leucocyte count. It was noted, however, that the large differences were mainly due to very few animals suffering from mononuclear leukaemia. The statistical significance of this finding disappeared following exclusion of one control and one high-dose female with extraordinarily high numbers of large unstained cells ($15.64 \times 10^3/\mu L$ or $114.97 \times 10^3/\mu L$, respectively, as compared to group means of 0.08 or $0.09 \times 10^3/\mu L$) . For both animals, it was confirmed by histopathology that they had this tumour. It is worth noting that total incidence of females with mononuclear cell leukaemia was significantly lower at the two upper dose levels (see Table 16) proving that the observation of large unstained cells was not indicative of an adverse effect of treatment.

At terminal necropsy after two years, but partly also in animals which died or had to be killed before, a number of changes were seen (Table 16). The clinical observations regarding the eyes (opacity in top-dose males), the skin (masses in females at 120 and 600 ppm) and the fur (loss of fur in top-dose females) were confirmed. In addition, a more frequent occurrence of spots on the liver was noted in high-dose males. On the other hand, a smaller number of animals with testis atrophy and softening of testis or epididymis was observed at the top dose level.

Table 16. Selected gross findings in the two-year feeding study in rats, based on terminal kill and premature deaths (n = 50 per sex and dose[§])

Sex, dose (ppm)	Males					Females				
	0	10	20	120	600	0	10	20[§]	120	600
Eye opacity	4	7	3	5	13*	1	7*	3	3	2
Loss of fur	2	4	2	3	3	7	5	8	9	26**
Skin masses	24	25	29	30	20	14	22	10	23*	27**
Spots on liver	3	1	0	3	10*	13	9	11	9	15
Testis atrophy	7	5	8	9	1*	-	-	-	-	-
Soft testis	18	20	12	17	9*	-	-	-	-	-
Soft epididymis	30	34	37	32	17*	-	-	-	-	-

[§] For this group $n = 49$

*$p < 0.05$, **$p < 0.01$, Fisher's exact test

Organ weights of heart and liver were increased in both males and females. A higher absolute and relative heart weight was noted, in both sexes, at the two upper dose levels whereas a significant increase in liver weight was observed in high-dose females and in mid- and high-dose males (Table 17). Further organ weight changes, such as higher absolute weights of kidneys and epididymis in males and of adrenals and kidneys in females, were confined to the highest dose level and partly reflected the higher mean body weight since relative organ weights were not affected. On the other hand, there were histopathological kidney findings in high-dose females (see Table 18). A higher thyroid weight in high-dose males perhaps reflected the follicular cell hyperplasia. An increase in thyroid weight was also observed in high-dose females but statistical significance was not achieved. Absolute and relative weight increase of the ovaries was not accompanied by histopathological changes.

Table 17. Mean organ weights in the two-year feeding study in rats at terminal kill (n = 10 per sex and dose)

Sex, dose (ppm)	Males					Females				
	0	10	20	120	600	0	10	20	120	600
Mean absolute liver weight (g)	10.11	9.75	10.51	11.61**	14.56**	5.97	6.27	6.40	6.55	8.31**
Mean relative liver weight (%)	2.44	2.46	2.52	2.66	3.25*	2.27	2.37	2.47	2.50	3.10**
Mean absolute heart weight (mg)	1126	1137	1174	1345**	1635**	804	831	836	928**	1142**
Mean relative heart weight (%)	0.27	0.29	0.28	0.31*	0.36**	0.31	0.32	0.33	0.36*	0.43**

*$p < 0.05$, **$p < 0.01$, Dunnett's or Dunnett-type test

Histopathological examination revealed a number of presumably treatment-related non-neoplastic findings in several organs (Table 18). Most prominent were skin perifolliculitis in high-dose females, a significant increase in medullary hyperplasia of the adrenals in mid- and high-dose males and a higher incidence of myocardial fibrosis in both sexes at the two upper dose levels, that is exacerbation by the treatment of a common finding. This fibrosis is an irreversible repair mechanism that is assumed to result from previous damage to the myocard, perhaps due to ischemia. A clear effect on the thyroid was seen in both sexes but was confined to the top dose level. In addition, kidneys and liver were target organs in females but not in males. Again, a larger increase of quite common findings was observed.

Table 18. Selected neoplastic and non-neoplastic histopathological findings in the two-year feeding study in rats, based on terminal kill and premature deaths (n = 50 per sex and dose)

Sex, dose (ppm)	Males					Females				
	0	**10**	**20**	**120**	**600**	**0**	**10**	**20**	**120**	**600**
Neoplastic findings										
Adrenals: pheochromocytoma (benign)	4	4	7	5	9	0	0	0	1	1
Adrenals: pheochromocytoma (malignant)	0	2	2	0	1	0	0	1	0	0
Mononuclear cell leukaemia	4	6	5	4	0	8	8	2*	2*	2*
Non-neoplastic findings										
Skin: perifolliculitis	0	0	0	1	1	4	5	5	8	28**
Heart: myocardial fibrosis	35	40	41	45*	48**	9	7	15	22**	41**
Liver: bile duct hyperplasia	50	50	49	50	50	25	25	31	41**	42**
Adrenals: medullary hyperplasia	15	18	18	25*	32**	7	4	6	6	13
Kidneys: chronic nephropathy	45	41	46	47	48	17	14	16	23	35**
Thyroid: follicular cell hyperplasia	0	0	0	0	27**	0	0	0	0	35**

*$p < 0.05$, **$p < 0.01$, Fisher's exact test

No statistically significant increase in any tumour type was reported in males or females. However, there was a numeric increase in benign pheochromocytoma in the adrenals of male rats receiving the high dose (Table 18). The actual study incidence in the high-dose male group was above the historical control incidence as reported from the performing laboratory. In seven similar studies on the same rat strain, incidence of pheochromocytoma varied from one to eight in the untreated male groups with a median of four. The total incidence in all males from the historical database (350 in total) was 8%, as compared to 18% in the actual group of concern. On the other hand, these benign tumours of the adrenal medulla did not progress to malignancy and no comparable increase was seen in females. Most likely, the numeric increase in pheochromocytoma was related to the rather frequent occurrence of medullary hyperplasia in male rats at the two upper dose levels. Both findings are regarded by some pathologists as a continuous histologic spectrum and it may be difficult to clearly distinguish hyperplasia from neoplasia (Chandra, Hoehnerhoff & Peterson, 2013). There is good evidence that prolonged exposure to some hormones, drugs or other agents, such as vitamin D, may cause or stimulate proliferative lesions of the adrenal medulla resulting in focal or diffuse hyperplasia that can progress to neoplasia. Different mechanisms may be involved, including the uncoupling of mitochondrial respiration as might be expected with pyflubumide considering its pesticidal mode of action (Tischler et al., 1999; Tischler et al., 1999; van Leeuwen et al., 2015; Furuya et al., 2017). On balance, these findings do not support a carcinogenic effect for pyflubumide in the rats but confirm severe organ toxicity to the adrenals at higher dose levels and suggest a possible MOA for the organ toxicity.

The NOAEL for chronic toxicity in this study was 20 ppm (equal to 0.7 and 0.9 mg/kg bw per day in males and females). This NOAEL was based on increased organ weights of heart and liver and on myocardial fibrosis in both sexes, on adrenal medullary hyperplasia in males and on bile duct hyperplasia in females, all occurring at the upper dose levels of 120 and 600 ppm. At the same dose levels, mean body weights and food intake were increased. No evidence of carcinogenicity was obtained up to the highest dose of 600 ppm (equal to 23 mg/kg bw per day in male rats and 29 mg/kg bw per day in females) which is considered the NOAEL for carcinogenicity in this study (Kuwahara, 2012).

2.4 Genotoxicity

Pyflubumide was evaluated in a minimum set of guideline-compliant genotoxicity studies including an in vivo micronucleus assay in mice. These studies, which were all negative, are summarized in Table 19 and are considered sufficient to exclude mutagenic potential.

Table 19. Summary of genotoxicity studies with pyflubumide

Type of study	Test system	Concentration range tested	Metabolic activation	Purity	Result	Reference
Reverse gene mutation test in bacteria (Ames test)	*S. typhimurium* (TA98, 100, 1535, 1537) and *E. coli WP2UvrA*	61.7–5000 mg/plate (± S9 mix)	+/−	95.8%	Negative	Wada, 2010
In vitro gene mutation test in mammalian cells (mouse lymphoma assay)	L5178Y $tk^{+/-}$ mouse lymphoma cells	10–150 µg/mL (3 h exposure, ± S9); 2.5–40 µg/mL (24 h exposure, –S9)	+/−	97.3%	Negative	Lloyd, 2017
Chromosome aberration mammalian cells in vitro	Chinese hamster lung cells (CHL/IU)	10–40 µg/mL (6 h exposure, +S9); 6.3–25 µg/mL (6 h exposure, –S9); 3–12/10 µg/mL (23/45 h exposure, –S9)	+/−	95.8%	Negative	Tsukushi, 2011a
In vivo bone marrow micronucleus assay	Male Slc/ICR mice	500–2000 mg/kg bw (Twice on two consecutive days)	Not needed	95.8%	Negative	Tsukushi, 2011b

(a) In vitro studies

A bacterial reverse gene mutation (Ames) assay was conducted in four *Salmonella typhimurium* strains (see Table 19) and in *Escherichia coli* strain WP2 *uvrA*. Pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was dissolved in DMSO and applied in two separate tests at concentrations of 61.7, 185, 556, 1667, and 5000 µg/plate (test 1) or 313, 625, 1250, 2500, and 5000 µg/plate (test 2). All experiments were run in triplicates, both with and without metabolic activation (S9 mix). All test plates were negative for genotoxicity. In contrast, appropriate positive controls caused a distinct increase in mutation frequency. No cytotoxicity was observed. However, some precipitation was noted at pyflubumide concentrations of 2500 µg/plate and above without metabolic activation and at the maximum concentrations of 5000 µg/plate in the presence of the S9 mix. On balance, pyflubumide proved negative in the Ames test (Wada, 2010).

Genotoxicity in mammalian cells was tested in a mouse lymphoma assay. L5178Y cells were exposed to pyflubumide (Lot no. 14NNI-0711.001K, purity 97.3%) either for three hours in the presence of S9 mix for metabolic activation, or in an additional experiment for 24 h without activation. Based on a previous range-finding experiment, nine concentrations were used for the definitive mutagenicity tests. In the 3 h test without activation, they ranged from 10–250 µg/mL. In the 3 h test in the presence of S9 mix, concentrations between 20 and 500 µg/mL were used. However, due to cytotoxicity resulting in a lower relative total cell growth, meaningful analysis for viability and 5-trifluorothymidine resistance (TFT, that was the marker for genotoxicity in this assay) could be performed only up to a concentration of 150 µg/mL, with and without activation. At this maximum analysed concentration, some precipitation

was observed. In the 24 h exposure testing, under non-activation conditions, concentrations from 2.5 up to 100 µg/mL were applied but evaluation was confined to concentrations up to 40 µg/mL at maximum, and even at this fairly low level, severe cytotoxicity was apparent. In spite of precipitation or cytotoxicity, no increase in mutant frequency was observed at any concentration. The positive control substances methyl methanesulfonate (for non-activation conditions) and benzo[a]pyrene as used in the experiment in the presence of S9 mix, gave the expected increases. Thus, pyflubumide was found non-mutagenic in this study up to concentrations causing precipitation or cytotoxicity (Lloyd, 2017).

In an in vitro chromosome aberration assay, Chinese hamster lung cells were exposed to pyflubumide (Lot no. 9HZ0013P, purity 95.8%) for six hours, both with or without metabolic activation, and then cultivated for another 17 h before harvesting. In additional experiments in the absence of S9 mix, cells were continuously treated for 23 or 45 h. The test concentrations ranged from 1.5–50 µg/mL in the different experiments. Due to a low viability in the treated cultures, the highest concentrations that could be evaluated were 40 µg/mL for the 6 h exposure with activation, 25 µg/mL for the 6 h exposure without activation, and 12 or 10 µg/mL in the trials over 23 or 45 h, respectively. A total of 200 metaphases per concentration were scored and the percentage of cells with structural chromosome aberration calculated. No evidence of clastogenicity was obtained under any of the test conditions. The positive control substances cyclophosphamide (with activation) and mitomycin C (in the absence of S9 mix) gave the expected increases in structural aberrations. Accordingly, pyflubumide was devoid of a clastogenic potential in this study. Furthermore, there were no increases in polyploidy in the cultures treated with pyflubumide.

It was acknowledged that the number of metaphases under examination was lower than requested by the OECD test guideline 473 (that is at least 300) in its 2016 version, but it must be taken into consideration that the test was run in compliance with the 1997 version in which 200 was recommended as sufficient. However, unequivocally negative studies which were guideline-compliant when performed should not be repeated just because of an update to the respective guideline. Therefore, the study is considered acceptable (Tsukushi, 2011a).

(b) In vivo studies

In an in vivo micronucleus assay, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered by oral gavage once daily on two consecutive days to groups of five male Slc/ICR mice at dose levels of 0, 500, 1000 or 2000 mg/kg bw. The vehicle was an aqueous solution of 0.5% CMC sodium salt also containing 0.2% Tween 80. In addition, further five males received a single intrapertitonial (ip) dose of 3 mg per kg bw of the positive control substance mitomycin C that had been dissolved in distilled water. The animals were killed 24 h after the final dose and bone marrow was obtained from femoral epiphyses. Bone marrow smears were prepared and 2000 immature erythrocytes per animal scored for the occurrence of micronuclei. In addition, 200 erythrocytes per animal were evaluated for the ratio between normochromatic and polychromatic cells. All this scoring was performed blind.

There were no unscheduled deaths and no clinical signs of toxicity were observed. There was no significant or dose-related increase in the frequency of micronucleated erythrocytes in the treated groups. In contrast, the positive control substance produced a marked increase.

The percentage of polychromatic (immature) erythrocytes at the two upper dose levels (57.1% and 56.8%, respectively) was higher than in the control and low-dose groups (46.6% and 47.9%, respectively) but the difference was not statistically significant and there was no clear dose response. Accordingly, alternative possibilities to confirm bone marrow exposure and, in consequence, the validity of the negative result must be considered. In the ADME studies (Yoshizane, 2010a and 2010b, see above), radioactive residues were detected in bone marrow, but these experiments had been performed in rats, not in mice. On the other hand, haematological findings in the short-term studies in mice (Inagaki, 2009; Kashimoto, 2009) suggest that the highly perfused bone marrow was in fact exposed in the micronucleus assay (Tsukushi, 2011b).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a reproduction study, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was continuously administered to two consecutive generations of male and female Crl:CD^{O}(SD) [IGS] rats via their food. Group size was 24 rats per sex and dose in both the F0 and F1 generations. Dietary concentrations were 0, 7.5, 15, 100, or 500 ppm. These nominal dose levels had been selected on the basis of a preliminary one-generation study which was briefly mentioned in the main study report but not made available to JMPR. It was noted, however, that this range-finding study, with 1000 ppm as the highest dose, was performed in a closely related rat strain, Slc:SD. Based on substance intake in the pre-mating and gestation phases in the two generations, the actual mean daily doses ranged from 0.41–0.46 mg/kg bw and from 0.65–0.68 mg/kg bw in low dose males and females respectively. At the low-intermediate dose level of 15 ppm, mean daily intakes of 0.78–0.92 mg/kg bw were calculated for males and of 1.28–1.36 mg/kg bw for females. The high-intermediate dose of 100 ppm corresponded to daily intakes of 5.27–6.22 mg/kg bw in males and of 8.59–9.23 mg/kg bw in females. At the high dose level, mean daily intakes of 26–31 and of 42–45 mg/kg bw were calculated for males and females respectively. For risk assessment purposes, the lowest figures had to be used but first rounded. Accordingly, the actually resulting mean daily intakes were 0.4, 0.8, 5.3, and 26 mg/kg bw.

The F0 (= P) generation rats were mated after at minimum 70 days on test within their treatment groups to produce the F1 litters. They were further administered the test substance for gestation and lactation periods and were killed after the weaning of F1 pups. Duration of substance administration to this parental generation was approximately 18–19 weeks in total. In each group, 24 males and 24 females were selected among 21–25-day-old F1 weanlings as parental animals for the F2 generation. These young adult F1 rats were bred within their respective treatment groups to produce F2 litters from at least 70 days after weaning onwards. Sibling mating was avoided. There was only one litter per generation. In both generations, litters were randomly culled to a maximum of eight pups, preferentially four males and four females, on lactation day 4. Following weaning of F2 pups at 21 days after birth, all F1 generation parental animals and F2 pups were killed.

All animals on study were monitored daily for mortality and clinical signs of toxicity. When being weighed, the adult rats of both generations were also examined for neurological signs such as excitement, convulsion, sedation, or abnormal gait. Endpoints under evaluation during the study included body weight, body weight gain, food consumption, observations of estrous cycle, reproductive indices, and offspring parameters, including sex ratio and time points of vaginal opening or preputial separation in the pups as landmarks of sexual maturation. Gross examination of all parental animals was conducted at scheduled termination. The following organs were taken and weighed: adrenals, brain, epididymis, heart, kidneys, liver, pituitary, prostate (ventral lobe), ovaries, seminal vesicle, spleen, testes, thyroid, and uterus. Testicular sperm heads and epididymal sperm were collected and counted. Motility of spermatids was also determined, as well as the percentage of sperm cells with abnormal morphology. In all control and high-dose parental animals from both the F0 and F1 generations, histopathological examination of the reproductive organs, of the adrenals, the heart, the lungs, the liver (in females only) and pituitary was performed. In addition the ovaries were examined in the high-intermediate dose groups. Primordial follicles were counted in control and high-dose F_1 females. Because of observed changes at the high dose level, histopathological examination of the lungs was extended to all groups in the F_1 generation and examination of thyroid to high-intermediate dose females. Animals found dead or those which were killed for humane reasons were necropsied if possible to determine the cause of death. Gross examination of pups was conducted and at weaning, one male and one female pup from each litter were subject to body weight and organ weight measurement of brain, heart, liver, thymus, spleen, and uterus. In these pups, heart, liver, thymus, and thyroid were subject to histopathology, along with the lungs in all groups and gross lesions.

Four premature deaths occurred among males from different groups but these cases were clearly not related to treatment. During the pre-mating or the gestation periods, there were no further clinical observations that could be attributed to administration of the test substance. However, a single high-dose F_1 dam had birth difficulties and exhibited abnormal nesting behaviour resulting in litter loss. It can neither be confirmed nor excluded that these observations were caused by the test substance. Apart from that case, no signs of parental toxicity were seen during lactation.

Significantly higher mean body weights than in the control groups were noted in F1 males and in F0 and F1 females receiving the high dose of 500 ppm. Increases were reflected in a higher body weight gain and might be, at least partly, explained by a higher food intake, even though the differences to the controls were not always statistically significant. At the dose level of 100 ppm, there was also some evidence of greater food consumption in females of both generation during gestation and lactation. Such effects on body weight and food intake are usually not regarded as adverse from a toxicological point of view, but were certainly treatment-related, being in line with similar observations in other rat studies (see above). Taking into consideration a possible impact on human health, they are at least unwanted.

Gross examination at terminal kill did not reveal any abnormalities in the F0 generation at any dose level, neither in males nor females. In the F1 adults, however, hypertrophy of the heart was frequently observed in both sexes at 500 ppm. The second remarkable and most-likely treatment-related findings, again in both sexes and with a clear dose response, were spots on the lungs at the two upper dose levels. In the control groups and at the lower dose groups, no such lesions of the heart or of the lungs were apparent (Table 20).

The heart was confirmed as a target organ since its mean weight was increased in both generations of male rats at 100 and 500 ppm. There were a few other significant organ weight findings such as a higher absolute thyroid weight in high-dose F1 males and a higher prostate weight in the F0 generation at 500 ppm; these were not accompanied by significant changes in relative organ weights but at least the thyroid is a known target organ of pyflubumide. In the high-dose males in the F1 generation, relative brain, testis and epididymis weights were reduced. Since these findings were not paralleled by decreases in absolute organ weights and since there were no histopathological changes, they were also attributed to the higher body weight in that group.

In females, absolute liver, heart and ovary weights were increased in the F0 generation at the two upper dose levels. A clear dose response was apparent and relative organ weights were also significantly higher in the same groups. In addition, mean absolute weights of uterus and thyroid were increased in top-dose females but were not accompanied by concomitant increases of relative organ weight. In the F1 generation significant changes were confined to the highest dose group. Increases in absolute and relative liver, heart, uterus and ovary weights were noted and attributed to treatment, whereas higher absolute organ weights of adrenals, pituitary, kidneys and thyroid rather reflect the higher body weight.

Histopathology revealed a number of presumably treatment-related findings in the lungs and the heart; these were apparent, however, only in the F1 generation (Table 20) at the two upper dose levels. In the F0 generation, in contrast, the only effect was the more frequent observation of a decrease in colloid area of the thyroid in both sexes, achieving statistical significance in high-dose females. A similar pattern of the same thyroid lesion was confirmed in the F1 generation.

Table 20. Selected gross and histopathological findings in the F1 generation in the reproductive study with pyflubumide in rats
(n = 24 per dose and sex; statistical analysis was performed only for animals producing live litters)

Sex, dose, (ppm)	Males					Females				
	0	**7.5**	**15**	**100**	**500**	**0**	**7.5**	**15**	**100**	**500**
Gross findings										
Heart: hypertrophic	0	0	0	0	13**	0	0	0	0	6*
Lungs: spots on surface	0	0	0	4*	12**	0	0	0	2	14**
Histopathological findings										
Lungs: haemorrhage	4	5	2	2	16**	4	1	0	4	17**
Lungs: alveolar wall thickened	0	0	0	6*	12**	0	0	0	3	15**
Lungs: alveolar distention	0	0	0	3	11**	0	0	0	1	9**
Lungs: brown pigment	0	0	0	2	14**	0	0	0	1	12**
Heart: myocardial fibrosis	5	NE	NE	NE	8	1	NE	NE	NE	4
Thyroid: decrease in colloid area	1	NE	NE	NE	3	2	NE	NE	1	9**

* $p < 0.05$, ** $p < 0.01$, Fisher's exact test

NE Organ not examined

Investigations on spermatogenesis (sperm count, motility and morphology) in adult males did not reveal any differences between the various groups. Likewise, follicle count in F1 females was not affected by treatment.

There were only a few significant alterations in reproductive parameters (Table 21). Mean duration of the estrous cycle was slightly increased at the top dose in the F0 generation and in the F1 generation from 100 ppm, but the mating index or the time to successful mating were not affected. This rather marginal change may have been treatment-related but is not regarded as adverse. Gestation length was significantly prolonged in the high-dose group in the F0 generation. This effect was considered adverse even though it was not confirmed in the F1 generation. It is worth noting that the single dam exhibiting abnormal labour and disturbances of nesting behaviour had had a normal gestation length. The viability index (the ratio of number of pups alive to the number of delivered pups) was significantly decreased on the day of birth in the same high-dose group of the F0 generation and the study author suspected that this could be due to the delay in parturition. This is only a hypothesis and cannot explain why the effect on gestation length was relatively small, compared to a clear decrease in viability. It is not clear from the study whether the lower viability index was due to stillborn pups or to those which died on day 0.

Other parameters such as fertility, number of implantations, or litter size were not altered, but sex ratio was skewed, for unknown reasons, in the high-dose group of the F1 generation. Because of its isolated occurrence, this finding is considered to have occurred by chance. Mean pup weights at birth were significantly increased, in both generations, at the top dose level. This finding cannot be explained by a smaller litter size since this was not significantly diminished (even though the lowest value was noted in the F0 generation at 500 ppm). The toxicological relevance of such an increase, in contrast to a decrease, is equivocal. On one hand, it cannot be excluded that this finding was related to the prolonged gestation. On the other hand, higher pup weights at birth might also reflect the higher body weight of the dams at the same dose level, which was considered treatment-related.

Table 21. Selected reproductive and offspring effects in the two-generation reproduction study with pyflubumide

Dose	0 ppm		7.5 ppm		15 ppm		100 ppm		500 ppm	
Generation	F_0	F_1	F_0	F_1	F_0	F_1	F_0	F_1	F_0	F_1
Mean length of estrous cycle (days)	4.0	4.0	4.0	4.0	4.0	4.1	4.0	4.2**	4.2**	4.2
Gestation length (days)	22.1	22.1	22.2	22.3	22.1	22.2	22.3	22.1	22.7**	22.4
Litter size	13.4	13.5	13.0	12.8	14.0	13.1	14.3	14.4	11.9	13.4
Viability index, day 0 (%)	99.1	99.4	99.1	97.9	97.6	98.0	94.9	99.3	93.5**	97.8
Sex ratio (expressed as % of male pups)	50.3	51.8	53.0	44.8	51.6	51.0	51.9	48.5	48.1	43.3*
Mean pup weight (g) on day 0, m/f	6.9 / 6.6	6.8 / 6.6	7.1 / 6.7	7.2 / 6.8	6.9 / 6.5	7.2 / 6.8	7.1 / 6.7	7.2 / 6.8	8.0**/ 7.6**	7.9**/ 7.5**
Mean pup weight (g) on day 21 (weaning), m/f	67.1 / 64.3	69.8 / 66.7	68.6 / 64.9	66.6 / 67.8	67.1 / 64.8	71.9 / 68.7	69.9 / 66.9	71.4 / 69.6	71.5**/ 68.7**	69.9 / 66.8
Preputial separation (day)	ND	40.0	ND	40.0	ND	40.0	ND	40.5	ND	40.9
Male pup body weight (g) at preputial separation	ND	216	ND	220	ND	218	ND	223	ND	230*

*$p < 0.05$, **$p < 0.01$, Dunnett's or Dunnet-type test

ND not determined in this group

Pup survival during lactation was not affected. The higher pup weight observed on day 0 continued in the F1 pups over the whole lactation period. In the F2 pups, by contrast, a significant increase over the control was observed for the last time on lactation day 7.

A possible effect on the sexual development of the pups was confined to the males and was relatively minor. A distinct delay in preputial separation in high-dose male pups cannot be excluded at the two upper dose levels even though the difference to the controls was not statistically significant. However, the mean pup body weight in these groups was higher when preputial separation was observed, achieving statistical significance at the top dose level (Table 21). This finding could well be linked to the later occurrence of preputial separation but, it should be borne in mind that the time at which this landmark is reached strongly depends on body weight development. As a result of higher body weights in F1 pups at the high dose level, preputial separation would be expected to occur a bit earlier, not later as was the case here. Both parameters are interrelated and it is difficult to assess which one is primary and which is secondary in this case. No firm conclusion can be drawn. In contrast, time of vaginal opening in female F1 pups was not affected.

In weanling F2 pups from the high-dose group, white spots on the lungs were noted. In both generations, there were increases in absolute and relative organ weights of the heart at the two upper dose levels and of the liver in the high-dose groups. Thymus weights, in contrast, were lower at 100 and 500 ppm. Histopathology revealed alveolar enlargement (distension) in male and female pups in both generations at the two upper dose levels resembling the findings in the F1 generation. At the maximum dose, lung haemorrhages were seen more often at least in female pups.

To conclude, the NOAELs in this two-generation reproduction study in rats were as follows:

- Parental toxicity: 15 ppm (equivalent to 0. 8 mg/kg bw per day), based on gross and histopathological findings in the heart and in the lungs and on organ weight changes at 100 ppm and above.
- Reproduction and fertility: 100 ppm (equivalent to 5.3 mg/kg bw per day), based on decreased viability index and slightly prolonged gestation at the high dose level of 500 ppm in the F0 generation.
- Offspring toxicity: 15 ppm (equivalent to 0. 8 mg/kg bw per day), based on pathological findings and organ weight changes in weanling pups at 100 ppm and above.

The pathological findings on the lungs in the F1 and F2 pups suggested an adverse effect on offspring which was still present when these animals reached adulthood. For their further characterization, a number of mechanistic studies were performed (see sub-section 2.6(c) below) (Sato, 2012).

(b) Developmental toxicity

Rat

Pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered by oral gavage to presumed-pregnant Crl:CD(S)[IGS] rats (group size 23 or 24) once per day from gestation days (GDs) 6–19. Dose levels were 0, 5, 30, and 200 mg/kg bw per day. The test substance was dissolved in a 1% aqueous solution of sodium CMC that was also applied to the vehicle control group. On GD 20, all dams were killed and subjected to caesarean section. Gravid uteri were removed and foetuses counted, sexed, and examined for external, visceral and skeletal abnormalities. The dams were necropsied and ovaries, uteri and placentas examined for parameters such as weight, number of corpora lutea, implants or evidence of early resorptions.

Among the dams, there were no unscheduled deaths and no clinical signs of toxicity were noted. The only consistent effect on body weight was a lower mean body weight gain in top-dose females (reduction by ca 30% compared to the control group) during GDs 6 and 9. This parameter was characterized by strong within-group variation and five of the dams receiving 200 mg/kg bw per day even lost weight. Food consumption was slightly, but significantly, reduced (by ca 10%) in the mid-dose group during GDs 6 and 9 and by up to 40% in the high-dose group during GDs 6 and 12. During the later stages of gestation, food intake and body weight gain were similar to the control values. Gross examination of the dams did not reveal any findings that could be attributed to treatment. The parameters gravid uterine weight, number of corpora lutea and implants, and the incidence of pre- or postimplantation losses was not affected. However, mean placenta weight in the dams receiving the high dose was around 11% higher than in the control group but a MOA has not been proposed.

There was no increase in resorptions or in the number of live foetuses. Sex ratio was not altered. However, the mean fetal weight was higher at 200 mg/kg bw per day than in any other group and the roughly 6% difference to the control group was statistically significant. There were no malformations that could be attributed to treatment but a significant increase in the total incidence of visceral variations was observed in the high-dose group. These variations comprised remnants of the thymus in the neck, dilated renal pelvis and left umbilical arteries which were, however, not significantly increased when considered as separate entities. Accordingly, the increase in total number is equivocal.

On balance, the maternal NOAEL in this study can be established at the mid-dose level of 30 mg/kg bw per day. The initial effect on food intake at this dose level was only marginal whereas the lower body weight gain and occasional body weight loss at 200 mg/kg bw per day can be regarded as an acute adverse effect. In addition, an increase in mean placenta weight at this dose level is, given the lack of further information to explain this effect, regarded as potentially adverse. In contrast to the opinion of the study author, the developmental NOAEL should be also set at 30 mg/kg bw per day because of the unusual increase in mean fetal weight at 200 mg/kg bw per day. Usually, an increase in fetal weight is not considered adverse and is often related to a lower number of fetuses per litter. However, this was not the case here and, taking into account that pyflubumide caused an increase in body weight in several rat studies, this finding is considered treatment-related. Since it was regarded as an unwanted effect in general, the same view should be taken here (Sato, 2010).

Rabbit

Study1

A range-finding experiment for a subsequent developmental toxicity study was performed in groups of eight pregnant Japanese White rabbit does which were administered pyflubumide (Lot no. 9HZ0013P, purity 95.8%) once a day by oral gavage from GDs 6 to 27. The dose levels were 0, 30, 100, 300, and 1000 mg/kg bw per day. The pregnant females were killed and fetuses obtained by caesarean section on GD 28.

Maternal toxicity became apparent from a dose of 100 mg/kg bw per day upwards. In this dose group, one doe aborted and two delivered their pups prematurely, just before scheduled termination on GD 28. Abortion was also observed in three females in the group receiving 300 mg/kg bw per day and in one doe in the top dose group. At this maximum dose level of 1000 mg/kg bw, two females were found dead during the treatment period. Body weight, body weight gain and food consumption were compromised at 100 mg/kg bw per day and above. Necropsy of the does which survived until scheduled termination revealed enlargement of the liver as well as higher relative heart and liver weights from a dose of 300 mg/kg bw per day upwards. Hair boluses was found in the stomachs of some of the does which had been found dead or aborted as well as in a few high-dose animals at termination.

The number and viability of fetuses was not affected at the two lower dose levels but both markedly decreased at 300 mg/kg bw per day. In addition, mean fetal weight was lower. At the maximum dose level of 1000 mg/kg bw per day, there were no live fetuses anymore, due to resorption and fetal death. There were no external or visceral malformations up to the dose of 300 mg/kg bw per day, the highest dose that could be evaluated for this parameter.

Based on these results, 80 mg/kg bw per day was selected as the maximum dose for the main developmental study in rabbits. Taking into account the pronounced maternal toxicity already found at 100 mg/kg bw per day, this choice seems appropriate (Hojo, 2009).

Study2

Pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered once daily by oral gavage to groups of 25 presumed-pregnant (artificially inseminated) Japanese White (Kbl:JW) rabbits from GD 6–27. Dose levels were 0, 5, 20, or 80 mg/kg bw per day. The test substance was dissolved in a 1% aqueous solution of sodium CMC that was also applied to the vehicle control group. On GD 28, all females were killed and subjected to caesarean section. Gravid uteri were removed and foetuses counted, sexed, and examined for external, visceral and skeletal abnormalities. The dams were necropsied and ovaries, uteri and placentas examined for parameters such as weight, number of corpora lutea, implants and evidence of early resorptions.

Among does, there were no unscheduled deaths and, up to the mid-dose level of 20 mg/kg bw per day, no clinical signs of toxicity or gross findings at necropsy were noted. In the group receiving 80 mg/kg bw per day, however, three does aborted on GDs 18, 25, or 27 after having experienced body weight losses. In addition, four females from this group delivered prematurely on GD 28. Body weight, body weight gain and food consumption were not affected in the groups receiving the low and mid doses. In the high-dose group, in contrast, mean food intake was generally reduced from GD 9 onwards. For three of the seven three-day intervals, the difference to the control group was even statistically significant. Whereas mean body weight in the high-dose group, at scheduled termination, was not different from the other groups, body weight gain over the treatment period was lower than in the control groups by about 40%, but the difference did not gain statistical significance, most likely because of a more than doubled SD which suggested strong interindividual variance.

In addition to the animal losses at the high doses, two control and three high-dose rabbits were found not to be pregnant. Total resorption of a complete litter was noted in one female each in the control, mid- and high-dose groups. Based on its occurrence in both control and treated group, this latter finding cannot be attributed to treatment. Gravid uterine weight, number of corpora lutea and implants and the incidence of preimplantation losses were not affected. Mean placenta weight in the does receiving the high dose, however, was significantly higher (by 12.7% in the mean) than in the control group. No MOA was proposed but the same effect had been observed in rats (Sato, 2010, see above).

No adverse effects on the fetuses were reported in any group. There was no increase in any morphological abnormalities. The number of live fetuses per litter, percent of resorptions, sex ratio and fetal weight were not affected even though it must be acknowledged that the number of litters that could be included in the evaluation was markedly lower at the top dose level because of abortions and premature deliveries. In fact, at scheduled termination, the number of females with live fetuses accounted for 22, 25, 24, and 14 in the control, low-, mid-, and high-dose groups, respectively.

In this guideline-compliant study in rabbits, the maternal NOAEL was 20 mg/kg bw per day, based on reduced food consumption and body weight gain, body weight losses in a few females, abortions and premature delivery. In addition, placenta weight was increased. No clinical signs were noted during the first three days of treatment and all but three high-dose females gained weight. Even in the animals which lost weight, the decrease was only minimal. Thus maternal toxicity in this study cannot be unequivocally considered an acute effect.

Since no developmental or teratogenic findings in the fetuses have been reported, the highest dose level of 80 mg/kg bw per day is considered the developmental NOAEL (Hojo, 2010).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, pyflubumide (Lot no. 14NNI-0711-001K, purity 97.3%), was administered once by oral gavage to male and female Crl:CD(SD) Sprague Dawley rats at doses of 0 (vehicle control), 500, 1000 or 2000 mg/kg bw. The test item had been dissolved in 0.5% CMC sodium with 0.2% Tween 80. The dose volume was 10 mL/kg bw. The groups comprised 10 animals per sex. Dose selection was based on the low toxicity in an acute oral toxicity study (Tsukushi, 2010a) and on a preliminary range-finding acute neurotoxicity study in which the same doses had been applied to groups of three male and female rats each (Barnett, 2018a).

Over the course of the 14 days that followed the observation period, the animals were frequently monitored for viability and general appearance. A FOB and motor activity (MA) assessments were conducted on all rats on study prior to dosing, approximately eight hours after dosing (day 0) and on days 7 and 14 after treatment. Body weights were recorded prior to dose administration, on the days when the FOB was conducted and at scheduled termination. On test day 16, the rats were killed, in situ, perfused with 10% neutral buffered formalin and gross examination for pathological changes carried out. Brain weight was determined in all rats. Five rats per sex from the control and high-dose groups were selected for subsequent histopathological examination of brain, Gasserian ganglia and associated trigeminal nerve tissue, eyes with retinas and optic nerves, spinal cord, peripheral nerves from hind limbs and skeletal muscle (gastrocnemius and soleus).

There were no unscheduled deaths and no clinical signs of toxicity occurred which could be attributed to test substance administration. Likewise, there were no gross lesions observed at necropsy in any group. However, mean body weight was lower in male rats in all treated groups from day 8 onwards even though the difference from the control group was consistently below 5% and was not statistically significant. Remarkably, there was no much difference between the three dose groups and a dose response can hardly be established. The decline in body weight was clearly due to a reduced body weight gain in male rats at all three dose levels from day 0 to day 7. Body weight gain was 30–40% lower than in the control group and for all groups the difference was statistically significant ($p < 0.01$ in Dunnett's test). However, once again there was no clear dose response since the reduction was most pronounced in the low-dose group. It is not known whether this effect resulted from lower food intake or impaired food utilization since food consumption was not determined and food efficiency not calculated. During the second half of the post-observational period (days 8–16), body weight gain in the treated groups was similar to the control group but this was apparently not sufficient to compensate for the lower initial weight gain. In females, by contrast, there were no consistent effects on body weight or its gain.

Table 22. Possible treatment-related findings on body weight gain and body temperature

Dose (mg/kg bw)	**0**	**500**	**1000**	**2000**	**0**	**500**	**1000**	**2000**
Sex	**Males**				**Females**			
Mean bw gain, days 1–8 (g)	51.20	30.80*	35.80*	33.90*	13.60	16.90	16.20	16.70
Mean bw gain, days 8–15 (g)	33.40	37.80	36.10	32.50	16.70	12.50	21.90	17.90
Body temp., day 1 (°C)	37.15	36.22*	36.11*	36.05*	37.18	36.64*	36.51*	36.37*

* $p < 0.01$, Dunnett's test

It is worth noting that slight body weight losses (2, 3, or 5 g on average) had been observed on the day after dosing in the range-finding experiment at dose levels of 500, 1000, and 2000 mg/kg bw, but in the preliminary study this effect was seen in female rats whereas males were not affected (Barnett, 2018a).

The FOB and MA did not reveal differences at any time point between the control and treatment groups that could be attributed to treatment. Occasional significant findings, such as higher mean forelimb grip strength in high-dose males at termination, were not considered substance-related and adverse because they were confined to a single timepoint and had not been observed before, that is closer to the application time. The only remarkable finding was a slight decrease in body temperature in male and female rats on the day of substance administration. The difference to the control groups was statistically significant at all three dose levels but body temperature in these treated groups was very similar and the dose response curve very flat. Seven days later, body temperature was the same as in the controls. This finding is regarded as an immediate response to substance administration but, since the reduction was slight and animals recovered fairly soon, it is not considered adverse or indicative of neurotoxicity.

Brain weight was not affected and neurohistopathology as performed on a limited number of control and high-dose animals did not reveal differences for males nor females, between the two groups such as would point to neurotoxic potential. The very few microscopic findings were considered by the consulting pathologists to represent common background lesions of rats. Most frequently observed was minimal nerve fibre degeneration within the trapezoid body of the ventral pontine region of the brain, part of the auditory system. In males, this degeneration was seen in three control and three high-dose rats. Similarly in females the incidence of 2/5 was the same in the control and top-dose goups.

On balance, the NOAEL for neurotoxicity in this acute study was 2000 mg/kg bw in both males and females. In contrast, a systemic NOAEL could not be established because of the adverse effect on body weight gain in all treated male groups. The lowest dose of 500 mg/kg bw is the LOAEL (Barnett, 2018b).

No separate short-term neurotoxicity study was performed. However, neurotoxicological parameters were investigated in the 28-day oral feeding study in rats (Horiuchi, 2007) that is reported above. The only effect was a lower forelimb and hindlimb grip strength in males receiving a dose of 2000 ppm (equivalent to 137.1 mg/kg bw per day) that can be attributed to a dramatically (by more

than 25%) reduced body weight. Functional observations were also included in the 90-day and one-year studies in rats (Kuwahara, 2009; Kuwahara, 2011b) revealing limited evidence of decreases in motor activity either in high-dose (1200 ppm) females in the 90-day or in high-dose (600 ppm) males in the one-year study (see above). These findings reflect a systemic effect, rather than any specifically neurotoxic effects. Histological examination of high-dose animals did not detect lesions in the brain, sciatic nerve or spinal cord.

(b) Immunotoxicity

No available studies for pyflubumide. There are no indications of immunotoxicity coming from the other studies.

(c) Mechanistic studies

A number of mechanistic studies were performed to investigate pharmacological properties of pyflubumide and to explain effects on the heart and the thyroid in various species and studies. Other studies aimed to clarify the mechanism of pulmonary lesions in rat pups in the multigeneration study. Most of these studies were not conducted under GLP and did not follow a specific guideline.

Pharmacological effects

Study 1

An investigative acute oral toxicity study was performed in male and female Fischer (F344/DuCrlCrlj) rats and in female Crlj:CD1(ICR) mice to investigate pharmacological effects of pyflubumide, clarify the possible mechanisms of toxicity and to obtain information on possible curative treatment. For this purpose, the test substance (Lot no. 9HZ0013P, purity 95.8%) was dissolved in 0.5% CMC sodium with 0.2% Tween 80 and administered once by oral gavage at a constant dosing volume of 20 mL/kg bw, but at different dose levels to groups of either male or female rats, or female mice. In the experiment on renal excretion in male rats, the animals received in addition, 2.5 mL/100 g bw of physiological saline. Apparently, no specific guideline but country-specific Japanese guidance for studies of this type was followed. In Table 23, a summary of the different study groups and parameters is given.

Table 23. Pharmacological study with pyflubumide in rats and mice - overview of study design

Objective	Parameters examined	Species, number and sex	Doses applied (mg/kg bw)
General condition	Occurrence of clinical signs (monitored 1, 2, 4, 6 and 24 h after dosing); body weight; FOB parameters; body temperature; pupil diameter	Rat, 5 females per group	0, 200, 600, 2000
Effects on nervous system	Spontaneous locomotor activity measured at 1, 2, 4, 6 and 24 h after dosing	Rat, 5 females per group	0, 200, 600, 2000
Effects on nervous system	Electric shock-induced seizures or convulsions, 2 h after dosing	Mouse, 8 females per group	0, 200, 600, 2000
Cardiovascular effects	Heart rate and systolic blood pressure measured at 1, 3, 6 and 24 h after dosing	Rat, 5 females per group	0, 80, 400, 2000
Haematology	Measurement of a wide range of parameters at 2 h after dosing	Rat, 5 males per group	0, 200, 600, 2000
Renal function/ urinary effects	Urine volume, urinary electrolytes, osmotic pressure (measured for the time periods 0–6 h and 6–24 h after dosing)	Rat, 5 M per group	0, 200, 600, 2000

There were no premature deaths in any group.

Thorough observation of female rats over 24 hs did not reveal any indications of clinical signs or behavioural abnormalities. FOB parameters (forelimb and hindlimb grip strength and landing foot splay) as well as pupil size were not affected up to the highest dose of 2000 mg/kg bw. All female rats

gained weight and mean weights did not differ between groups, neither prior to dosing nor after 24 h. In another experiment in female rats which had been administered the same doses, spontaneous motor activity was not affected by treatment at any dose level.

In female mice, the current threshold (in mA) for an electroshock to induce clonic or extensive tonic convulsions was not altered by prior treatment with different doses of pyflubumide as compared to the control group which had received the vehicle only.

Systolic blood pressure in female rats was not affected in any dose group. A significant increase in heart rate in rats receiving 80 or 400 mg pyflubumide/kg bw three or six hours after dosing was not dose-related and not observed at the maximum dose level. Therefore, this incidental finding cannot be attributed to treatment even though it is similar to what was observed during the feeding studies in dogs.

Administration of pyflubumide at doses up to 2000 mg/kg bw to male rats had no effect on any haematological parameter (RBC, white blood cell or coagulation). However, only a few effects (impairment of coagulation, immediate haemolysis) could have been detected at this very early (2 h after administration) measurement time. Furthermore, no baseline data from the same animals are available which might have facilitated a comparison.

In contrast, there were clear and dose-related effects on renal parameters. Urine volume was significantly decreased in "saline-loaded" male rats in all treated groups. In line with that, sodium, potassium and chloride excretion were reduced, mostly in line with dose, and the sodium/potassium ratio was skewed since sodium excretion decreased more than that of potassium (Table 24). The osmotic pressure of urine, in contrast, was not affected.

Table 24. Effects of a single dose of pyflubumide on urinary volume and electrolyte concentrations in male rats (n = 5 per dose group); mean values with SD as obtained for the entire 24-hour post observation period

Dose (mg/kg bw)	Urine volume (mL/100 g bw)	Na^+ excretion (µEq/100 g bw)	K^+ excretion (µEq/100 g bw)	Na^+/K^+ ratio	Cl^- excretion (µEq/100 g bw)
0	5.7 ± 0.6	715 ± 43	952 ± 108	0.76 ± 0.11	791 ± 20
200	3.7 ± 0.9**	381 ± 64**	707 ± 76**	0.54 ± 0.07**	353 ± 68**
600	3.0 ± 0.7**	274 ± 88**	589 ± 92**	0.47 ± 0.13**	257 ± 81**
2000	2.9 ± 0.6**	230 ± 58**	602 ± 54**	0.38 ± 0.08**	224 ± 41**

* $p < 0.01$, Dunnett's test

Overall, in this investigative single-exposure study in rats and mice, the only finding was an effect on urine volume and electrolyte concentrations seen in male rats from the lowest dose tested of 200 mg/kg bw. No information on toxicity mechanisms or options for possible treatment in poisoning incidents could be obtained (Tsuchiyama, 2012a).

Study 2

In an additional study to further examine the effects on renal function and to establish a NOAEL for such effects, pyflubumide (Lot no. 9HZ0013P, purity 95.8%) was administered by oral gavage as a single dose to male Fischer (F344/DuCrlCrlj) rats. Two groups of five males each received the test compound at dose levels of 6, 20, 60, or 200 mg/kg bw. Two control groups of the same size were administered only the vehicle; an aqueous solution of 0.5% CMC sodium with 0.2% Tween 80. In addition, all animals were given a 2.5 mL/100 g bw oral dose of physiological saline. In the first set of test groups ("pilot groups"), urine was collected over the first six hours after dosing, whereas blood was sampled 6.5 hours post-dosing when the animals were killed. In the second set of study groups ("main groups"), urine was collected over 24 h and blood was taken 24.5 h post-dosing.

As in the previous study, (Tsuchiyama, 2012a), urine volume and concentrations of Na^+, K^+ and Cl^- ions were measured as well as the osmotic pressure. Excretion of the three elements and the Na^+: K^+ ratio was calculated. In addition, the sodium, potassium and chloride ion concentrations were determined in blood serum.

Urine volume excreted over the first six hours and for the whole 24 h period was significantly reduced in the groups receiving 60 and 200 mg/kg bw even though there was no clear dose response. It

seems that the total decrease was mainly due to a lower excretion of urine during the first six hours. A lower urine volume was observed for the first six hours also at the low dose levels of 6 and 20 mg/kg bw but the effect was marginal and had no significant impact on total 24 h excretion. Significant decreases in sodium, potassium and chloride excretion were noted in all treated groups for the first six hours post-dosing even though the differences were not always statistically significant or strictly dose-related (Table 25). An altered sodium:potassium ratio as observed in the previous study was seen for the first six hours in the 20 mg/kg bw group and over the 24 h period only at the top dose. When the entire 24 h post-observation period is taken into consideration, however, effects were less pronounced and confined to the highest (sodium excretion, Na^+:K^+ ratio) or the two upper dose levels (Cl^- excretion), whereas potassium excretion was similar to the control group. Osmotic pressure was not affected at all.

Table 25. Effects of a single dose of pyflubumide on urinary volume and electrolyte concentrations in male rats (n = 5 per dose group); mean values as obtained for the first 6 hours post dosing and the entire 24-hour post-observation period

Dose (mg/kg bw); Time (h)	Urine volume (mL/100 g bw)		Na^+ excretion (µEq/100 g bw)		K^+ excretion (µEq/100 g bw)		Na^+/K^+ ratio		Cl^- excretion (µEq/100 g bw)	
	0–6 h	0–24 h	0–6 h	0–24 h	0–6 h	0–24 h	0–6 h	0–24 h	0–6 h	0–24 h
0	2.8	6.2	159	770	121	992	1.36	0.78	223	856
6	2.1*	5.1	76	712	84	967	0.96	0.74	129**	737
20	2.2	5.5	55*	740	90	1058	0.63**	0.71	128**	783
60	1.3**	4.6*	21**	605	39**	906	0.50**	0.66	53**	586*
200	1.5**	4.7*	15**	526*	43**	876	0.34**	0.59**	52**	491**

* $p < 0.05$, ** $p < 0.01$, Dunnett's test

Electrolyte determinations in serum revealed a single significant finding only. Potassium concentration was increased at the highest dose level by about 6 % (after 6.5 h) and 8 % (after 24.5 h) over the control mean. Sodium and choride concentrations were not affected.

On balance, an acute impact of pyflubumide on renal function was confirmed, but was less pronounced than in the previous study in which higher concentrations were tested. Since the effects at doses below 60 mg/kg bw were only transient, a dose of 20 mg/kg bw per day can be considered the NOAEL.

These findings suggest an impairment of renal functions when high doses of pyflubumide are applied by bolus administration. It is worth noting that urine volume in dietary studies in rats and dogs was increased rather than decreased (see for example Kuwahara, 2011b, or Ishikawa, 2011), which might also indicate an effect on renal function, but a different one. Moreover, apart from an increase in chronic nephropathy in high-dose females in the two-year study in rats (Kuwahara, 2012), there was no further evidence in the available routine dietary toxicological studies that the kidney might be a target organ of pyflubumide. Thus, this presumed renal effect as revealed by the pharmacological studies might be not relevant to the expected (dietary) route of human exposure (Tsuchiyama, 2012b).

Mechanistic studies on possible mode of action for heart effects in the rat

Intravenous administration of a single dose of pyflubumide at 5 mg/kg bw to four male Fischer (F344/DuCrlCrlj) rats resulted in an increased heart rate and in a decreased systolic and diastolic blood pressure. The latter effect was thought to be due to vascular dilatation. The hypothesis was brought forward that the enlargement and organ weight increase of the heart in several studies might be due to these pharmacological effects, similar to those observed with various medical drugs such as hydralazine or minoxidil (Toga, 2009).

In a subsequent in vitro study, anatomical specimens from the right heart atrium and from the thoracic aorta were excised from untreated male Fischer (F344/DuCrlCrlj) rats. These specimens were prepared and, following equilibration, exposed in Magnus tubes for more than 10 minutes to various concentrations of pyflubumide and five of its plasma metabolites (Metabolites B, C, D, F, and U, see Table 5 and metabolite list in the Annex 1). Two parameters were investigated. The frequency of

autonomous pulsation of the atrial tissue was not altered by the active ingredient or its metabolites up to the highest tested concentration of 10 μM. In contrast, relaxation of the aortic sample (pre-contracted by exposure to 1 μM phenylephrine), as measured by reduction in vascular tension, was observed after exposure to 10 and 30 μM pyflubumide and four of its metabolites (only pyflubumide-RfOH, metabolite U, was not active). The metabolite pyflubumide-NH-RfOH (metabolite D) which was also identified in rat plasma and in rat milk, but not in urine or faeces, was much more potent already causing reduction in aortic tension by more than 50% at a concentration of of 1 μM. The relaxation was regarded as indicative of vasodilatation. The study's author attributed the lower blood pressure, higher heart rate and enlargement of the heart, as previously observed in studies in rats and dogs, to this vascular effect (Toga, 2011).

One might also assume that the morphologic changes caused by pyflubumide in the adrenals in, for example, the long-term rat study (Kuwahara, 2012) could have contributed to cardiotoxicity. In addition to the significant increase in medullary hyperplasia in male rats at the two upper dose levels, the incidence of benign pheochromocytoma in the high-dose male group was also greater even though statistical significance was not achieved. This type of tumour has been shown to induce cardiomyopathy in humans and also in rats, most likely due to a stimulating effect of catecholamines (released by the tumour) on the myocard. Among other findings, an increase in heart weight and interstitial and replacement fibrosis has been described (Chandra, Hoehnerhoff & Peterson, 2013), that is findings that had also become apparent with pyflubumide. However, this hypothesis does not explain the clinical–functional and morphological heart findings in the dog studies since, in this species, adrenal lesions were apparent only in the cortex, not in the medulla. Moreover, the main symptom of a higher release of catecholamines, both acute and chronic, is an increase in blood pressure (Greim et al., 2009). With pyflubumide, however, a contrary decrease in blood pressure was observed after intravenous application to rats (Toga, 2009) or in male dogs receiving a dietary dose of 2500 ppm in the initial phase of a 90-day study (Ishikawa, 2010). Therefore, this alternative hypothesis cannot be accepted.

Mechanistic studies on thyroid effects in the rat

A number of experiments were performed in male Fischer (F344/DuCrlCrlj) rats to investigate the effects of pyflubumide on the thyroid and its function, with special emphasis on hormone production.

Study 1

In a first trial, groups of six animals received a dietary dose of 1200 ppm for either 3, 7, or 28 days. Unfortunately, food consumption was not recorded and a mean daily intake not calculated in this study. However, based on the approach usually taken by JMPR, this dietary dose may be considered equivalent to a dose of 60 mg/kg bw per day. Three control groups of the same size were fed untreated basal diet for the same periods. At termination, blood was sampled and the concentrations of the hormones T_3, T_4 and TSH measured. Animals were killed and thyroid glands removed, weighed and microsome fractions prepared to determine TPO activities. In addition, a positive control group received a single dose of 150 mg/kg bw of the known TPO inhibitor 1H-1,2,4-triazole-3-thiol (MTZ) by oral gavage. These rats were terminated three hours after dosing. For TPO activity measurement, frozen thyroids taken from three animals per group were combined, minced and homogenized before microsome fractions were prepared. Total activity in the thyroid was calculated on the basis of protein concentration (expressed in guaiacol units) and organ weight. Each sample was analysed in duplicate and the mean value for the group used for evaluation.

No premature deaths and no clinical signs of toxicity were observed. Body weight was not affected but thyroid weight was reduced after three days of pyflubumide administration. In contrast, thyroid weight was markedly increased after treatment for 7 or 28 days. In parallel, significant decreases in T_4 were observed in these groups whereas a reduction in T_3 was confined to the seven-day exposure. Levels of TSH were increased at all time points, suggesting an immediate feedback response in the organism to the reduction of thyroid hormone synthesis. All these hormone changes were most pronounced after seven days of pyflubumide administration, despite ongoing organ weight increase, suggesting that stimulation by TSH was partly effective. The reductions in T_3 and T_4 as well as the increase in TSH confirmed similar findings in a previous 28-day feeding study in rats receiving 2000 ppm (Horiuchi, 2007, see earlier).

Thyroid peroxidase activities were clearly reduced in the treated groups, exceeding by far even the effect of the positive control substance after seven days (Table 26).

Table 26. Summary of thyroid findings (mean values) in the mechanistic study with pyflubumide

Exposure	3 days		7 days		28 days		Single dose
Concentration	0 ppm	1200 ppm	0 ppm	1200 ppm	0 ppm	1200 ppm	MTZ
Thyroid weight (mg)	10	7*	8	23#	10	35*	7*
T_3 (ng/dL)	246.80	218.36	223.69	160.38#	156.57	151.90	
T_4 (ng/dL)	5.60	5.55	5.77	4.75##	5.77	5.53*	
TSH (ng/mL)	5.65	7.28*	6.63	41.88#	6.31	15.75*	
TPO activity (mGU/thyroid)§	7.70	1.95	10.85	0.15	16.35	3.53	2.15

* $p < 0.01$, Student's t-test or Aspin Welch test

# $p < 0.05$, ## $p < 0.01$, Dunnett's or Steel test

§ Not subject to statistical analysis because of the availability of only two measured activity values per group

Study 2

In a second experiment, groups of six male rats received dietary doses of 20, 120, or 600 ppm of pyflubumide for seven days in order to evaluate dose relationship of thyroid toxicity. These dose levels were considered equivalent to mean daily doses of 1, 6 or 30 mg/kg bw. The parameters examined were the same as in the first trial (Study 1, above). Mean thyroid weight was significantly increased by 37.5% at 600 ppm but not at 20 or 120 ppm. Compared to the control group TPO activities were not affected at 20 ppm but were by around 50% lower in the group receiving 120 ppm and by more than 75% in the 600 ppm group. However, there were no significant alterations in the hormone levels of T_3, T_4 or TSH up to 600 ppm. Since these groups were also compared to the 1200 ppm group in the first seven-day experiment, the statistical tests were different from those in the 3- or 28-day experiments in which only one dose group receiving 1200 ppm was included.

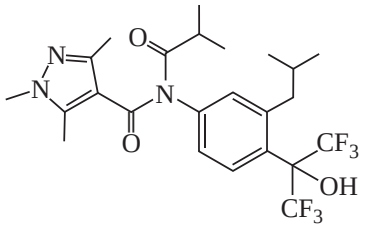
On balance, there is good evidence that the thyroid effects such as hyperplasia and hypertrophy which were observed in several toxicological studies in different species may be explained by a strong impact of pyflubumide on TPO activity. The resulting reduction of T_3 and T_4 is responsible for continuous stimulation of the thyroid by elevated TSH levels. This is a mechanism of relevance to humans. At 20 ppm (equivalent to 1 mg/kg bw per day), no TPO inhibition was noted but this dose is considered a NOEL because TPO inhibition at 120 ppm (equivalent to 6 mg/kg bw per day) was not associated with any adverse effects on the thyroid since organ weight was not increased at this dose, and hormone levels were not altered. Based on organ weight increase from 600 ppm (equivalent to 30 mg/kg bw per day), the NOAEL in this mechanistic study was established at 120 ppm (equivalent to 6 mg/kg bw per day). An impaired thyroid function might have contributed to the body weight increase in some of the routine studies in rats (Inagaki, 2012a).

Study 3

In another study, thyroid microsomal fractions were obtained from male Fischer (F344/DuCrlCrlj) rats and exposed in vitro to pyflubumide and five of its rat metabolites at a concentration of 10 µM by means of the guaiacol method to determine the TPO activity compared to the vehicle control dimethyl sulfoxide (DMSO). The TPO inhibitor 1H-1,2,4-triazole-3-thiol and propylthiouracil (PTU, another known TPO inhibitor) were employed as positive control substances. The initial measurements revealed strongest inhibition of TPO activity by the metabolites pyflubumide-RfOH (Metabolite U, by 63% as compared to DMSO), pyflubumide-NH-RfOH (Metabolite D, by 36%), and pyflubumide -NH-1H-RfOH (Metabolite F, by 27%) whereas the parent compound (10% less active), its NH- form (Metabolite B, −13%) and pyflubumide-NH-1H (Metabolite C, −13%) had no clear effects. Further testing at different concentrations was confined to the two most active metabolites. Metabolite U exhibited some inhibition of TPO activity over the whole concentration range tested, from 0.03 µM up to 10 µM. Inhibition by more than 50% (similar to 0.1 µM MTZ or PTU) was observed consistently from 0.3 µM upwards even though there was no dose response. In contrast, inhibitory activity of Metabolite D was confined to the maximum concentration of 10 µM and not reproducible at 3 µM. In a further in vitro experiment by means of the ^{125}I-thyroglobulin method, exposure to Metabolite U (the only one tested) resulted in a lower T_4 formation from iodinated tyrosine, but the metabolite was less potent than PTU.

This mechanistic study suggested that the metabolite pyflubumide-RfOH (Metabolite U, Fig. 3) was the main inhibitor of thyroid peroxidase at least in vitro. This metabolite has a very similar structure to the parent compound. In the ADME study with pyrazole-radiolabelled pyflubumide (Yoshizane, 2010a), it was proposed as an intermediate (UP1) as depicted in Fig. 2 but was not actually identified in the excreta (see Table 5). In contrast, it was found as a plasma metabolite, accounting for as much as 8–13% of the total radioactivity in the different dose groups of the ADME study that used the pyrazole label (Yoshizane, 2010a). According to the sponsor's information from the metabolite list (Annex 1), it accounted for 4.5% of the total administered dose.

Figure 3. Chemical structure of pyflubumide-RfOH (Metabolite U)



It remains open the extent to which this metabolite may have contributed to TPO inhibition in vivo (Inagaki, 2012a) and to the effects on thyroid in vivo which were mostly seen just at high dose levels. However, because of this possibility, albeit theoretical, Metabolite U is regarded as toxicologically relevant (Inagaki, 2012b).

Studies on lactational effects of pyflubumide on rat pups

Because of the lung lesions in F1 offspring in the two-generation study in rats (Sato, 2012), further studies were performed to investigate reproducibility, relevance and mechanism. The lesions comprised thickened alveolar wall, alveolar distention, haemorrhages and pigmentation (see Table 20). Their incidence was markedly increased in both sexes at 500 ppm but some lesions were already observed at 100 ppm at least in males. It was suspected by the sponsor that these effects might be due either to prenatal exposure or to exposure via the milk.

Study 1

Lactating Sprague Dawley (Crl:CD) rats were administered [pyrazole-3(5)-^{14}C]-radiolabelled pyflubumide as a single dose of either 1 or 100 mg/kg bw by oral gavage. Two rats per dose were used; they had already been on lactation for 10 days before. At 1, 3, 6, 9, 12, 24, 48, 72, and 96 h after dosing, milk was analysed for the abundance of radioactivity, of the parent compound and its metabolites. For comparison purposes, blood samples were also drawn.

Pyflubumide was rapidly transferred into the milk. The maximum radioactivity of 2.0 (low dose) or 132.2 µg equiv./g (high dose) was measured nine hours after administration. When compared to radioactive residues nine hours after application of a single dose of 100 mg/kg bw in the ADME study with the pyrazole label (Yoshizane, 2010), the amount in milk was higher than the concentrations measured in any organ or tissue except the GIT or, in females, in fat. Later on, radioactive concentrations in milk declined fast with a half-life of around half a day at both dose levels. This temporal course was similar to that observed in plasma, but radioactivity in milk consistently far exceeded the plasma values. For the first 24 h, milk : plasma ratios ranged from 5 to > 14, depending on time point of measurement and dose level. The AUC in milk was 6.9 times (1mg/kg bw) or 7.5 times (100 mg/kg bw) higher than in plasma. Besides the parent compound, four major and at least four minor metabolites could be identified (for more, including quantitative, information, see section 1.2 on biotransformation). This study provided convincing proof that milk transfer of pyflubumide is a relevant route of elimination in lactating females and that significant exposure of pups via the milk can be assumed (Murata, 2012).

Study 2

An extended cross-fostering study was performed in mated female Sprague Dawley (Crl:CD) rats. Eight dams per group either received basal diet throughout the period of the study, or were administered pyflubumide at a dietary concentration of 500 ppm (mean daily intake not calculated) from presumed day 0 of gestation until weaning of the litters on day 21 after parturition. Immediately after delivery,

litters from these two groups were exchanged. Accordingly, the pups with potential prenatal exposure were reared by foster dams which had not been given the test substance during gestation whereas the pups delivered by untreated dams were nursed by treated dams and, thus, were exposed postnatally. A naïve control group of the same size was allowed to nurse their own pups, that is, without exchange for cross fostering. A smaller group of four dams received 500 ppm of pyflubumide during gestation and lactation, delivered and nursed their pups until weaning. In addition, two positive control groups of three mated dams each were included, kept on basal diet, and, after delivery, pups from these groups received subcutaneous injections of either dexamethasone (DEX) or propylthiouracil (PTU) at daily doses of 0.25 µg or 100 µg from postpartum days 3–25. Dams were regularly monitored for mortality and clinical signs, and weighed. Food consumption and reproductive parameters were determined. Pups were counted, sexed and weighed. On day 26, all pups were weaned, killed and the lungs, excised for histopathology.

There were no deaths among the mated females and no clinical signs were observed which could be attributed to treatment. Body weight, food intake and reproductive performance were not affected in the dams in any group. Likewise, pup viability and sex ratio were not altered by treatment with pyflubumide and there were no significant differences in pup weight (gain) as compared to the negative control group. Pups receiving the subcutaneous injections of PTU, however, gained much less weight.

A significant increase in pulmonary lesions was observed in the groups which had received either the postnatal or the prenatal and postnatal dietary doses, but not in the group with exposure only in utero. The positive control substances gave the expected response (Table 27).

Table 27. Incidences of pulmonary lesions in rat pups

Group of pups	Dose (ppm) gestation	Dose (ppm) lactation	No. of litters /no. of pups	Alveolar dilatation (% affected)	Haemorraghes (% affected)
Control	0	0	8 / 62	13	13
Prenatal exposure	500	0	8 / 57	14	9
Postnatal exposure	0	500	7 / 49	96*	39*
Prenatal + postnatal exposure	500	500	4 / 26	92*	65*
DEX	-	0.25 µg/rat per day	3 / 21	100*	67*
PTU	-	100 µg/rat/day	3 / 21	100*	76*

* $p < 0.01$, Fisher's probability test

On balance, the lung lesions in pups were attributed to postnatal exposure to pyflubumide via the milk (Inagaki, 2012c).

Study 3

Eight Sprague Dawley rat pups per group received pyflubumide once a day by oral gavage from postnatal days (PNDs) 4–13 at dose levels of 0.4, 2, 10, or 50 mg/kg bw per day. Six more groups were administered one of the following three metabolites at dose levels of 2 or 50 mg/kg bw per day: pyflubumide-NH (Metabolite B); pyflubumide-NH-1-H (Metabolite C); or pyflubumide-NH-RfOH (Metabolite D). A control group of the same size received the vehicle, an aqueous solution of 0.5% CMC containing 0.1% Tween 80. The pups were monitored daily for mortality and clinical signs and weighed on PNDs 4, 7, 10, 14, 21 (weaning) and 26 (terminal kill). At termination, lungs were excised and subjected to histopathology.

A few premature deaths were observed in the groups exposed to the metabolites D (one high-dose pup on PND 11) and B (pyflubumide-NH form), in which group three low- and two high-dose pups were found dead on PND 19, that is six days after the final dose. The cause of death could not be established in any of these cases. In the other groups no mortality and no clinical signs were noted. In the group receiving the parent pyflubumide, mean body weight was decreased at 50 mg/kg bw per day towards the end of the study (PNDs 21 and 26).

The incidence of alveolar dilatation was significantly increased in the groups receiving pyflubumide at 10 mg/kg bw per day and above. With the metabolites, a similar effect was noted in the groups receiving Metabolites B and D, but was confined to the 50 mg/kg bw dose. Because of the wide

dose spacing, however, the NOAEL for all three compounds causing alveolar dilatation was the same, 2 mg/kg bw per day. No significant difference in the occurrence of lung haemorrhages was observed in any group but the study author speculated that the exposure period might have been too short to elicit this effect that had been seen in the multigeneration study by Sato (2012) (Inagaki, 2012d).

Study 4

Sprague Dawley rat pups (eight per treatment group) were administered pyflubumide on two consecutive days by oral gavage at dose levels of 10 and 50 mg/kg bw per day. The pups were treated either on PNDs 4 and 5, on PNDs 6 and 7, on PNDs 8 and 9, on PNDs 10 and 11, or on PNDs 12 and 13. A naïve control group of 16 pups did not receive anything. On PND 26, all pups were killed, the lungs excised and prepared for histopathological examination. During the study, the pups were observed for clinical signs of toxicity and regularly weighed.

No premature deaths and no clinical signs were observed and a possible effect on body weight was confined to a slight decrease in the group receiving the high dose on PNDs 4 and 5. A significant increase in alveolar dilatation was noted in the groups receiving the high dose of 50 mg/kg bw on days 4/5 and 6/7, but not later. In both groups, five out of eight pups were affected, as compared to three out of 16 in the control group. A trend towards an increase was also seen in the group receiving 50 mg/kg bw on days 12/13, in which three of the eight pups exhibited alveolar dilatation (Inagaki, 2015).

(d) Toxicity of metabolites

Pyflubumide acid metabolite 1,3,5-trimethylpyrazole-4-carboxylic acid (Metabolite H in the Table in Annex 1) is a significant plant metabolite but has been found in rat metabolism only in traces (see Table 5, Metabolite 7). It was tested for acute oral toxicity in female Fischer (F344/DuCrlCrlj) rats. For this purpose, the test item (purity 99.9%) was suspended in an aqueous solution of 0.5% CMC sodium containing 0.2% Tween 80 and applied, as the first step of the experiment, by single oral gavage to three animals at a dose level of 2000 mg/kg bw in a dosing volume of 10 mL/kg bw. Since no deaths or clinical signs of toxicity occurred, the procedure was repeated at the same dose level in an additional three animals.

There was no mortality. No clinical signs were observed with the possible exception of reduced defaecation in two animals in the second group on the day following dosing. Body weights were not affected and no gross abnormalities were observed upon necropsy.

On balance, the metabolite was of very low acute oral toxicity with a median lethal dose (LD_{50}) greater than 2000 mg/kg bw (Koga, 2013a).

With the same metabolite, an Ames test was performed on *Salmonella typhimurium* strains TA100, 1535, 98, and 1537, and in *Escherichia coli* strain WP2uvrA. Testing was conducted in triplicate by means of the preincubation method, both in the presence and absence of S9 mix for metabolic activation. The acid metabolite of pyflubumide was dissolved in DMSO which was also used as the negative vehicle control. Appropriate positive control substances were included in this study.

In all tester strains, concentrations of up to 5000 μg/plate could be applied since cytotoxicity or precipitation were not observed. There was no increase in revertant colonies, proving that the metabolite was devoid of a genotoxic potential in this test system. In contrast, the positive control substances gave the expected increases (Fujishima, 2013a).

The pyflubumide aniline isobutyryl metabolite 3'-isobutyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]isobutylanilide (Metabolite L) is a significant plant metabolite but has only been found at trace levels in GIT contents of rats. Metabolite L was tested for acute oral toxicity in female Fischer (F344/DuCrlCrlj) rats. For this purpose, the test item (purity 97.2%) was suspended in an aqueous solution of 0.5% CMC sodium containing 0.2% Tween 80 and applied, as the first step of the experiment, by single oral gavage to three animals at a dose level of 2000 mg/kg bw in a dosing volume of 10 mL/kg bw. Since no deaths or clinical signs of toxicity occurred, the procedure was repeated at the same dose level in an additional three animals.

There was no mortality. No clinical signs were observed. Body weights were not affected and no gross abnormalities were observed upon necropsy.

On balance, the metabolite was of very low acute oral toxicity with an LD_{50} greater than 2000 mg/kg bw (Koga, 2013b).

Once again using Metabolite L, an Ames test was performed in *Salmonella typhimurium* strains TA100, 1535, 98, and 1537, and in *Escherichia coli* strain WP2uvrA. Testing was conducted in triplicate by means of the preincubation method, both in the presence and absence of S9 mix for metabolic activation. The aniline isobutyryl metabolite of pyflubumide was dissolved in DMSO which was also used as the negative vehicle control. Appropriate positive control substances were included in this study.

In all experiments with the metabolite, concentrations of up to 5000 µg/plate were tested since cytotoxicity was not observed. However, precipitation was reported to occur in all tester strains at concentrations of 1250 µg/plate and above. The highest concentration that could be applied without causing at least some precipitation was 625 µg/plate. There was no increase in revertant colonies proving that the metabolite was devoid of a genotoxic potential in this test system. In contrast, the positive control substances gave the expected increases (Fujishima, 2013b).

For toxicity of other metabolites (pyflubumide-NH, pyflubumide-NH-RfOH and pyflubumide-RfOH, Metabolites B, D, and U respectively), some information may be found in the sub-section on mechanistic studies, but in general the database on metabolites is very limited. On the other hand, many of them are structurally closely related to the parent compound (see also the list of metabolites in the Annex 1 as well as Figs 2 and 3).

3. Observations in humans

Nothing was available. Pyflubumide is a new compound for which no information on possible adverse effects on human health has been submitted.

Comments

Biochemical aspects

Following oral administration to rats of ^{14}C-radiolabelled pyflubumide as a single low dose of 1 mg/kg bw, the compound was rapidly absorbed (T_{max} 6 h) but only partially so. Based on urinary (< 6%) and biliary (ca 43%) excretion, cage wash, tissue and carcass residues after 72 hours, absorption accounted for ca 52% of the applied dose. Absorption of a single dose of 100 mg/kg bw is expected to be only marginally lower, however, a final conclusion cannot be drawn because this high dose was not administered to bile-cannulated rats. The absorbed portion was widely distributed throughout the body, with highest concentrations found in liver and kidneys, adrenals, bone marrow and fat. Elimination was nearly complete at the low- and high-dose levels after seven days, with faeces being the main route of elimination, accounting for 90% or more (Yoshizane, 2010a, b, c). In nursing rats, excretion of pyflubumide and of some of its metabolites via the milk was demonstrated. The milk : plasma radioactivity ratio was approximately 10 : 1, and the AUC was up to 7.5 times higher in milk than in plasma (Murata, 2012).

Extensive metabolism of pyflubumide was observed, at least of the systemically available portion. The main metabolic pathways comprised deacylation of the nitrogen atom, followed by hydroxylation and demethylation, whereas cleavage of the molecular backbone of pyflubumide was very limited. Eight or nine metabolites were identified in urine, faeces, plasma or milk, but each of these displayed a different mix of metabolites. In bile there were 12 metabolites. Main metabolites (exceeding 10% of administered dose in either excreta or plasma in ADME studies) were pyflubumide-NH (Metabolite B), pyflubumide-NH-1-H-RfOH (Metabolite F) and pyflubumide-NH-1-H-3′(3-OH)-RfOH (Metabolite R). The unchanged parent compound was mainly detected in the GI tract and faeces, representing the non-absorbed part, but to a small extent also in milk.

The impact of sex, dose, or position of radiolabel on toxicokinetics or metabolism was low.

Toxicological data

In rats, the acute oral and dermal LD_{50} was > 2000 mg/kg bw (Tsukushi, 2010a, b) whereas the inhalation LC_{50} was above 5.23 mg/L in a 4h, nose-only exposure experiment (Fukuyama, 2010). Pyflubumide was not irritating to the skin nor to the eyes of rabbits (Munechika, 2010a, b) and proved negative for skin sensitization in a local lymph node assay (Munechika, 2011).

Oral (feeding) short-term toxicity studies with pyflubumide were performed in mice (28-day and 90-day), rats (28-day and 90-day), and dogs (90-day and one-year). In the mouse, the main target organs of toxicity were the liver and the haematopoietic system. In the rat, the main target organs of toxicity were the liver, thyroid, haematopoietic system and heart. In the dog, the main target organs of toxicity were the heart, liver and adrenals.

In a 28-day study in mice, the dietary dose levels were 0, 20, 200 and 2000 ppm (equal to 0, 3.0, 32, and 297 mg/kg bw per day in males, 4.1, 40, and 396 mg/kg bw per day in females). A further group receiving 10 000 ppm was prematurely terminated due to excessive toxicity. The dose of 20 ppm (equal to 3.0 mg/kg bw per day) was the NOAEL, based on increased liver weight with associated histopathology in both sexes, resulting from 200 ppm (equal to 32 mg/kg bw per day) (Inagaki, 2009).

In a 90-day study in mice, dietary doses of 0, 40, 400 and 4000 ppm (equal to 0, 5.3, 51, and 505 mg/kg bw per day in males, 0, 6.4, 64, and 596 mg/kg bw per day in females) were administered. The NOAEL was 400 ppm (equal to 51 mg/kg bw per day), based on liver toxicity (increased liver weight with associated histopathology and clinical chemistry parameters), slight effects on red blood parameters with an increase in spleen weight, follicular cell hypertrophy of the thyroid and eosinophilic changes in the zona fasciculata of the adrenals at 4000 ppm (equal to 505 mg/kg bw per day) (Kashimoto, 2009).

In a 28-day study in the rat, dietary dose levels of 0, 200 and 2000 ppm (equal to 0, 17, and 137 mg/kg bw per day in males, 0, 17, and 140 mg/kg bw per day in females) were fed to the animals. A further group receiving 20 000 ppm was prematurely terminated due to excessive toxicity. Treatment-related and adverse effects were seen at all dose levels, with effects on liver, heart and thyroid from the lowest dose of 200 ppm. A NOAEL could not be identified and the LOAEL was 200 ppm (equal to 17 mg/kg bw per day) (Horiuchi, 2007).

In a subsequent 90-day study in rats, animals were administered dietary doses of 0, 20, 200 and 1200 ppm (equal to 0, 1.2, 12, and 72 mg/kg bw per day in males, 0, 1.4, 14, and 81 mg/kg bw per day in females). An additional control group and second high-dose group were included to assess their recovery when fed an untreated diet for four weeks after dosing had ceased. The NOAEL was 20 ppm (1.2 mg/kg bw per day) based on increased heart weights in both males and females at 200 ppm (equal to 12 mg/kg bw per day). The adverse findings were only partly reversible during the recovery period (Kuwahara, 2009).

In a 90-day feeding study in dogs, the dose levels were 0, 40, 300 and 2500 ppm (equal to 0, 1.2, 9.1, and 77 mg/kg bw per day in males, 0, 1.3, 9.5, and 75 mg/kg bw per day in females). The NOAEL was 300 ppm (equal to 9.1 mg/kg bw per day). Adverse, treatment-related effects were confined to the top dose of 2500 ppm (equal to 75 mg/kg bw per day) and consisted of cardiotoxicity (increased heart weights with associated histopathology and functional changes), increased liver weights with associated histopathology, clinical chemistry parameters and rare histological kidney findings (Ishikawa, 2010).

A one-year study was performed in dogs using dietary dose levels of 0, 40, 300 and 2000 ppm (equal to 0, 1.1, 8.0, and 54 mg/kg bw per day in both sexes). The NOAEL was 40 ppm (equal to 1.1 mg/kg bw per day) based on histopathological changes in the adrenals (hypertrophy, lipid depletion and thickening of the zona fasciculata) at 300 ppm (equal to 8 mg/kg bw per day) (Ishikawa, 2011).

In the 18-month study on mice, pyflubumide was administered at dietary doses of 0, 40, 400 or 1600 ppm (equal to 0, 4.4, 45, and 176 mg/kg bw per day in males, 0, 4.0, 43, and 178 mg/kg bw per day in females). The NOAEL was 400 ppm (equal to 43 mg/kg bw per day) based on lower body weight in females, on increased organ weights of liver and spleen and on histopathological findings in liver, adrenals, spleen and thyroid, which were observed either in one or both sexes at 1600 ppm (equal to 176 mg/kg bw per day). Increased tumour incidences were noted for the liver and the lymph nodes at the same maximum dose. Benign liver adenomas were increased in males only, with no progression to

carcinoma noted. A possible MOA has not been investigated. Marginal increases in haemangiosarcomas of mesenteric lymph nodes (statistically significant in a test for trend in males, and above laboratory historical control data in both sexes) were observed at 1600 ppm (Kuwahara, 2011a). Relevance to human risk of haemangiosarcoma in mice is generally considered low. On the other hand, no MOA has been proposed and/or investigated. Additional uncertainty comes from the small number of animals in the low- and mid-dose groups in which mesenteric lymph nodes had been examined microscopically. Overall, pyflubumide was carcinogenic in mice and a NOAEL for carcinogenicity of 400 ppm (43 mg/kg bw per day) was indicated by the study.

In a one-year chronic toxicity study in rats, pyflubumide was administered at dietary concentrations of 0, 10, 20, 120, and 600 ppm (equal to 0, 0.4, 0.9, 5.1 and 26 mg/kg bw per day in males, 0, 0.5, 1.1, 6.4, 32 mg/kg bw per day in females). The NOAEL was 20 ppm (equal to 0.9 mg/kg bw per day), based on the effects on heart (weight), liver (bile duct hyperplasia), red blood cell parameters, kidney (urinary casts and tubular basophilic changes), ovary (weight) and skin (loss of fur) at 120 ppm (equal to 5.1 mg/kg bw per day) (Kuwahara, 2011b).

In a separate two-year carcinogenicity study in rats, pyflubumide was administered at dietary concentrations of 0, 10, 20, 120 and 600 ppm (equal to 0, 0.4, 0.7, 4.5, and 23 mg/kg bw per day in males, 0, 0.5, 0.9, 6, and 29 mg/kg bw per day in females). The NOAEL for chronic toxicity in this study was 20 ppm (0.7 mg/kg bw per day) based on effects on liver (weight with associated bile duct hyperplasia), heart (weight, with associated fibrosis), adrenals (medullary hyperplasia) at 120 ppm (equal to 4.5 mg/kg bw per day). No evidence of carcinogenicity was obtained in the rat and the NOAEL for carcinogenicity was 600 ppm (equal to 23 mg/kg bw per day), the highest dose tested (Kuwahara, 2012).

The Meeting concluded that pyflubumide is carcinogenic in mice but not in rats.

Pyflubumide was tested for genotoxicity in an adequate range of studies in vitro (Wada, 2010; Tsukushi, 2011a; Lloyd, 2017) and in vivo (Tsukushi, 2011b) which were all negative.

The Meeting concluded that pyflubumide is unlikely to be genotoxic.

In the view of the lack of genotoxicity, in the absence of carcinogenicity in the rat and since the higher incidence of tumours in the mouse was confined to the highest dose, far above expected human exposure, the Meeting concluded that pyflubumide is unlikely to pose a carcinogenic risk to humans via exposure from the diet.

In a two-generation study, pyflubumide was administered to rats at dietary dose levels of 0, 7.5, 15, 100 or 500 ppm (equal to 0, 0.4, 0.8, 5.3, and 26 mg/kg bw per day for males, 0, 0.7, 1.3, 8.6, and 42 mg/kg bw per day for females). The NOAEL for parental effects was 15 ppm (equal to 0.8 mg/kg bw per day), based on increased organ weights of heart, thyroid, liver, and ovaries and histopathological findings in the heart at the dose levels above. A reproductive toxicity NOAEL of 100 ppm (equal to 5.3 mg/kg bw per day) was established because of prolonged gestation and a lower pup viability index on the day of birth at the maximum dose level of 500 ppm (equal to 26 mg/kg bw per day) even though these effects were confined to the first generation. The offspring NOAEL was 15 ppm (equal to 0.8 mg/kg bw per day), based on gross and histological lung lesions in F1 and F2 pups from 100 ppm (equal to 5.3 mg/kg bw per day) (Sato, 2012).

In a developmental study in rats, pyflubumide was administered by oral gavage at dose levels of 0, 5, 30, and 200 mg/kg bw per day. The maternal NOAEL was 30 mg/kg bw per day since body weight gain and food consumption were reduced at the top dose level. A few of the dams even lost some body weight. In addition, placenta weights were increased. The developmental NOAEL of 30 mg/kg bw per day was based on a significantly higher mean fetal weight at the next higher dose. There was no increase in malformations or individual variations (Sato, 2010).

In a developmental study in rabbits, pyflubumide was administered by oral gavage at doses of 0, 5, 20, and 80 mg/kg bw per day. The maternal NOAEL was 20 mg/kg bw per day, based on abortions and premature delivery, lower body weight gain (or even body weight loss), reduced food intake and higher placenta weights in the high-dose group. No effects on the fetuses were observed in rabbits up to the highest dose of 80 mg/kg bw per day which was thertefore considered the developmental NOAEL (Hojo, 2010).

The Meeting concluded that pyflubumide is not teratogenic.

In an acute neurotoxicity study in rats in which gavage doses of 0, 500, 1000, and 2000 mg/kg bw were administered, a systemic NOAEL could not be established since body temperature on the day of dosing was reduced in all treated male and female groups, from the lowest dose onwards. In addition, body weight gain was decreased in all male groups over the first week of the post-observation period even though no clear dose response was observed. However, the maximum dose of 2000 mg/kg bw was considered the NOAEL for neurotoxicity (Barnett, 2018). A separate neurotoxicity study with repeated administration was not submitted, but no concern was identified from the available studies.

The Meeting concluded that pyflubumide is not neurotoxic.

An immunotoxicity study was not submitted, but no concern was identified from the available studies.

The Meeting concluded that pyflubumide is not immunotoxic.

A number of mechanistic studies were performed to further investigate the effects on the heart and thyroid as observed in many studies in different species, and effects on lungs seen in rat offspring.

With regard to the heart gross and histopathological findings as well as the higher organ weight and clinical signs (tachycardia, lower blood pressure) observed in various studies in rats and/or dogs, mechanistic studies in the rat were carried out by single intravenous administration (Toga, 2009) and on excised rat tissue (Toga, 2011). These suggested a mode of action that is considered plausible: pyflubumide or its metabolites cause vasodilatation with subsequent decrease in blood pressure. As a reflex response, heart action is increased, resulting in tachycardia and, following long-lasting maintenance of these pathophysiological conditions, morphological heart changes may ensue. This mechanism was considered relevant to humans.

Thyroid effects such as organ weight increase or follicular cell hyperplasia could be clearly attributed to inhibition of TPO resulting in a lower availability of iodine, reduced concentrations of circulation T_3 and T_4 and, because of hormonal feedback regulation, an increase in TSH release (Inagaki, 2012a, b).

It could be demonstrated that pyflubumide and a number of its metabolites are excreted by lactating rat females to a significant extent via the milk (Murata, 2012). In cross-fostering experiments, it was shown that the lung lesions in rat pups, as observed in the two-generation study, can be clearly attributed to postnatal exposure via the milk but were not due to in utero exposure (Inagaki, 2012c). Young pups exhibited similar lung changes after repeated gavage application of a dose of 10 mg/kg bw per day or above from PND four to 13 (Inagaki, 2012d). It seems that there is a critical window of sensitivity for this effect. Alveolar dilatation was already observed when the test substance was applied by oral gavage administration to pups at a dose level of 50 mg/kg bw on two consecutive days, provided the pups were not older than seven days (Inagaki, 2015).

Toxicological data on metabolites and/or degradates

The pyflubumide plant metabolites 1,3,5-trimethylpyrazole-4-carboxylic acid (Metabolite H) and 3′isobutyl-4′-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]isobutylanilide (Metabolite L) were of low acute oral toxicity since the LD_{50} in rats, in both cases, was greater than 2000 mg/kg bw (Koga, 2013a, b). In addition, these two metabolites proved negative in the Ames test (Fujishima, 2013a, b). If an evaluation of these metabolites becomes necessary, the threshold of toxological concern (TTC) approach (Cramer class III) is considered appropriate and applicable.

A mechanistic study revealed that the metabolite pyflubumide-NH-RfOH (Metabolite D) had a higher potency than the parent compound in causing vasodilatation of aorta specimens in vitro Toga, 2011). When administered by oral gavage to rat pups from PND 4–13 at a dose level of 50 mg/kg bw per day, it caused alveolar dilatation, similar to the parent compound (Inagaki, 2012d). In ADME studies, this metabolite was detected following single oral application of 100 mg/kg bw of pyflubumide, at a rate of up to 8.4% of the applied dose in rat plasma (Yoshizane, 2010a, b) but was not found in excreta. In addition, it accounted for up to 7% of total residues in milk following single oral administration of the same dose to rats (Murata, 2012).

The NH- form of pyflubumide (Metabolite B, that is the acaricidal compound), also caused alveolar dilatation in rat pups when administered by oral gavage from PND 4–13 at a dose level of 50 mg/kg bw per day, meaning that is was of similar potency with regard to this effect as the parent compound (Inagaki, 2012d). This is of particular relevance since it was the main metabolite in rat milk, accounting for up to 58% of total milk residues (Murata, 2012). In the ADME studies, at a dose level of 100 mg pyflubumide/kg bw, it had been detected only in faeces at rates between 11% and 19% of the applied total dose, but not in urine, whereas it occurred in plasma only in traces (Yoshizane, 2012a). With regard to vasodilatation in vitro, Metabolite B was of similar potency to the parent (Toga, 2011). Metabolite B has a similar structure to the parent compound. On balance, it appears reasonable to apply the ADI and ARfD as established for pyflubumide to this metabolite also. With regard to its excretion via the milk it should be taken into consideration that the ARfD is based on, and the ADI at least supported by, the reproduction study. Metabolite B can be assumed to have been tested in that study.

The metabolite pyflubumide-RfOH (Metabolite U) proved a potent inhibitor of TPO in vitro) Inagaki, 2012b), but was inactive in an in vitro test for vasodilatation (Toga, 2011). This metabolite is a proposed intermediate in rat metabolism. It was identified as a minor metabolite in plasma, accounting for up to 4.5%, but was not found in excreta or milk (Yoshizane, 2010a, b; Murata, 2012). Based on its very close structural similarity to the parent compound, this metabolite is assumed not to be more toxic than its parent. Accordingly, ADI and ARfD of pyflubumide are applicable to Metabolite U, too.

Microbiological data

Not available.

Human data

Not available since pyflubumide is a new compound.

The meeting concluded that the existing database on pyflubumide is adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI for pyflubumide of 0–0.007 mg/kg bw that was derived from the NOAEL of 0.7 mg/kg bw per day based on findings in liver, heart, and adrenals in the two-year study of toxicity and carcinogenicity in rats, using a safety factor of 100. This was supported by the parental and offspring NOAELs in the two-generation study in rats (0.8 mg/kg bw per day) and by the NOAEL in the one-year study in dogs (1.1 mg/kg bw per day).

The upper range of the ADI provides a margin of over 25 000 to the LOAEL for liver adenomas and haemangiosarcomas in mice.

The Meeting established an ARfD of 0.008 mg/kg bw on the basis of the offspring NOAEL of 0.8 mg/kg bw per day for lung lesions which have been shown to occur as an acute effect in the two-generation rat study, using a safety factor of 100.

The ADI and ARfD are applicable to the metabolites pyflubumide-NH (Metabolite B) and pyflubumide-RfOH (Metabolite U).

Levels relevant to risk assessment of pyflubumide

Species	Study	Effect	NOAEL	LOAEL
Mouse	90-day study of toxicity	Toxicity	400 ppm, equal to 51 mg/kg bw per day	4000 ppm, equal to 505 mg/kg bw per day
	18-month chronic/ carcinogenicity study	Toxicity	400 ppm, equal to 43 mg/kg bw per day	1600 ppm, equal to 176 mg/kg bw per day
		Carcinogenicity	400 ppm, equal to 43 mg/kg bw per day	1600 ppm, equal to 176 mg/kg bw per day
Rat	Acute neurotoxicity study[b]	Neurotoxicity Toxicity	2000 mg/kg bwc -	- 500 mg/kg bw
	90-day study of toxicity	Toxicity	20 ppm, equal to 1.2 mg/kg bw per day	200 ppm, equal to 12 mg/kg bw per day
	One-year study of chronic toxicity[a]	Toxicity	20 ppm, equal to 0.9 mg/kg bw per day	120 ppm, equal to 5.1 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equal to 0.7 mg/kg bw per day	120 ppm, equal to 4.5 mg/kg bw per day
		Carcinogenicity	600 ppm, equal to 23 mg/kg bw per day[c]	-
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	100 ppm, equal to 5.3 mg/kg bw per day	500 ppm, equal to 26 mg/kg bw per day
		Parental toxicity	15 ppm, equal to 0. 8 mg/kg bw per day	100 ppm, equal to 5.3 mg/kg bw per day
		Offspring toxicity	15 ppm, equal to 0. 8 mg/kg bw per day	100 ppm, equal to 5.3 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	30 mg/kg bw per day	200 mg/kg bw per day
		Embryo/foetal toxicity	30 mg/kg bw per day	200 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	20 mg/kg bw per day	80 mg/kg bw per day
		Embryo/foetal toxicity	80 mg/kg bw per day[c]	
Dog	Thirteen-week study of toxicity[a]	Toxicity	300 ppm, equal to 9.1 mg/kg bw per day	2500 ppm, equal to 77 mg/kg bw per day
	One-year study of toxicity[a]	Toxicity	40 ppm, equal to 1.1 mg/kg bw per day	300 ppm, equal to 8.0 mg/kg bw per day

[a] Dietary administration [b] Gavage administration. [c] Highest dose tested in study.

Acceptable daily intake (ADI) for pyflubumide, pyflubumide-NH and pyflubumide-RfOH expressed as pyflubumide

0–0.007 mg/kg bw

Acute reference dose (ARfD) for pyflubumide, pyflubumide-NH and pyflubumide-RfOH expressed as pyflubumide

0.008 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Epidemiological, occupational health or other human observational data if they become available.

Critical end-points for setting guidance values for exposure to pyflubumide

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max}, 6 h) but incomplete (52% at low dose of 1 mg/kg bw)
Dermal absorption	No data
Distribution	Widely distributed, highest residues in liver, kidney, adrenals, bone marrow and fat
Potential for accumulation	Limited evidence for retention in fat
Rate and extent of excretion	Nearly complete within 7 days, mainly via faeces (≥90%); biliary excretion accounting for main part of absorbed dose (43%), urine less important (< 6%); excretion via milk also proven (milk:plasma ratio about 10:1)
Metabolism in animals	Extensive with 8–12 (some unique) metabolites occurring in the different matrices
Toxicologically significant compounds in animals and plants	Pyflubumide, *N*-deisobutylated pyflubumide (P-NH, "NH- form", Metabolite B), pyflubumide-RfOH (Metabolite U)
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.23 mg/L (four-hour nose-only exposure)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Dermal sensitization	Not sensitizing (local lymph node action)
Short-term studies of toxicity	
Target/critical effect	Adrenals (histopathological lesions in cortex and medulla)
Lowest relevant oral NOAEL	1.1 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Heart (organ weight, histopathology); thyroid (organ weight, histopathology); adrenals (histopathology); liver (histopathology, organ weight); bw increase in rats
Lowest relevant NOAEL	0.7 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice[a]
Genotoxicity	No evidence of genotoxicity in vitro or in vivo[a]
Reproductive toxicity	
Target/critical effect	Reproductive toxicity: prolonged gestation and lower viability index at birth Offspring toxicity: histological lung lesions in rat pups due to lactational exposure Parental toxicity: increased weight of heart, liver, thyroid and ovary, myocardial fibrosis, increased body weight gain and food intake
Lowest relevantparental NOAEL	0.8 mg/kg bw per day
Lowest relevant offspring NOAEL	0.8 mg/kg bw per day
Lowest relevant reproductive NOAEL	5.3 mg/kg bw per day

Developmental toxicity	
Target/critical effect	Maternal: higher placenta weight in rats and rabbits, reduced body weight and food intake in rats and rabbits, abortions in rabbits Developmental: higher fetal weight in rats; none in rabbits
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	30 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	2000 mg/kg bw (i.e. no specific neurotoxic potential up to highest dose tested); not established for systemic effects (LOAEL 500 mg/kg bw)
Subchronic neurotoxicity NOAEL	No data, no evidence from routine studies
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data; no concern from routine studies
Studies on toxicologically relevant metabolites	
Pyflubumide-NH (Metabolite B)	Alveolar dilatation in very young rat pups after gavage application of 50 mg/kg bw per day from PND 4–13; NOAEL 2 mg/kg bw per day Significant excretion via milk demonstrated in rats
Pyflubumide-RfOH (Metabolite U)	Inhibition of TPO but no vasodilatation in vitro
Human data	Not available for this new compound

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI[a]	0–0.007 mg/kg bw	Two-year, (rat)	100
ARfD[a]	0.008 mg/kg bw	Two-generation study; offspring toxicity (rat)	100

[a] Applies to pyflubumide, pyflubumide-NH, pyflubumide-RfOH, expressed as pyflubumide

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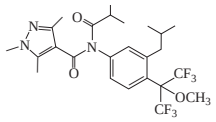
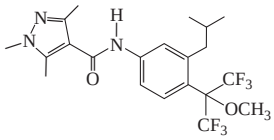
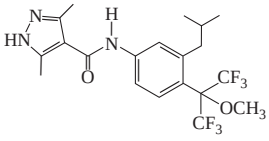
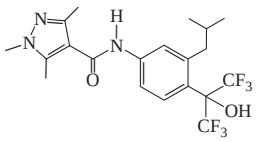
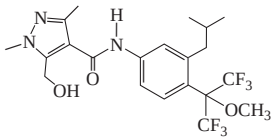
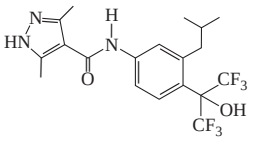
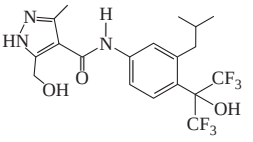
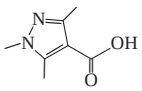
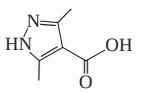
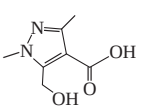
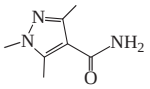
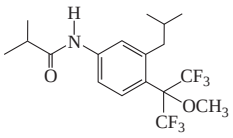
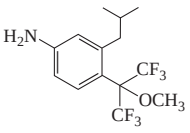
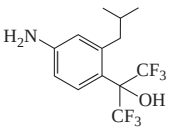
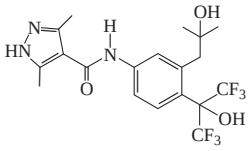
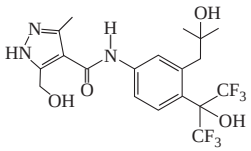
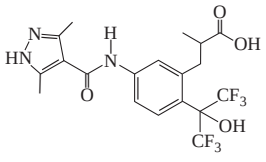
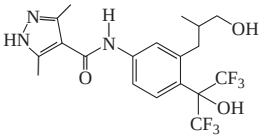
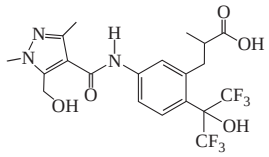
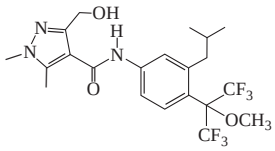
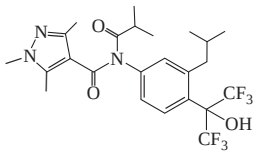
Annex 1 – List of metabolites

[*The information in Annex 1 was submitted by the sponsor. It has not been checked by the monographer or reviewer, but the chemical diagrams have been redrawn to ensure legibility.*]

Name or Code IUPAC name (MW)	Structure	Found in:
Pyflubumide (NNI-0711) 3'-Isobutyl-*N*-isobutyryl-1,3,5-trimethyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl) ethyl] pyrazol-4-carboxanilide (535.52)		Rat (urine: 0.02% AR; faeces: 1.81-42.63% AR; GI contents: 15.66% AR) Apple (fruit: 19.1-92.0% TRR; leaves: 17.4-95.7% TRR) Eggplant (fruit: 89.6-98.4% TRR; leaves: 89.9-99.3% TRR) Spinach (83.4-100.0% TRR) HTH (70.5-97.0% TRR) Soil (4.0-104.9% AR) Hydrolysis (Tier I, day 5: 0.2-30.9%) Photolysis (0.1-99.4% AR)
NNI-0711-NH (Metabolite B) 3'-Isobutyl-1,3,5-trimethyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]pyrazol-4-carboxanilide (465.43)		Rat (faeces: 3.15-17.35% AR; plasma: 1.29% AR; GI contents: 14.74% AR) Apple (fruit: 1.2-17.6% TRR; leaves: 1.9-14.7% TRR) Eggplant (fruit: 0.37-1.16% TRR; leaves: 0.39-1.30% TRR) Spinach (0.2-3.2% TRR) HTH (3.0-19.4% TRR) Soil (0.4-82.0% AR) Hydrolysis (Tier I, day 5: 51.9-94.6%) Photolysis (0.6-39.5% AR)
NNI-0711-NH-1-H (Metabolite C) 3'-Isobutyl-3,5-dimethyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]pyrazol-4-carboxanilide (451.40)		Rat (plasma: 2.83% AR) Photolysis (0.2-3.7% AR)

Name or Code **IUPAC name** **(MW)**	**Structure**	**Found in:**
NNI-0711-NH-RfOH (Metabolite D) 3'-Isobutyl-1,3,5-trimethyl-4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl]pyrazol-4-carboxanilide (451.41)		Rat (plasma: 8.38% AR) Eggplant (fruit: 0.02% TRR; leaves: 0.02-0.13% TRR) Soil (0.1-1.5% AR)
NNI-0711-NH-5-CH_2OH (Metabolite E) 5-(Hydroxymethyl)-3'-isobutyl-1,3-dimethyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl] pyrazol-4-carboxanilide (481.43)		Eggplant (leaves: 0.02% TRR) Soil (0.5-6.6% AR)
NNI-0711-NH-1-H-RfOH (Metabolite F) 3'-Isobutyl-3,5-dimethyl-4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]pyrazol-4-carboxanilide (437.38)		Rat (urine: 0.03-0.17% AR; faeces: 1.04-13.49% AR; plasma: 34.93% AR; GI contents: 5.87% AR; bile: 0.57% AR [also glucuronide in bile, 0.51% AR])
NNI-0711-NH-1-H-5-CH_2OH-RfOH (Metabolite G) 5-(Hydroxymethyl)-3'-isobutyl-3-methyl-4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl] pyrazol-4-carboxanilide (481.39)		Rat (faeces: 0.35-4.31% AR; plasma: 1.73% AR; GI contents: 1.57% AR; [also glucuronide in bile, 1.19% AR])
NNI-0711-acid (Metabolite H) 1,3,5-Trimethylpyrazole-4-carboxylic acid (154.17)		Rat (urine: 0.04-0.12% AR) Eggplant (fruit: 0.02-0.32% TRR; leaves: 0.07-0.27% TRR) Spinach (0.8% TRR) HTH (6.7-8.8% TRR) Soil (0.1-2.6% AR) Hydrolysis (Tier I, day 5: 4.7-14.4%) Photolysis (21.0-58.7% AR)
NNI-0711-acid-1-H (Metabolite I) 3,5-Dimethylpyrazol-4-carboxylic acid (140.14)		Rat (urine: 0.04-0.13% AR) Photolysis (0.2-2.7% AR)
NNI-0711-acid- 5-CH_2OH (Metabolite J) 5-(hydroxymethyl)-1,3-dimethylpyrazol-4-carboxylic acid (170.17)		Rat (urine: 0.02% AR)

Name or Code IUPAC name (MW)	Structure	Found in:
NNI-0711-amide (Metabolite K) 1,3,5-Trimethylpyrazole-4-carboxamide (153.18)		Photolysis (1.0-11.5% AR)
NNI-0711-aniline isobutyryl (metabolite L) 3'-Isobutyl-4'-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl] isobutylanilide (385.34)		Rat (GI contents: 0.21% AR) Eggplant (fruit: 0.63-1.03% TRR; leaves: 0.44-0.82% TRR) HTH (5.3-10.2% TRR) Soil (0.8-5.4% AR) Hydrolysis (Tier I, day 5: 6.7-20.7%) Photolysis (0.3-47.3% AR)
NNI-0711-aniline (Metabolite M) 3-Isobutyl-4-[2,2,2-trifluoro-1-methoxy-1-(trifluoromethyl) ethyl] aniline (315.25)		Photolysis (0.3-1.8% AR)
NNI-0711-aniline RfOH (Metabolite N) 2-(4-Amino-2-isobutylphenyl)-1,1,1,3,3,3-hexafluoropropan- 2-ol (315.25)		Rat (faeces: 1.11% AR; GI contents: 0.89% AR)
NNI-0711-NH-1-H-3'-(2-OH)-RfOH (Metabolite O) 3'-(2-Hydroxy-2- methylpropyl)-3,5-dimethyl- 4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl] pyrazol-4-carboxanilide (453.38)		Rat (urine: 0.08-0.11% AR; faeces: 1.25-8.98% AR; plasma: 9.37% AR; GI contents: 2.25% AR; bile: 0.46% AR [also glucuronide in bile, 0.50% AR])
NNI-0711-NH-1-H-5-CH_2OH-3'-(2-OH)-RfOH (Metabolite P) 5-(Hydroxymethyl)-3'-(2-hydroxy-2-methylpropyl)-3-methyl-4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl] pyrazol-4-carboxanilide (469.38)		Rat (urine: 0.03-0.12% AR; faeces: 1.37-5.23% AR; plasma: 1.07% AR; GI contents: 0.77% AR; bile: 0.26% AR [also glucuronide in bile, 0.31% AR])
NNI-0711-NH-1-H-3'-(2-COOH)-RfOH (Metabolite Q) 3-{5-(3,5-Dimethylpyrazol-4-carbonylamino)-2-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl] phenyl}-2-methylpropionic acid ()		Rat (urine: 0.09-0.63% AR; faeces: 2.74-10.35% AR; GI contents: 9.24% AR; bile: 7.74% AR)

Name or Code **IUPAC name** **(MW)**	**Structure**	**Found in:**
NNI-0711-NH-1-H-3'-(3-OH)-RfOH (Metabolite R) 3'-(3-Hydroxy-2-methylpropyl)-3,5-dimethyl-4'-[2,2,2- trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl] pyrazol- 4-carboxanilide (453.38)		Rat (urine: 0.03-0.33% AR; faeces: 0.93-9.44% AR; plasma: 12.63% AR; GI contents: 7.31% AR; bile: 0.44% AR [also glucuronide in bile, 1.23% AR])
NNI-0711-NH-5-CH_2OH-3'-(2-COOH)-RfOH (Metabolite S) 3-{5-[5-(Hydroxymethyl)-1,3-dimethylpyrazol-4-carbonylamino]-2-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl] phenyl}-2-methylpropionic acid (497.39)		Rat (urine: 0.06-0.20% AR; faeces: 0.76-1.41% AR; GI contents: 1.38% AR; bile: 1.55% AR [also glucuronide in bile, 0.69% AR])
NNI-0711-NH-3-CH_2OH (Metabolite T) 3-(Hydroxymethyl)-3'-isobutyl-1,5-dimethyl-4'-[2,2,2-trifluoro-1-methyoxy-1-(trifluoromethyl) ethyl] pyrazol-4-carboxanilide (481.43)		Soil (0.1-0.9% AR)
NNI-0711-RfOH (Metabolite U) 3'-Isobutyl-N-isobutyryl-1,3,5-trimethyl-4'-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl] pyrazol-4-carboxanilide (521.49)		Rat (plasma: 4.50% AR)

AR Applied Radioactivity / Dosed Radioactivity /Administered Dose

HTH High Temperature Hydrolysis

TRR Total Radioactive Residue

PYRIDATE

First draft prepared by
R. Cope[1] and J.Zarn[2]

[1] Australian Pesticides and Veterinary Medicines Authority, Armidale, Australia

[2] Federal Office of Public Health, Switzerland

Explanation

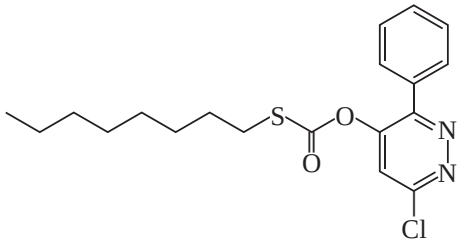
Pyridate is the ISO-approved common name for *O*-6-chloro-3-phenylpyridazin-4-yl *S*-octyl thiocarbonate (IUPAC), with the Chemical Abstract Service number 55512-33-9. It is a proherbicide of the pyridazine class that is converted to the active chemical, pyridafol (CL-9673), in the plant. Pyridafol is as an electron transport inhibitor at the photosystem II.

Pyridate has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

Some of the critical studies do not comply with good laboratory practice (GLP), as the data were generated before the implementation of GLP regulations. Also, many of the critical studies are old and non-compliant with current testing standards. Overall, however, the Meeting considered that the database was adequate for risk assessment.

No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Figure 1. Structure of pyridate



Evaluation for Acceptable Intake

1. Biochemical aspects

1.1. Absorption, distribution and excretion

(a) Oral route

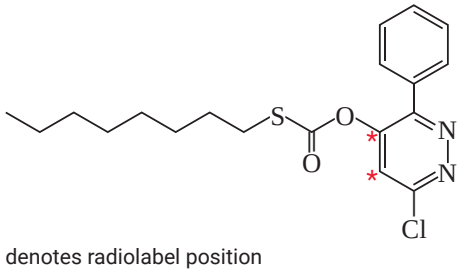
Rat

Study 1

A study in CD-remote Sprague Dawley rats performed using [^{14}C]-radiolabelled pyridate (Fig. 2) and in compliance with GLP demonstrated the rapid absorption and elimination of radioactivity following single gavage administration of 20 mg/kg body weight (bw) and 200 mg/kg bw of pyridate, and following multiple gavage doses each of 20 mg/kg bw of pyridate. In all cases the elimination of pyridate was greater than, or equal to, 94% within 96 h following dosing. Based on the extent of excretion of urinary radioactivity, it is estimated that more than 80% of the AD was absorbed and eliminated within 24 h of a single low dose of 20 mg/kg bw. This was confirmed in studies using bile duct-cannulated rats (see below). Repeated oral dosing at 20 mg/kg bw per day for 14 days did not influence the rate of excretion. Plasma radioactivity levels peaked at 1–2 h following oral dosing at 20 or 200 mg/kg bw, then decreased rapidly thereafter. Plasma concentrations at one hour after dosing were dose-proportional in the 20 to 200 mg/kg body weight range. No biologically important sex differences in the toxicokinetic profile were detected.

Following oral dosing at 20 or 200 mg/kg bw, radioactivity was rapidly distributed to the liver, kidney, fat, brain, gastrointestinal tract (GIT) and plasma. The highest levels of radioactivity were found in the plasma, GIT, liver and kidneys, reflecting the route of administration and routes of excretion. Concentrations of radioactivity in fat and brain were an order of magnitude lower than plasma levels. There was no evidence of accumulation nor did repeated oral dosing affect the distribution of radioactivity. Plasma and tissue radioactivity were at, or near, background levels at 96 h post dosing. An evaluation of excretion in bile duct-cannulated animals (three/sex) was made after the administration of a single low dose (20 mg/kg bw). Administration of single oral doses of 600 mg/kg bw resulted in disproportionately high plasma and tissue radioactivity and altered toxicokinetics compared and with the levels observed at lower doses. This was associated with saturation of urinary excretion (Cameron et al., 1988).

Figure 2. Positions of pyridate ^{14}C-labels (from Cameron et al., 1989)



* denotes radiolabel position

Study 2

A gavage study performed in Sprague Dawley rats investigated the toxicokinetics of pyridate and its metabolite pyridafol (SAN 1367H, CL 9673). Groups of rats were administered:

- single doses of [^{14}C] radiolabelled pyridate at dose levels of 20 or 200 mg/kg bw,
- pyridafol at dose levels of 11, 20 or 110 mg/kg bw (11 and 110 mg/kg bw are stated to be pyridafol molar-equivalent dose levels), or
- single doses of [^{14}C] radiolabelled pyridate or pyridafol at dose levels of 20 or 11 mg/kg bw respectively, following 14 consecutive daily non-labelled doses.

Toxicokinetic parameters were assessed for the two groups, using alternate sampling of tail vein blood at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 96, 120 and 168 h following dosing. Mass balance investigations were performed using samples of urine (taken at 4, 8, 12 and 24 h and subsequently at 24 h intervals to 168 h), faeces and cage wash (12 and 24 h and then at 24 h intervals to 168 h), carcass and skin (at 168 h). Distribution in tissues (bone; femur), brain, carcass, fat, GIT (emptied), kidneys, liver, lung, muscle, ovaries/testes, skin and spleen) was investigated at 1, 6, 24 and 96 h after dosing.

In the preliminary study, groups of rats were administered single oral doses of 200 mg/kg bw pyridate or pyridafol.

In the pyridate-dosed groups, the majority of the administered radioactivity was excreted in both sexes within 48 h. In males, the majority of the administered radioactivity was recovered in the urine (78.8% of the dose within 48 h), with much smaller quantities detected in the faeces (8.8%) and cage wash (5.7%), and a minimal amount (0.04%) detected in expired carbon dioxide. The mean total recovery of radioactivity in males was 80.2% in urine, 5.6% in cagewash, 10.9% in faeces and 0.07% as expired carbon dioxide; 0.09% was detected in the carcass. In females, the majority of the administered radioactivity was recovered in the urine (67.8% of the dose within 48 h), with much smaller quantities detected in the faeces (11.5%) and cage wash (3.5%), and a minimal amount (< 0.01%) detected in expired carbon dioxide. The mean total recovery of radioactivity in females was 75.9% in urine, 4.5% in cagewash, 15.0% in faeces and < 0.01% as expired carbon dioxide; 0.1% was detected in the carcass.

In the pyridafol-dosed groups, the majority of the administered radioactivity was excreted in both sexes within 72 h. In males the majority of the administered radioactivity was identified in the urine (89.3% of the dose within 72 h) with much smaller quantities detected in the faeces (5.6%) and cage wash (2.5%) and a minimal amount (0.32%) detected in expired carbon dioxide. The mean total recovery of radioactivity in males was 90.0% in urine, 2.7% in cagewash, 6.3% in faeces and 0.34% as expired carbon dioxide; 0.1% was detected in the carcass. In females, the majority of the administered radioactivity was identified in the urine (82.0% of the dose within 72 h) with much smaller quantities detected in the faeces (4.5%) and cagewash (8.4%), and a minimal amount (0.1%) detected in expired carbon dioxide. The mean total recovery of radioactivity in females was 83.3% in urine, 8.6% in cage wash, 15.0% in faeces and 0.9% as expired carbon dioxide; 0.23% was detected in the carcass.

In the main study, the pharmacokinetic parameters calculated from whole blood reflect those for plasma, indicating a lack of binding to blood cells or plasma proteins. The parameters calculated for whole blood are presented in Table 1. Both pyridate and pyridafol were rapidly absorbed. Area under curve (AUC) values for whole blood and plasma were greater in females than males in the low-dose groups, for both single and multiple dosing. Repeated dosing did not substantially alter the pharmacokinetic parameters, with the exception of an increase in C_{max} and a corresponding decrease in the half-life, which was more pronounced for pyridate than for pyridafol. The absorption of pyridate and pyridafol at the high dose level was as rapid as seen at the low dose level; T_{max} was generally within 0.54 h of dosing. At the high dose level the sex-related differences in AUC were less pronounced than those seen in the low-dose groups. AUC values calculated for the single-dose groups were comparable for pyridate and pyridafol. In contrast, however, repeated dosing resulted in significantly higher blood and plasma AUCs for pyridate compared to pyridafol. Comparison of the elimination rate constants for blood and plasma after single dosing shows no significant differences between male and female rats of the same dose groups. Following repeated dosing, elimination rate constants in males were higher than those in corresponding females. A comparison of blood and plasma clearance between male and female rats of all dose groups reveals higher values for male rats.

Table 1. Summary of pharmacokinetic parameters for pyridate and pyridafol in the rat

Treatment	Sex	Dose level (mg/kg bw)	Sample time (h)	T_{max} (h)	C_{max} (µg/g)	$t_{½}$ (h)	K_{el} (h^{-1})	CL ($g\ h^{-1}$)	VD (g)	AUC_{last} (µg h/g)
Pyridate	M	20	0–120	1.6	5.6	17.4	0.04	85.6	2131	62.2
SOLD			0.5–168	0.5	8.6	12.4	0.06	66.5	1179	81.1
Pyridate	F	20	0–120	2.2	8.5	16.3	0.04	30.8	747	162.2
SOLD			0.5–168	1.5	9.2	18.7	0.04	19.5	567	290.9
Pyridafol	M	20	0–120	1	9.7	9.1	0.08	66.4	841	89.5
SOLD			0.5–168	0.5	13.3	6.0	0.12	57.5	497	97.6
Pyridafol	F	20	0–120	2.8	12.8	11.1	0.07	19.4	315	252.2
SOLD			0.5–168	0.8	15.5	12.8	0.06	24.3	455	196.8
Pyridafol	M	11	0–120	2.2	5.8	7.1	0.10	51.8	529	55.2
SOLD			0.5–168	0.8	8.2	5.6	0.15	46.1	360	60.1
Pyridafol	F	11	0–120	1.6	12.9	11.4	0.07	18.4	319	137.6
SOLD			0.5–168	1.1	8.9	12.2	0.07	26.2	441	98.6
Pyridate	M	20	0–120	1	16.0	3.6	0.23	68.0	336	104.5
MOLD			0.5–168	1.7	14.1	9.2	0.21	77.2	1304	85.6
Pyridate	F	20	0–120	1	29.4	14.5	0.05	20.7	429	245.4
MOLD			0.5–168	1.7	31.3	6.5	0.17	26.8	220	184.3
Pyridafol	M	11	0–120	1	10.5	2.6	0.33	63.9	212	60.5
MOLD			0.5–168	0.5	10.4	9.0	0.14	74.1	947	51.3
Pyridafol	F	11	0–120	1	20.4	6.6	0.12	21.9	217	123.8
MOLD			0.5–168	0.5	21.3	8.2	0.14	32.1	387	84.7
Pyridate	M	200	0–120	2	39.6	-	-	-	-	862
SOHD			0.5–168	7.2	33.6					1183
Pyridate	F	200	0–120	2	35.7	-	-	-	-	1282
SOHD			0.5–168	11	45.8					2055
Pyridafol	M	200	0–120	1.6	26.3	-	-	-	-	404
SOHD			0.5–168	11	35.0					1164
Pyridafol	F	200	0–120	10	35.1	-	-	-	-	1608
SOHD			0.5–168	1	40.5					929
Pyridafol	M	110	0–120	2	32.1	-	-	-	-	552
SOHD			0.5–168	1	33.4					657
Pyridafol	F	110	0–120	10	37.0	-	-	-	-	1234
SOHD			0.5–168	6	35.8					890

Treatments: SOLD Single oral low dose MOLD Multiple oral low dose SOHD Single oral high dose

T_{max} Time to reach the maximum concentration C_{max} Maximum concentration $t_{½}$ half-life

K_{el} Elimination constant CL Clearance VD Volume of distribution

AUC_{last} Area under the concentration–time curve from 0–168 h

Source: Bounds et al., 1997a

The excretion profiles of pyridate and pyridafol are shown in Table 2. The total recovery of radioactivity was high in all groups, ranging between 92% and 102%. Excretion profiles were similar for all groups, with more than 67% of the AD (> 82% when cage wash values are included) excreted in the urine. A smaller proportion (5.3–14%) of the administered dose (AD) was excreted in the faeces. Less than 1% of the AD remained in the carcass at 168 h. Overall, the majority of the administered radioactivity (> 70%) was excreted within 48 h of dosing in all dose groups. The proportion of the AD excreted in the urine and the low levels of residual radioactivity present in the carcass at 168 h indicate that pyridate and pyridafol are well absorbed and rapidly excreted in rats of both sexes.

Table 2. Summary of the excretion profiles of pyridate and pyridafol in the rat

Treatment	Sex	Dose (mg/kg bw)	Major recovery	Radioactivity (%) 0–168 h			Carcass	Recovery (%)
				Urine	Faeces	Cage wash		
Pyridate SOLD	M	20	0–48h	78	8.5	8.7	0.2	95
Pyridate SOLD	F	20	0–48h	72	6.8	17	0.6	97
Pyridafol SOLD	M	20	0–48h	81	7.7	7.9	0	96
Pyridafol SOLD	F	20	0–48h	72	6.4	17	0.2	95
Pyridafol SOLD	M	11	0–48h	77	9.0	11	0.2	97
Pyridafol SOLD	F	11	0–48h	68	7.5	19	0.8	95
Pyridate MOLD	M	20	0–72h	73	14	9.6	0.1	97
Pyridate MOLD	F	20	0–48h	81	11	5.6	0.2	98
Pyridafol MOLD	M	11	0–48h	80	6.9	15	0	102
Pyridafol MOLD	F	11	0–48h	76	8.5	12	0.1	97
Pyridate SOHD	M	200	0–48h	79	9.4	9.6	0.1	98
Pyridate SOHD	F	200	0–72h	76	9.9	9.8	0.2	96
Pyridafol SOHD	M	200	0–24h	81	9.0	5.2	0.1	95
Pyridafol SOHD	F	200	0–24h	74	8.3	9.2	0.2	92
Pyridafol SOHD	M	110	0–24h	83	5.6	5.8	0.1	95
Pyridafol SOHD	F	110	0–24h	79	5.4	8.9	0.1	93

Treatments: SOLD Single oral low dose MOLD Multiple oral low dose SOHD Single oral high dose

Source: Bounds, 1997a

Radioactivity was detected in all tissues and organs investigated in both sexes at up to 24 h following dosing, indicating rapid distribution. By 96 h after dosing most tissues and organs showed levels of radioactivity comparable to the background range, consistent with rapid elimination. With the exception of the GIT, skin and carcass, only the liver (in both sexes at 1 h and 6 h after dosing) and lungs (in females at 1 h) contained greater than 1% of the AD. The tissue distribution of radioactivity in rats administered 11 or 20 mg/kg bw pyridafol was similar to that seen for pyridate. Tissues containing the highest levels of radioactivity at 1, 6 and 24 h after dosing were the GIT, liver and kidneys. At 96 h after dosing, the majority of tissues showed very low levels of radioactivity, corresponding to less than 0.01% of the AD.

The tissue distribution of radioactivity following the administration a single high dose of 200 mg/kg bw pyridate or pyridafol was similar, as was the tissue distribution following a single high dose of 110 mg/kg bw pyridafol. Tissues containing the highest levels of radioactivity at 1, 6 and 24 h after dosing were identified as the GIT, liver and kidneys, reflecting the routes of absorption and excretion.

At 96 h after dosing, the majority of tissues showed very low levels of radioactivity, equivalent to less than 0.01% of the AD.

The tissue distribution of radioactivity in rats pretreated for 14 days prior to a single dose of radiolabelled material was comparable for pyridate (20 mg/kg bw) and pyridafol (11 mg/kg bw). The tissues containing the highest residues of radioactivity at 1, 6 and 24 h after dosing were identified as the GIT, liver, kidneys, carcass and skin. The majority of tissues showed levels of radioactivity equivalent to less than 0.01% of the dose at 24 h after dosing, indicating that elimination was more rapid than following a single dose.

This study demonstrates that single oral doses of 20 or 200 mg/kg bw pyridate are rapidly and quantitatively absorbed; maximum plasma concentrations were attained within 0.5–3 h. Levels of plasma radioactivity declined with half-lives of between 12 h and 19 h. At the low dose level, female rats showed significantly higher AUC values than males. Repeated dosing did not affect the toxicokinetic parameters, with the exception of an increase in the C_{max} and a corresponding decrease in half-life. Based on levels of radioactivity in the urine and carcass, it can be calculated that 72.6–81.2% of the AD is absorbed. Including cage wash values, the extent of absorption is calculated to be between 82.8% and 89.7% of the AD. The majority of the absorbed radioactivity was excreted in the urine (72–81%), with a much smaller proportion excreted in the faeces (6.8–14%), largely within 48 h of dosing. Less than 1% of the dose remained in the carcass at 96 h after dosing, irrespective of dose level, pretreatment or sex. The tissues containing the highest levels of radioactivity at 1, 6 and 24 h after dosing were the GIT, liver and kidneys, reflecting the routes of absorption and excretion. At 24 h (repeated dosing) or 96 h after dosing, the majority of tissues had levels of radioactivity equivalent to less than 0.01% of the AD. No major differences in the tissue distribution of radioactivity were observed between the various groups. The pretreatment of rats therefore appeared to induce a more rapid elimination of radioactivity. Data indicate comparable toxicokinetic properties for pyridate and pyridafol (Bounds et al., 1997a).

Dog

The absorption, distribution, metabolism and excretion of [^{14}C] radiolabelled pyridate were also investigated in a limited study in dogs, using groups of one dog/sex and dosing with pyridate at oral dose levels of 32, 80 and 200 mg/kg bw. The study was compliant with GLP. In females, higher plasma levels of radioactivity and increased urinary excretion (75% compared to 40–45% in males) were seen at 32 and 80 mg/kg bw. These findings suggest a higher level of systemic exposure in female dogs, however, no firm conclusion can be drawn due to the small group size used in this study. Peak plasma concentrations of radioactivity were observed at between 2 h and 12 h in dogs in the mid- and high-dose groups. Pyridate-derived radioactivity was readily eliminated from the plasma with the levels at 48 h following dosing equivalent to less than 10% of peak levels. By 96 h following dosing, plasma pyridate-derived radioactivity levels were less than 1% peak levels. In the animal dosed at 200 mg/kg bw, whole blood pyridate-derived radioactivity levels generally paralleled plasma levels (albeit at slightly lower levels). Approximately 86% to 95% of the administered radioactivity was recovered in the urine, faeces, cage wash and vomit by 96 h after oral dosing (Cameron et al., 1988).

(b) Intravenous route

Rat

In a GLP-compliant study using intravenous dosing, single doses of [^{14}C]-pyridate (2.5 mg/kg bw) or [^{14}C]-pyridafol (5 mg/kg bw) were administered to groups of rats. The C_{max} and AUC ranges from rats dosed with pyridafol at 5 mg/kg bw were approximately 2–3 times greater than for rats dosed with pyridate at 2.5 mg/kg bw; for both substances the values were greater for female than for male rats. Radioactivity levels in blood decreased more rapidly in rats administered pyridafol, compared to rats dosed with pyridate; half-lives of 4.77.3 h and 7.1–13.3 h are calculated for pyridafol and pyridate, respectively. Elimination rate constants were consequently higher for pyridafol (0.12–0.19 h^{-1}) than for pyridate (0.05–0.1 h^{-1}). High clearance rate constants (36–81 g/h) and low volumes of distribution (233–1319 mL) indicated that neither pyridafol nor pyridate are significantly bound to blood proteins. The ranges observed for kinetic parameters following intravenous dosing were comparable to those observed following a single oral low dose.

The total recovery of radioactivity was 89.0–90.6% for pyridate and 96.1–97.1% for pyridafol. The administered radioactivity was rapidly excreted in the urine, with >77% of the recovered radioactivity present in the 0–24 h urine samples and cage wash for all groups. The total excretion of radioactivity in the faeces accounted for a smaller proportion (5–11%) of the administered dose; the majority of the faecal excretion occurred between 12 h and 48 hours. Excretion profiles were similar for all groups and are comparable to those seen following the administration of a single low oral dose of pyridate or pyridafol (Bounds *et al*., 1997b).

Using blood AUC data from this study and values from the study by the same laboratory using oral dosing (Bounds et al. 1997a; reported above), the extent of bioavailability (*F*) following oral administration can be estimated (Table 3). Data indicate the extensive oral absorption of pyridate and pyridafol; oral bioavailability was slightly higher in females compared to males for both pyridate and pyridafol.

Table 3. Calculated bioavailability, F, of pyridate and pyridafol after oral dosing

Dose level (mg/kg bw)	*F* for males		*F* for females	
	Pyridafol	Pyridate	Pyridafol	Pyridate
11	1.01	-	1.66	-
20	0.89	1.11	1.72	2.01
110	1.01	-	1.42	-
200	0.73	1.57	0.95	1.47

Source: Bounds et al., 1997a, b

Toxicokinetic data for pyridate and pyridafol following intravenous dosing are comparable to data obtained following single oral dosing at low levels and show only slight differences in bioavailability for either sex; data for females indicates a slightly higher level of bioavailability. Faecal radioactivity identified for rats administered a single intravenous dose indicates a degree of biliary excretion. It can be concluded that the majority of faecal radioactivity identified in rats administered a single oral dose is likely to represent absorbed material excreted in the bile. The oral absorption of pyridate (and pyridafol) is therefore likely to be high.

1.2. Biotransformation

Rat

Pyridate was found to be metabolized rapidly and extensively following oral dosing. The metabolism of pyridate proceeds via an initial hydrolysis step to form pyridafol (CL9673, SAN 1367H), which was identified as the major component in plasma. Pyridate was only found in small quantities in plasma and only in the one-hour plasma samples. Pyridafol formed by the initial hydrolysis step is subsequently metabolized by conjugation with glucuronic acid. Pyridafol and the *O*-glucuronide conjugate of pyridafol represent approximately 12–19% and 23–50% of the total urinary radioactivity, respectively. A third significant urinary component, representing approximately 26–37% of the sample radioactivity, was tentatively identified as hydroxylated pyridafol. Faecal metabolites were identified as unchanged pyridate (10–35% of the sample radioactivity) and pyridafol (19–59% of the sample radioactivity) (Cameron, 1988). Data for the dog are of limited reliability due to the small group size, but indicate a different pattern of metabolism. In contrast to the rat, the *N*-glucuronide of pyridafol was identified as the main conjugated urinary metabolite in the dog. Pyridafol accounted for approximately 18–23% of the total urinary radioactivity (Cameron, 1989).

Samples from the study of Bounds et al. (1997a) were analysed for the presence of metabolites of pyridate and pyridafol. The metabolite profile was qualitatively similar for all dose groups but showed some quantitative differences. Approximately 40–70% of the administered radioactivity was excreted as pyridafol and phenyl-hydroxylated pyridafol (M1), which were identified as the two major urinary metabolites. The glucuronic acid conjugate of pyridafol (M2) and the sulfate conjugate of pyridafol (M3) were also identified as urinary metabolites in this study. The glucuronide was more prominent in

females of all dose groups, whereas the sulfate conjugate was quantitatively more important in males. All other urinary metabolites accounted for less than 3% of the AD. Metabolism was generally found to be more rapid in the repeated low-dose groups compared to the single oral low-dose groups. Most notably, hydroxylation on the phenyl group and formation of the glucuronide conjugate of pyridafol were more prominent in the repeat-dose groups. Unchanged pyridate was not identified in any urine sample. A total of 30–83% of the faecal radioactivity was identified; the major faecal metabolite was identified as phenyl-hydroxylated pyridafol (M1), accounting for up to 4.4% of the AD. Levels of this metabolite were slightly higher in female compared to male rats. The proportion of unchanged pyridate excreted in the faeces was higher in the high-dose groups compared to the low-dose groups.

Table 4. Urinary metabolites of pyridate and pyridafol (all values as % of administered dose)

Compound	Group		Total	Pyridafol	M1	M2	M3	M4	M5	M6	M7	M8
Pyridate	SOLD 20 mg/kg bw	M	77.6	32.1	25.6	3.9	9.4	0.1	1.7	0.3	0.6	0.5
		F	71.6	21.8	22.4	16.4	5.2	0.1	0.8	0.1	2.0	0.5
	SOHD 200 mg/kg bw	M	72.8	26.3	27.2	9.4	5.5	0.1	1.2	0.3	0.7	0.5
		F	80.7	20.5	39.3	13.0	3.6	0.0	0.2	0.1	2.3	0.5
	MOLD 20 mg/kg bw	M	80.6	22.8	31.0	10.2	8.7	0.3	1.5	1.1	1.6	0.5
		F	73.7	14.4	37.6	12.2	3.6	0.4	0.7	0.5	2.2	0.5
Pyridafol	SOLD 11 mg/kg bw	M	76.2	39.5	23.7	1.6	5.5	1.0	0.9	0.9	0.5	0.5
		F	64.9	21.5	31.4	3.4	2.4	0.3	0.0	0.7	0.8	0.5
	SOLD 20 mg/kg bw	M	72.6	45.0	21.4	0.6	5.0	0.4	1.3	0.9	0.2	0.5
		F	78.7	27.6	33.1	5.5	2.9	0.2	0.3	0.2	0.5	0.5
	SOHD 110 mg/kg bw	M	78.3	29.0	22.6	18.4	5.1	0.1	0.3	0.6	1.5	0.5
		F	75.1	18.7	35.5	14.1	2.5	0.0	0.0	1.2	2.4	0.5
	SOHD 200 mg/kg bw	M	79.3	35.9	30.1	2.6	1.5	ND	1.0	0.9	0.9	0.5
		F	74.4	17.1	42.8	8.7	1.0	ND	0.6	0.8	2.2	0.5
	MOLD 11 mg/kg bw	M	82.7	20.3	39.5	7.9	8.7	0.5	0.0	3.0	1.8	0.5
		F	78.2	10.8	42.7	12.5	3.5	0.7	0.0	1.5	2.7	0.5

Treatments: SOLD Single oral low dose MOLD Multiple oral low dose SOHD Single oral high dose

M/F Male/female

ND Not detected

Source: Mewes, 1997a

Table 5. Faecal metabolites of pyridate and pyridafol (all values as % of administered dose)

Compound	Group		Total	Extractable	Pyridate	Pyridafol	M1
Pyridate	SOLD 20 mg/kg bw	M	7.6	4.9	1.3	1.5	3.3
		F	5.8	5.6	0.5	1.4	2.6
	SOHD 200 mg/kg bw	M	13.8	11.7	3.9	4.2	2.9
		F	10.2	9.2	1.4	2.6	3.8
	MOLD 20 mg/kg bw	M	6.2	4.1	0.7	0.8	3.6
		F	7.4	5.6	0.8	1.0	4.4

Compound	Group		Total	Extractable	Pyridate	Pyridafol	M1
Pyridafol	SOLD 11 mg/kg bw	M	8.6	4.5	ND	0.9	2.3
		F	8.2	5.9	ND	0.7	4.1
	SOLD 20 mg/kg bw	M	5.8	3.0	ND	0.6	1.3
		F	4.1	2.7	ND	0.6	1.3
	SOHD 110 mg/kg bw	M	9.5	4.6	–	1.5	1.6
		F	9.0	7.2	–	2.1	4.0
	SOHD 200 mg/kg bw	M	6.4	4.3	–	0.7	2.4
		F	8.6	5.8	–	0.7	3.8
	MOLD 11 mg/kg bw	M	4.9	2.4	–	0.2	1.2
		F	5.2	3.9	–	0.3	2.2

Treatments: SOLD Single oral low dose MOLD Multiple oral low dose SOHD Single oral high dose

M/F Male/female

ND Not detected

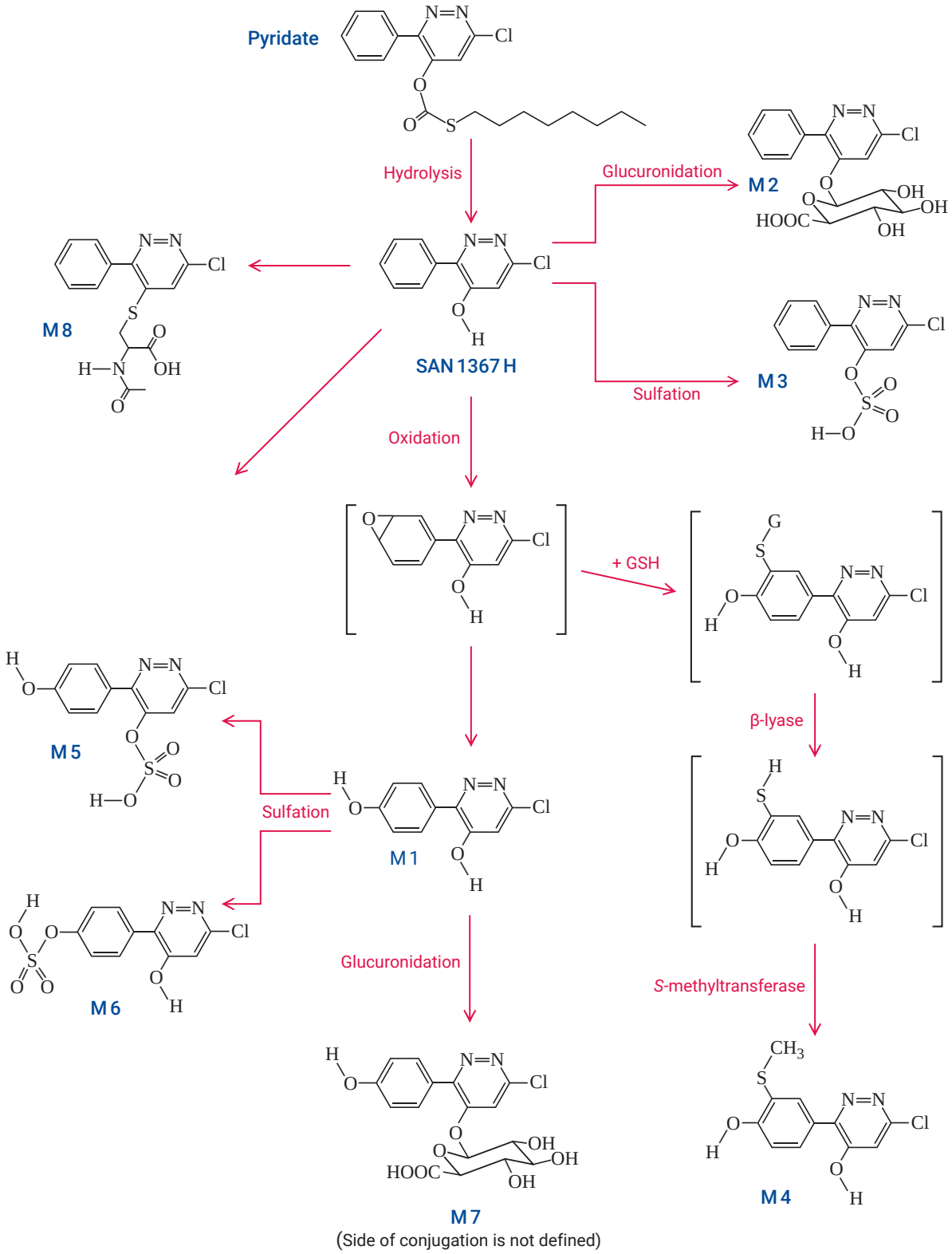
Source: Mewes, 1997a

The results of this study demonstrate that pyridate is almost completely metabolized in the rat with the initial metabolic step being the hydrolysis of pyridate to form pyridafol (SAN 1367H, CL-9673), presumably with liberation of the *n*-octylthiocarbonyl sidechain. Pyridafol is then further metabolized by conjugation with either glucuronic acid or sulfate (excreted in the urine), or by hydroxylation on the phenyl group (excreted in the urine and faeces). Minor metabolites were identified as:

- the sulfate conjugate of pyridafol (M5, M6),
- the glucuronic acid conjugate (M7) of pyridafol,
- the conjugation of M1 with glutathione, then subsequent hydrolysis and methylation of the resulting thiol (M4),
- the conjugation of pyridafol with glutathione and subsequent hydrolysis and acetylation of the free amino group (M8).

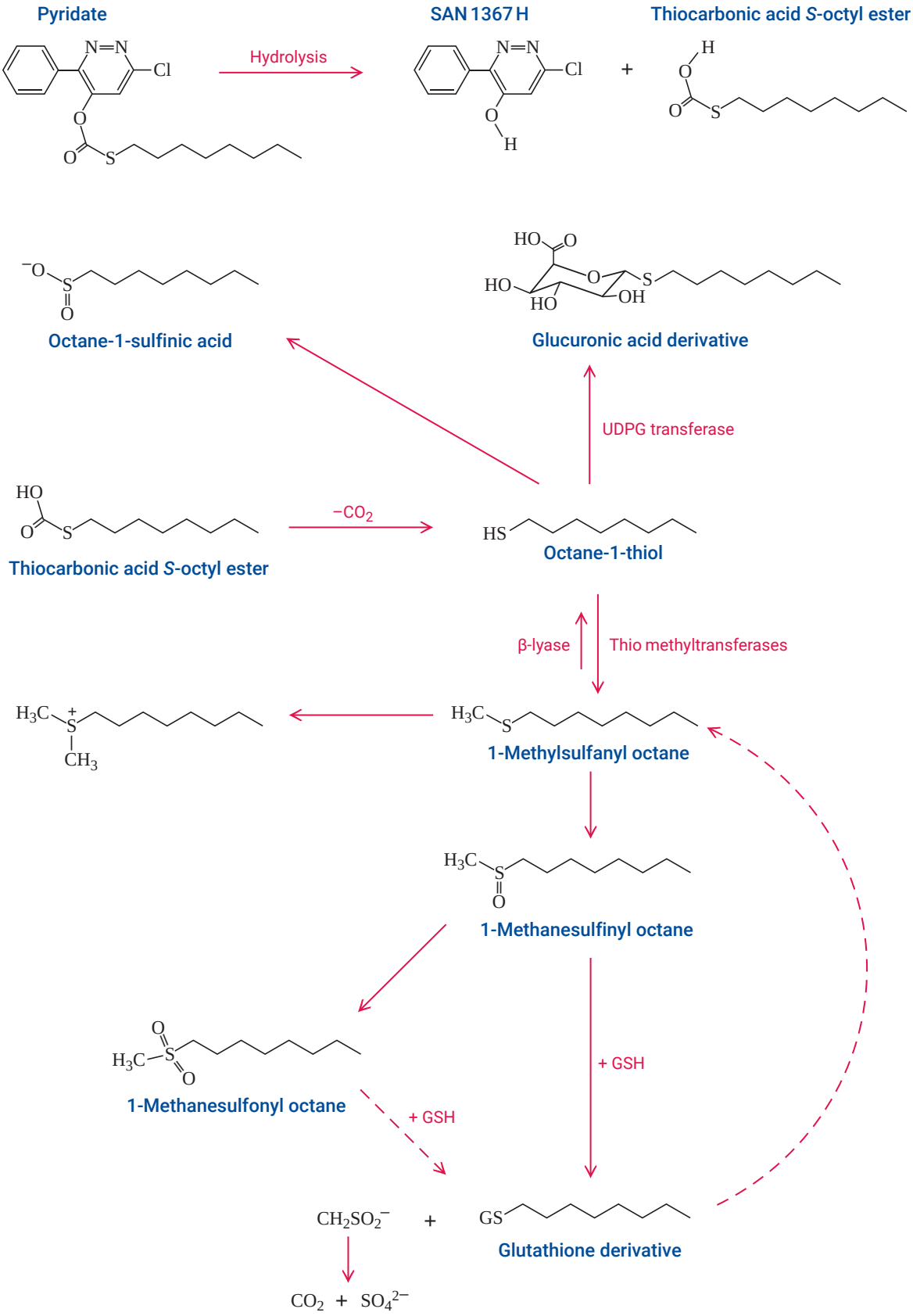
CL-9869 was not detected in urine or faeces. Therefore no evidence was found that biotransformation in rats includes methylation of the hydroxyl group of pyridafol. The metabolism of pyridafol was found to be comparable to that of pyridate, following the initial hydrolysis step. A proposed metabolic pathway is shown below in Fig.3 (Mewes, 1997a).

Figure 3. Proposed metabolic pathway for pyridate and pyridafol in the rat



A theoretical assessment of the metabolic fate of the thiocarbonic acid *S*-octyl ester was conducted. The *S*-octyl thiocarboxylate is unstable and is likely to undergo rapid decarboxylation resulting in the formation of the mercaptan octan-1-thiol and carbon dioxide. The octan-1-thiol metabolite may be further metabolized through thiol methylation to form 1-methylsulfanyl-octane and possibly further methylation with subsequent oxidation of the sulfur atom. Minor pathways may be glucuronidation of the thiol or oxidation to octane-1-sulfinic acid. A tentative metabolic pathway of the noctylthiocarbonyl sidechain is shown below in Fig. 4 (Mewes *et al.*, 1997b).

Figure 4. Proposed metabolic pathway for the n-octylthiocarbonyl sidechain in the rat



2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

All studies used test methods that are inconsistent with current standards. In the acute oral toxicity studies, signs of toxicity (including dyspnoea, sedation, lethargy, ataxia, uncoordinated movements, paddling movements, piloerection and hunched posture) were observed at dose levels of 1000 mg/kg bw and above. Signs of toxicity were reversible in surviving animals. The results of older and more recent studies report comparable results; studies performed using a variety of dosing vehicles (aqueous carboxymethyl cellulose, corn oil and PEG 200) also report similar findings. In contrast to the acute oral toxicity stuydies, deaths occurred following oral gavage dosing at 500 mg/kg bw in the rat acute neurotoxicity study (Diehl, 2016) and following the first oral gavage dose of 400 mg/kg bw in the rat developmental study (Becker et al., 1986). This likely correlates with the saturation of urinary excretion in rats.

Twenty-four hours of occlusive dermal exposure of rabbits to undiluted pyridate induced reversible weight loss and slight to moderate erythema and oedema between two and 15 days after treatment. In the acute inhalation toxicity studies, signs of toxicity (including dyspnoea, sedation, curved body position and ruffled fur) were observed at dose levels equal or greater than 2.7 mg/L.

Table 6. Summary of acute toxicity studies with pyridate

Species	Strain	Sex	Route (vehicle)	Purity	LD_{50} or LC_{50}	Reference
Mouse[a,b,d]	NMRI	M/F	Oral gavage (5% aqueous gum arabic)	93.1%	>10 000 mg/kg bw (M/F)	Ullmann, Sacher & Vogel, 1987
Rat[a,b,c]	Wistar	M/F	Oral gavage (PEG 400)	90.3%	5993 mg/kg bw (M) 3544 mg/kg bw (F) 4690 mg/kg bw (M/F)	Ullmann & Sacher, 1984
Rat[a,b,c]	Wistar	M/F	Oral gavage (PEG 400)	93.1%	4174 mg/kg bw (M) 2961 mg/kg bw (F) 3588 mg/kg bw (M/F)	Ullmann, Sacher & Vogel, 1988
Rat[a,c]	Wistar	M/F	Oral gavage (corn oil)	90.4%	> 2800 mg/kg bw (M) 2371 mg/kg bw (F)	Pels Rijcken, 1996a
Rat[a,c]	Wistar	M/F	Oral gavage (1% aqueous CMC)	90.4%	> 2800 mg/kg bw (M) 2092 mg/kg bw (F)	Pels Rijcken, 1996b
Rat[a,c,e]	Wistar	M/F	Oral gavage (PEG 200)	90.4%	> 2000 mg/kg bw (M/F)	Pels Rijcken, 1996c
Rat[a,b,g,h]	Wistar	M/F	Inhalation	–	> 4.37 mg/L (M/F)	Ullmann, Zbinden & Chevalier, 1983
Rabbit[a,b,e,f]	NZW	M/F	Dermal (no vehicle)	90.3%	> 2000 mg/kg bw (M/F)	Ullmann, Sacher & Chevalier, 1984

M/F Male/female CMC Carboxymethyl cellulose LC_{50}/LD_{50} Median lethal concentration/dose

[a] Conducted in accordance with GLP

[b] Analytical certificate not supplied

[c] Vehicle-only control group not included in the study design

[d] Vehicle-only control group included in the study design

[e] Limit dose test design. A single dose of 2000 mg/kg bw used

[f] Test material applied to the skin under an occlusive dressing; current guidelines indicate that a porous gauze dressing should be used

[g] Four hour aerosol nose-only exposure used

[h] Based on gravimetrical determination approximately 36–39% of particles at the 4.37 mg/L concentration were within a range of < 1–3 µm, but aerosol characterization inconsistent with modern standards

(b) Dermal irritation

In an older study that was non-compliant with current testing standards, occlusive exposure of intact and abraded skin of six rabbits (unknown strain) to pyridate (batch and purity not stated) for 24 h produced mild to moderate dermal erythema (scores 1–3 on a scale of 1–4 over the period of 24–72 h following the start of exposure) and oedema (scores 1–2 on a scale of 1–4 over the period of 24–72 h following the start of exposure). Neither the erythema nor the oedema at the application sites had resolved by study termination at 72 h following the start of exposure. No other adverse effects on the treated skin were observed (Kynoch & Liggett, 1976a).

In a more recent study that was compliant with modern standards, 4 h of semi-occlusive skin exposure to pyridate (batch no. CL11344/AR995; purity 89.8%) in three male and three female New Zealand White rabbits resulted in slight to well defined erythema in all animals within 30 to 60 minutes. Well defined erythema was present in all animals by 24 h following exposure (score 2 for all animals on a scale of 1–4). This progressed to moderate to severe erythema (score 3 on a scale of 1–4) in two out of the six rabbits by 48–72 h following treatment. The mean 24–72 h erythema score for all six animals was 2.2 on the scale of 1–4 that was used. Slight erythrema was still present in all six rabbits by 14 days following exposure. Slight oedema was present in two out of six rabbits at 48–72 h following treatment. This was fully reversed by greater than or equal to 4 days. Between days 7 and 10 desquamation was detected in five out of six rabbits. Desquamation persisted for 14 days following treatment in two out of six rabbits. All animals survived for 14 days following treatment (Shults, Brock & Laveglia, 1995a).

Based on the overall weight of evidence, pyridate is a mild skin irritant to rabbits.

(c) Ocular irritation

In an older study that was non-compliant with modern test standards, instillation of 0.1 mL of pyridate (batch and purity not stated) into the ocular conjunctival cul de sacs of six rabbits of unknown strain (one eye per rabbit with the opposite eye serving as untreated controls) resulted in conjunctival "dullness" in one out of six of the animals on days 1–7 following exposure. The conjunctival change in the affected animal progressed to scattered areas of opacity (score of 1 on a scale of 1–4) by days 14 and 21. Transient conjuctival redness (score 1 on a scale of 1–4) also occurred in a second rabbit but only on days 1 and 2 following exposure. No other adverse effects on the eyes were observed. All rabbits survived for 14 days following treatment (Kynoch & Liggett, 1976b).

In a more recent study that was compliant with modern test standards 0.1 mL of pyridate (batch no. CL11344/AR995; purity 89.8%) was instilled into the right ocular conjunctival cul de sacs of three male and three female New Zealand White rabbits (the left ocular conjunctival cul de sacs serving as untreated controls). The conjunctival cul de sacs were not washed out at 24 h following treatment. Minimal conjunctival redness and/or discharge occurred in four out of six rabbits at 1 h following exposure (score 1 on a scale of 1–4) and in five out of six rabbits at 24 h following treatment (score 1 on a scale of 1–4). These effects were fully reversed by 48 h following exposure. No adverse effects on the cornea (including negative fluorescein staining tests) or iris were observed in any animal at any time point. Split-lamp biomicroscopy was not performed and is unnecessary given the study findings. The mean 24–72 h score for conjunctival redness was 0.22. All rabbits survived for 14 days following treatment (Shults, Brock & Laveglia, 1995b).

Based on the weight of evidence, pyridate is a mild eye irritant in rabbits.

(d) Dermal sensitization

In an older guinea pig maximisation test that was non-compliant with current test standards ten male albino Hartley Dunkin strain guinea pigs were induced by intradermally injecting with 0.1 mL of 1% v/v pyridate (batch and purity not stated; non-irritant) in liquid paraffin with or without Freund's complete adjuvant. One week later these animals were topically re-exposed by occlusive application of 0.4 mL of pyridate for 48 h. Two weeks later the guinea pigs were topically challenged by occlusive application of 50% (v/v in liquid paraffin; non-irritant) pyridate for 24 h. All ten animals had developed erythema and oedema at the challenge sites by 24 h after challenge. Severe erythema and oedema (score 4 on a 1–4 scale) occurred in five out of ten guinea pigs at 72 h post challenge. Slight to moderate dermal reactions

occurred in the remaining five animals at this time point. Pyridate was considered to be a sensitizer in this study. The study lacked a vehicle-only control group (Kynoch, 1976).

In an older Beuhler test in male and female albino guinea pigs (conducted in a manner that was broadly consistent with modern standards) repeated, open topical induction using 0.5% to 10% pyridate (batch no. 2669427; purity 93.1%; in ethanol; non-irritant) displayed skin sensitization potential following open topical challenge with 3% and 10% pyridate (Ullman & Kups, 1988b).

Based on the weight of evidence, pyridate is considered to be a skin sensitizer.

2.2. Short-term studies of toxicity

(a) Oral administration

Mouse

In a study that was not compliant with modern test standards, groups of ten male and ten female Swiss mice were administered pyridate (batch and purity not reported) in the diet for 28 days at concentrations of 0, 1000, 3000 and 10 000 ppm (equivalent to 200, 600 and 2000 mg/kg bw per day). The general condition and behaviour of all animals and their food intake were observed daily. Individual body weights were determined weekly. Retro-orbital blood samples for haemoglobin (Hb) determination were collected from all animals on study day 26. On the completion of 28 days of treatment all mice were euthanized. Gross anatomic pathology evaluations were performed on all mice. Kidney, spleen, liver and lung weights were measured for all animals. Microscopic anatomic pathology evaluations were limited to the liver, kidneys and lungs from the high-dose group and the controls. The statistical methods used were not consistent with modern standards. No deaths, behavioural abnormalities in behaviour or general condition were observed during the study. Small (< 10%) decreases in mean body weights were evident in all pyridate-treated males and in females fed ≥ 3000 ppm. No effects on food consumption were detected. Mean Hb concentration was marginally, but significantly ($p < 0.01$ based on an inappropriate statistical test) higher in males administered 10 000 ppm. The relevant normal range for the laboratory performing the Hb assay was not provided. It is difficult to conclusively interpret the relevance of this finding since full complete blood counts and clinical chemistry evaluations were not performed. Mean absolute liver weight was significantly increased ($p < 0.01$ based on an inappropriate statistical test) in females administered 10 000 ppm pyridate. Mean relative liver weights were significantly elevated ($p < 0.01$ based on an inappropriate statistical test) in both sexes of mice at 10 000 ppm. Mean relative spleen weights were also elevated in this group, significantly so ($p < 0.01$ based on an inappropriate statistical test) in females Spleen weights were also notably (but not significantly) higher in females administered 3000 ppm. There were no gross effects of treatment in any group; histopathology of mice at the highest dose level did not reveal any treatment-related findings.

The no-observed-adverse-effect level (NOAEL) for this study was 1000 ppm (equivalent to 200 mg/kg bw per day) due to increased spleen weight in females and decreased mean body weight gain in males at 3000 ppm (Til et al., 1980). The NOAEL value is considered to be of reduced reliability since the design and reporting of the study was not in accordance with modern standards.

Rat

Study 1

In a study that was not compliant with current test standards groups of ten male and ten female Wistar rats were administered pyridate (batch no. and purity not reported) in the diet for 28 days at concentrations of 0, 1000, 3000 or 10 000 ppm (equivalent to 100, 300 and 1000 mg/kg bw per day). Body weights were recorded weekly; food consumption was measured daily and food utilization calculated. Tail-tip blood samples were collected on study days 25 (males) and 26 (females). These were assessed for a limited range of haematological parameters (erythrocyte and leucocyte counts, Hb concentration and haematocrit) in control and high-dose animals only. The study was terminated following 28 days of treatment. Gross anatomic pathology evaluations were performed on all animals and the organ weights of the kidneys, thymus, spleen, liver and lungs recorded. Histopathology was performed on the liver, kidneys and lungs from control and high-dose animals. The statistical methods used were inconsistent with modern standards. Relevant study findings are presented in Table 7. No deaths, abnormalities

in behaviour or general condition were observed during the study. Reduced weight gain and food consumption were recorded for both sexes administered dietary concentrations of 1000 and 3000 ppm (in two types of diets: a base diet and a semipurified diet), resulting in significantly reduced mean bodyweights ($p < 0.05$; based on Student's *t*-test which is considered statistically inappropriate) at all time points. Mean terminal body weights of both sexes at 3000 ppm were markedly (46% lower in males and 32% lower in females) lower than controls. Small, but significant haematology changes ($p < 0.05$; based on Student's *t*-test or the Wilcoxon test, which are considered statistically inappropriate) were observed in animals consuming 1% pyridate. However a biologically consistent pattern of changes occurred in both the animals fed the base diet and animals fed the semi-purified diets. Accordingly these changes are unlikely to be attributable to feeding pyridate. While the absolute weights of the kidneys, thymus, spleen, liver and lungs were significantly reduced in animals fed 1% pyridate, these changes were ascribed to the lower body weights in these animals. When both diet types are considered, significantly increased relative kidney and lung weights consistently occurred in males fed 1% pyridate, but this is based on $p < 0.05$ in the Student's *t*-test, which is considered statistically inappropriate. Likewise, when both diet types are considered significantly increased relative lung weights consistently occurred in males fed 1% pyridate, but this is based on $p < 0.05$ in a Student's *t*-test which is not considered statistically appropriate for multiple comparisons. When both dietary groups are considered the changes in kidney and liver weights were not associated with any consistent microscopic anatomic pathological changes.

Table 7. Findings in the 28-day study in rats receiving pyridate in the diet

	Males				Females			
Dose level (ppm)	**0**	**1000**	**3000**	**10 000**	**0**	**1000**	**3000**	**10 000**
Mean body weight								
Base diet, day 28 (g)	201.4	200.6	189.2*	108.5***	146.6	141.8	135.2**	99.0***
Semi-purified diet), day 28 (g)	210.6	210.6	200.2*	100.1***	149.9	146.6	140.2*	84.3***
Mean body weight gain								
Base diet (g/animal)	150	149	137	56	95	92	85	48
Semi-purified diet (g/animal)	158	157	148	47	99	95	88	33

*significantly different to controls; $p < 0.05$, **$p < 0.01$, ***$p < \approx 0.001$
based on Student's t test or Wilcoxon test (not considered statistically appropriate for multiple comparisons)

Source: Til et al., 1979

A NOAEL of 1000 ppm (equivalent to 100 mg/kg bw per day) was determined for this study, based on reduced body weight and food consumption at dietary concentrations ≥ 3000 ppm in this study (Til et al., 1979). The NOAEL value is considered to be of lower reliability since the design and reporting of the study was not in accordance with modern standards.

Study 2

A further 28-day that was not compliant with current test standards was performed to investigate potential differences in sensitivity to the toxicity of pyridate in two different rat strains. Groups of ten male and ten female Wistar (Cpb:Wu) and Sprague Dawley rats were administered pyridate (batch and purity not reported) in the diet for 28 days at concentrations of 0, 3000 and 10 000 ppm. Dietary concentrations are calculated to be equivalent to intakes of approximately 300 and 1000 mg/kg bw per day based on standard conversion factors. Animals were observed daily for mortality and clinical signs. Body weights were recorded weekly; food consumption was measured daily and food utilization calculated. Tail-tip blood samples taken towards the end of the study (on day 24) were assessed for Hb concentration. Additional haematological and clinical chemistry parameters were not measured. Gross necropsy was performed on all animals, and weights of the kidneys, thymus, spleen, liver and lungs recorded. No microscopic anatomic pathology was performed. The statistical methods used were not consistent with modern standards. No deaths occurred during the study period. No behavioural abnormalities were noted, however a number of rats of both strains were reported to show signs of emaciation during the first two weeks of treatment. Weight gains by both sexes from both rat strains

administered 10 000 ppm pyridate were markedly and significantly reduced; a less marked effect on weight gain was seen at 3000 ppm. Body weight effects reflected reduced food consumption. There was no obvious difference in strain sensitivity. Mean Hb concentration was slightly (but significantly, $p < 0.05$; based on the Wilcoxon test which is considered to be statistically inappropriate) lower in female Wistar rats administered 10 000 ppm; more marked effects were seen in female Sprague Dawley rats, with significant ($p < 0.05$) reductions in Hb concentration seen at both 3000 and 10 000 ppm. A slight (but not significant) reduction in Hb concentration was also seen in male Sprague Dawley rats at 10 000 ppm. In both strains, mean absolute weights of the kidneys, thymus, spleen, liver and lungs were lower in all treated groups compared with the control group. These findings are considered to reflect the lower terminal body weights in the treated groups compared to controls. Relative weights of the spleen were higher in females of both strains administered 10 000 ppm; relative liver weights were higher in female Sprague Dawley rats at 3000 and 10 000 ppm. There were no gross effects due to treatment in any group. Due to its experimental design limitations (insufficient dosing groups, large dose intervals, lack of microscopic anatomic pathology) the study was not considered to be sufficiently robust for the determination of a reliable toxicological threshold. Accordingly a NOAEL has not been determined (Til & de Groot, 1980).

Study 3

A 90-day study with a 28-day recovery period was performed in CD rats using pyridate (batch EOA-Knr: 2429966/2556520; purity 92%) at gavage dose levels of 0 (corn oil vehicle), 62.5, 177 and 500 mg/kg bw per day. The study design was not compliant with current testing standards. Ten rats/sex per group were sacrificed after treatment for four weeks; ten rats/sex were sacrificed after treatment for 90 days. A further 15 rats/sex were investigated to assess the reversibility of effects after 28 days without treatment following a 90-day treatment period. An additional high-dose group of ten rats/sex administered 500 mg/kg bw per day (increased to 600 mg/kg bw per day after two weeks) was also incorporated into the study design; clinical chemistry, haematology and histopathological investigations were not performed on rats of this ascending dose group, however microscopic anatomic pathology evaluations were performed on all gross lesions. Total and free triiodothyronine (T_3) and thyroxine (T_4), plasma and erythrocyte cholinesterase activities were measured in selected animals from all dose groups. Brain cholinesterase activity was measured at necropsy. Gross necropsy was performed on all rats; however full histopathological evaluation was limited to decedents, controls and high-dose level rats at scheduled necropsy. Investigations in the intermediate-dose groups were limited to the livers, lungs and kidneys from all rats at 62.5 and 177 mg/kg bw per day; and the spleen, mesenteric lymph nodes and mammary glands (females) from rats administered 62.5 and 500 mg/kg bw per day at the end of recovery period. Appropriate statistical test methods were used. Relevant study findings are summarized in Tables 8, 9 and 10. Increased pyridate-associated premature mortality during the dosing phase (but not during the recovery phase) of the study occurred at dose levels of greater than or equal to 177 mg/kg bw per day. The premature mortality rate among the high-dose females was at least 2.5-fold that of the high-dose males. A definitive cause of the premature mortality was not established. Most of the affected rats had pyridate-associated GI lesions. However the severity of these lesions was not considered to be of sufficient severity to result in death. Signs of toxicity (hypoactivity beginning approximately one hour post dosing and lasting for at least two hours in many affected rats; salivation) were seen at dose levels of ≥ 177 mg/kg bw per day, with a higher incidence in the highest dose levels of 500 and 500→600 mg/kg bw per day. Pyridate-associated hypoactivity tended to decrease as the experiment progressed. Ataxia was also observed at the highest dose levels of 500 and 500→600 mg/kg bw per day. These clinical signs were reversible during the recovery period. Mean terminal body weights of both sexes administered 500 and 500→600 mg/kg bw per day were significantly reduced; evidence of recovery was seen at 500 mg/kg bw per day following 28 days without treatment. Clotting times (prothrombin time, activated partial thromboplastin time) were transiently increased in males dosed with 500 mg/kg bw per day. However no other evidence of an adverse hypocoagulable state was observed over the course of the study. In females dosed at 500 mg/kg bw per day treatment had small effects on red blood cell parameters. Observed were: reduced erythrocyte count, reduced Hb concentration, reduced mean corpuscular Hb concentration and increased mean corpuscular volume (an overall pattern consistent with mild macrocytic hypochromic anaemia), effects consistent with mild anaemia. Small dose-related effects on red blood cell parameters (increased mean corpuscular volume, increased mean corpuscular Hb) were detected in males dosed at ≥ 177 mg/kg bw per day). At the end of the 28-day

recovery period, a slight (but statistically significant) increase in mean cell Hb concentration persisted in males at 500 mg/kg bw per day. Total leucocyte count was also significantly reduced in males dosed with 500 mg/kg bw per day pyridate following treatment for 90 days. Elevated serum total protein and serum albumen were observed in males dosed at 500 mg/kg bw per day for five weeks. This is suggestive of dehydration in these animals. Significantly ($p < 0.05$) elevated total serum bilirubin was observed following five weeks and 14 weeks of dosing at 500 mg/kg bw per day in males, and after five weeks of dosing at ≥ 177 mg/kg bw per day in females. These changes may reflect changes in erythrocyte lifespan and/or hepatotoxicity. Increased serum alkaline phosphatase (ALP) and alanine transaminase (ALT) (approaching or exceeding Hy's Law criteria) were present in both sexes following 14 weeks of dosing at 500 mg/kg bw per day. These findings are suggestive of hepatocellular membrane leakage and cholestasis. However microscopic anatomic pathology correlates were not detected. Low serum potassium levels were observed in seven out of nine females following 14 weeks of dosing at 500 mg/kg bw per day. Increased serum albumen (without concurrent changes in serum total protein), reduced serum globulin and an increased albumen:globulin ratio were observed in both sexes after 14 weeks of dosing at 500 mg/kg bw per day. Findings after the recovery period were limited to reduced glucose and total protein concentrations in males at 500 mg/kg bw per day. Urinary pH was reduced in females at 500→600 mg/kg bw per day. Other statistically significant ($p < 0.05$) changes were observed. However these changes are considered to be unrelated to treatment with pyridate.

Table 8. Findings in the 90-day study in rats receiving pyridate in the diet

Dose	**Males**					**Females**				
mg/kg bw per day	**0**	**62.5**	**177**	**500**	**500→600**	**0**	**62.5**	**177**	**500**	**500→600**
Premature mortality (incidence)	0/45	0/45	1/45[a]	4/45	1/10	0/45	1/45[a]	1/45	10/45[a]	4/10
Clinical signs										
Hypoactivity	–	–	+	+	+	–	–	+	+	+
Salivation	–	–	+	+	+	–	–	+	+	+
Week 13 mean body weight (g)	537	529	514	388**	355**	281	279	268	240**	235**
Mean body weight after recovery (g)	535	557	545	482	-	290	286	286	266	-
Haematology: week 5										
Prothrombin time (s)	14.0	13.9	14.3	15.7*	-	14.0	14.0	13.8	13.9	-
APTT (s)	21.6	22.8	22.9	31.6**	-	21.6	21.8	17.9**	21.1	-
Haematology: week 14										
RBC (10^6/µL)	8.64	8.65	8.83	8.43	-	8.46	8.38	8.65	7.75**	-
Hb (g/dL)	15.7	45.8	16.1	16.3	-	16.3	15.9	16.4	15.4**	-
MCV (fL)	56.6	57.6	58.7*	60.1**	-	57.4	57.8	57.6	61.0**	-
MCH (pg)	18.2	18.3	18.3	19.3**	-	19.3	19.1	18.9	19.9	-
MCHC (g/dL)	32.4	31.8	31.4*	32.3	-	33.7	33.1	33.1	32.7*	-
WBC (10^3/µL)	15.9	15.2	16.3	11.8*	-	8.9	8.6	9.6	8.5	-
Haematology: week 18 (recovery)										
MCH (pg)	19.4	19.6	20.2	20.4*	-	20.8	20.5	21.52	21.1	-

Dose	Males					Females				
mg/kg bw per day	0	62.5	177	500	500→600	0	62.5	177	500	500→600
Clinical chemistry: week 5										
Creatinine (mg/dL)	0.6	0.6	0.6	0.6	-	0.6	0.6	0.6	0.7*	-
Albumin (g/dL)	3.58	3.60	3.56	3.79**	-	3.72	3.69	3.94	3.90	-
Total protein (g/dL)	6.27	6.33	6.20	6.59*	-	6.61	6.54	6.81	6.67	-
AST (u/L)	59.2	53.0	54.0	58.5	-	57.0	59.3	57.6	48.8*	-
T. Bil (mg/dL)	0.13	0.15	0.16	0.18**	-	0.16	0.16	0.22**	0.21*	-
Clinical chemistry: Week 14										
BUN (mg/dL)	12.3	12.8	12.5	11.5	-	15.2	13.5	13.7	11.6**	-
Albumin (g/dL)	3.67	3.73	3.77	3.99**	-	4.08	4.01	4.13	3.91	-
ALP (u/L)	68.6	73.7	68.7	97.4	-	33.9	44.6	46.5	75.6**	-
ALT (u/L)	29.9	31.4	37.9	46.0*	-	27.6	23.8	26.8	33.6	-
Potassium (meq/L)	6.34	6.32	6.26	6.68	-	8.46	7.74	8.09	6.74**	-
T. Bil (mg/dL)	0.19	0.20	0.21	0.27**	-	0.22	0.21	0.22	0.25	-
Globulin (g/dL)	3.52	3.30	3.35	3.08**	-	3.43	3.52	3.43	3.06*	-
A:G ratio	1.05	1.13	1.13	1.31**	-	1.20	1.14	1.21	1.29*	-
Clinical chemistry: Week 18 (recovery)										
Glucose (mg/dL)	250.9	261.3	235.0	207.0*	-	185.6	208.8	202.89	173.8	-
Creatinine (mg/dL)	0.7	0.6	0.7	0.7	-	0.7	0.7	0.7	0.6*	-
Total protein (g/dL)	7.09	7.15	7.08	6.78**	-	7.64	7.50	7.67	7.31	-
Total T_4 (ng/mL)	29.46	27.82	29.65	12.65**	13.90**	22.86	24.08	22.82	-	16.07*
Free T_4 (pg/mL)	28.16	26.32	26.38	10.95**	11.93**	14.18	15.25	15.54	-	12.22

[a] includes one death due to dosing accident

APTT Activated partial thromboplastin time RBC Red blood cell MCV Mean corpuscular volume
MCH Mean corpuscular haemoglobin MCHC Mean corpuscular haemoglobin concentration
WBC White blood cell count AST Aspartate transaminase T. Bil Total bilirubin
BUN Blood urea nitrogen A:G ratio Albumin:globulin ratio

*significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Henck et al., 1987

Measurement of cholinesterase activity revealed a marked inhibition of plasma cholinesterase activity in females only at ≥ 177 mg/kg bw per day. This finding is not considered to be of toxicological significance in the absence of similar effects on brain and erythrocyte cholinesterase activity. Free and total T_4 levels were significantly reduced at 500 and 500→600 mg/kg bw per day in both sexes; no effect of treatment was apparent on T_3 levels.

Increases in liver and kidney weight were seen in females at ≥ 177 mg/kg bw per day; kidney weight was increased in males in all dose groups. Absolute testis and brain weights in males were significantly decreased at 500 mg/kg bw per day, but not at 500→600 mg/kg bw per day. Adrenal weight was increased in females at ≥ 177 mg/kg bw per day and in males of the 500 and 500→600 mg/kg bw per day group. Relative thyroid weight was significantly decreased in females at 62.5 and 177 mg/kg bw per day; a similar finding was not observed at 500 mg/kg bw per day though. In males, relative thyroid weight was significantly higher at 500 mg/kg bw per day but not at 500→600 mg/kg bw per day.

Table 9. Organ weights in the 90-day study in rats receiving pyridate in the diet

	Dose (mg/kg bw per day)	0	62.5	177	500	500→600
Males	Heart (g)	1.64	1.64	1.64	1.33**	1.35**
	Liver (g)	18.0	17.7	16.6	12.1**	12.6**
	Spleen (g)	0.794	0.795	0.755	0.480**	0.500**
	Thymus (g)	0.376	0.333	0.284**	0.138**	0.150**
	Brain (g)	2.07	2.09	2.07	1.94**	1.98
	Testes (g)	3.61	3.44	3.51	3.21**	3.34
	Thyroids (g)	0.026	0.022	0.023	0.021	0.018*
	Pituitary (g)	0.0163	0.015	0.014	0.012**	0.011*
	Adrenals (g)	0.056	0.056	0.054	0.075**	0.074**
	Liver (% body weight)	3.33	3.52	3.32	3.44	3.79**
	Thymus (% body weight)	0.07	0.066	0.057	0.047**	0.045**
	Kidneys (% body weight)	0.717	0.797**	0.826**	0.934**	0.974**
	Thyroids (% body weight)	4.78	4.45	4.52	5.95*	5.40
	Adrenals (% body weight)	10.4	11.3	108	21.9**	22.4**
	Adrenal (rel. to brain wt)	2.71	2.717	2.594	3.856**	3.753**
Females	Heart (g)	0.97	0.97	0.95	11.07	1.03
	Liver (g)	1.90	7.87	8.41	8.62	8.95
	Spleen (g)	0.415	0.473	0.446	0.368	0.374
	Thymus (g)	0.240	0.260	0.221	0.113**	0.113**
	Brain (g)	1.87	1.88	1.88	1.82	1.82
	Ovaries (g)	0.074	0.073	0.077	0.081	0.094
	Thyroids (g)	0.020	0.014**	0.014**	0.016*	0.016
	Pituitary (g)	0.016	0.014*	0.016	0.014*	0.013**
	Adrenal (g)	0.070	0.065	0.062*	0.097**	0.091
	Liver (% body weight)	2.95	3.05	3.29**	3.83**	4.20**
	Thymus (% body weight)	0.089	0.101	0.086	0.050**	0.053**
	Kidneys (% body weight)	0.738	0.783	0.833*	0.944**	1.036**
	Thyroids (% body weight)	7.57	5.46**	5.42**	7.09	7.70
	Adrenals (% body weight)	26.5	25.2	24.2	43.4**	42.7*
	Adrenal (rel. to brain wt)	3.755	3.466	3.283*	5.363**	4.988

*significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Henck et al., 1987

Histopathology revealed the presence of basophilic material in the germinal follicles of mesenteric lymph nodes at dose levels of ≥ 177 mg/kg bw per day. An increase in spleen haemosiderin deposition was seen in males at dose levels of ≥ 177 mg/kg bw and in females at ≥ 500 mg/kg bw per day. An increase in the degree of mammary gland hyperplasia was seen in females administered 500 and 500→600 mg/kg bw per day.

Table 10. Histopathology in the 90-day study in rats receiving pyridate in the diet

Dose	Males					Females				
(mg/kg bw per day)	0	62.5	177	500	500/600	0	62.5	177	500	500/600
Mesenteric lymph nodes: basophilic material	0/20	0/20	2/19	8/17	4/9	0/20	0/19	1/20	6/14	2/6
(After recovery)	2/15	-	-	8/15	-	0/15	-	-	0/11	-
Spleen pigmentation (mean haemosiderin rank)	14.4	20.4	61.5	90.3	87.8	32.7	52.5	52.7	70.9	90.0
(After recovery)	46.9	-	-	-	80.9	45.0	-	-	-	59.3
Mammary hyperplasia (group mean rank) [a]	-	-	-	-	-	46.10	38.79	46.68	74.71**	88.33**

[a] All individual animal glands were ranked sequentially in order of increasing extent of hyperplasia then the group mean rank was calculated and the Fisher exact test used to test for intergroup differences.

*significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Henck et al., 1987

A NOAEL of 62.5 mg/kg bw per day was determined for this study based on premature mortality, clinical signs and histopathological findings at dose levels of greater than or equal to 177 mg/kg bw per day (Henck et al., 1987).

Study 4

In a second 90-day, dose escalation study in rats, pyridate (Batch no. 2759523, 11344/AR22; purity 92.51%) was administered in the diet for 13 weeks to groups of CD rats (ten/sex) at concentrations of 0, 400 and 1200 ppm. The study design was not compliant with current testing standards. Rats at an additional dietary concentration were treated for 13 weeks with increasing concentrations of 3600 ppm (weeks 1–6), 4600 ppm (weeks 7–8) and 8000 ppm (weeks 9–13). Based on observations at 8000 ppm, an additional group was fed 6000 ppm, with a pair-fed control group. Detailed histopathology was performed on all animals from the control and 6000 ppm groups. Appropriate statistical methods were used. There was no mortality and no clinical signs were observed which were considered to be related to the administration of pyridate. Overall weight gain was reduced in both sexes at the highest dietary concentrations (6000 ppm and 3600→4600→8000 ppm). Blood urea concentrations were elevated in both sexes at the higher dietary concentrations; urinary pH was significantly lower than in the control group and Hb levels were significantly decreased in females. Urinalysis revealed the presence of reducing substances in both sexes administered 3600→4600→8000 ppm. Mean liver and kidney weights were elevated in females at 600 ppm; mean liver weight was also higher in females administered 3600→4600→8000 ppm. These changes in organ weights lacked any histopathology correlates. Histopathology revealed mineral deposition in the mesenteric lymph nodes of both sexes administered 3600→4600→8000 ppm. Because of the dose escalation methodology the determination of a sub-chronic NOAEL was not considered to be appropriate (Danks, 1991).

Dog

Study 1

In a 90-day study, groups of Beagle dogs (four/sex) were administered pyridate (batch No. Knr: 2556520; purity 92%) in gelatine capsules at dose levels of 0, 20, 60 and 200 mg/kg bw per day. The study design was compliant with current test standards. Appropriate statistical methods were used. Premature mortalities occurred at the highest dose level of 200 mg/kg bw per day (three females, all males). Premature mortality was correlated with emesis, anorexia and dehydration. It was noted in the study report that the pyridate technical used in the study has a persistent foul mercaptan-like odor. Clinical signs were observed in all dose groups (Table 11). At 20 mg/kg bw per day, emesis was observed in one male and two females. At 60 mg/kg bw per day, clinical signs were observed in both sexes and included emesis (all animals), salivation (two males and two females), ataxia (one male and two females), mydriasis (three males and one female) and nystagmus (one female). Clinical signs observed at 200 mg/kg bw per day

included emesis, hypoactivity, opisthotonus, muscle fasciculations, ataxia, nystagmus and mydriasis (all animals); head swing (one male and one female); salivation (one female); and head tilt (two females). The clinical signs observed at 20 and 60 mg/kg bw per day were reversible. Clinical signs generally decreased in severity with increasing exposure period.

Table 11. Mortality and clinical signs in the 90-day study in dogs receiving pyridate by capsule

Dose (mg/kg bw per day)	Males				Females			
	0	20	60	200	0	20	60	200
Clinical signs								
Mortality	0	0	0	4	0	0	0	3
Emesis	0	1	4	4	0	2	4	4
Ataxia	0	0	1	4	0	0	2	4
Hypoactivity	0	0	0	4	0	0	0	4
Opisthotonus	0	0	0	4	0	0	0	4
Fasciculations	0	0	0	4	0	0	0	4
Head swing	0	0	0	1	0	0	0	1
Head tilt	0	0	0	0	0	0	0	2
Nystagmus	0	0	0	4	0	0	1	4
Mydriasis	0	0	3	4	0	0	1	4
Salivation	0	0	2	0	0	0	2	1

Source: Tomkins, 1987

Following 12 weeks of pyridate treatment mean body weights were reduced ($p > 0.05$; ≤ 6% compared with control animals) in male dogs administered 60 mg/kg bw per day and in the single surviving female dog administered 200 mg/kg bw per day. These changes were correlated with reduced body weight gains in these animals. Food consumption was reduced in both sexes at the highest dose level. Serum AST activity was reduced in females at 200 mg/kg bw per day. Serum ALT and AST activities were reduced in dogs treated at 60 and 200 mg/kg bw per day; significantly in some groups. Reductions in the activities of these serum enzymes are not considered to be of toxicological significance. A tendency to higher serum alkaline phosphatase activity seen in males at 200 mg/kg bw per day reflects the higher pretest value for this parameter and is not related to treatment. Plasma cholinesterase activity was reduced in females at 200 mg/kg bw per day. Erythrocyte cholinesterase activity was reduced in females at 200 mg/kg bw per day, however the value does not attain statistical significance ($p > 0.05$). Erythrocyte cholinesterase activity was significantly increased in males at 60 mg/kg bw per day, however the toxicological significance of this finding is unclear. Relative liver weights were generally higher in treated groups; findings do not consistently attain statistical significance but (in females) show a clear dose–response relationship. Myelin digestion chambers (minimal change) were observed in some of the pyridate-treated dogs. However these changes were not considered to represent a pyridate-associated effect and were not considered to be present in sufficient number and severity to be associated with the clinical signs observed in the study. A NOAEL of 20 mg/kg bw per day was determined for this study based on the presence of emesis and clinical signs consistent with an effect on the nervous system, seen at higher dose levels (Tomkins, 1987).

Study 2

In a second 90-day dog study, groups of Beagle dogs (five /sex) were administered pyridate (batch No. 2759523, 11.344/AR22; purity 92.51%) in gelatine capsules at dose levels of 0, 40, 80 and 120 mg/kg bw per day. The study was compliant with current test standards and appropriate statistical methods were used. No deaths occurred in this study. Relevant study findings are summarized in Tables 12, 13 and 14. Clinical signs (marked ataxia often leading to prostration, hypoactivity, opisthotonus, emesis, salivation, pallor, cold to touch, dry nose, tremor, hunched posture and vacant expression) were observed in the 80 mg/kg bw per day group and to a more marked extent in dogs administered 120 mg/kg bw per day pyridate. Clinical signs at 40 mg/kg bw per day were limited to head shaking in one male, observed following the first dose only. Clinical signs generally decreased in severity over the study period.

Table 12. Findings in the 90-day study in dogs receiving pyridate by capsule

Dose (mg/kg bw per day)	Males				Females			
	0	40	80	120	0	40	80	120
Clinical signs								
Emesis	0	0	0	5	0	0	1	5
Ataxia	0	0	1	5	0	0	4	5
Vacant expression	0	0	1	0	0	0	0	0
Hypoactivity	0	0	2	2	0	0	2	5
Opisthotonus	0	0	0	5	0	0	0	5
Hunched posture	0	0	0	5	0	0	3	5
Tremor	0	0	0	1	0	0	0	3
Head shaking	0	1	0	0	0	0	1	0
Mydriasis	0	0	0	0	0	0	2	0
Prostration	0	0	0	4	0	0	0	5
Salivation	0	0	2	5	0	0	2	5

Source: Vandaele, 1990

While weight gain was reduced in females at 120 mg/kg bw per day (correlated with reduced food consumption) no clear effect of treatment was seen on weight gain in males. Haematology revealed effects on red blood cell parameters: reduced packed cell volume (PCV), Hb concentration and erythrocyte count, and increased MCV. These effects were seen in both sexes at 80 and 120 mg/kg bw per day. An increase in the number of erythrocyte Heinz bodies was seen in dogs of both sexes at 80 and 120 mg/kg bw per day; mild reticulocytosis was also apparent at 120 mg/kg bw per day. Clinical chemistry showed a significant reduction in ALT activity in all treated groups, but this finding is not considered to be of toxicological significance. ALP and AST activities were not affected at any concentration. Higher albumin concentrations were seen in both sexes at the highest dose level. Mean absolute ovary weight was significantly higher in females at 120 mg/kg bw per day. Relative liver and kidney weights were increased in both sexes at 80 and 120 mg/kg bw per day.

Table 13. Findings in the 90-day study in dogs receiving pyridate by capsule

Dose (mg/kg bw per day)	Males				Females			
	0	40	80	120	0	40	80	120
Haematology: week 6								
PCV (%)	40	38	35**	36*	42	40	35**	35**
Hb (g/dL)	13.9	13.4	12.2**	13.1	14.5	13.9	12.2**	12.4**
RBC (10^6/µL)	5.91	5.62	5.05**	5.23**	6.19	5.82	5.10**	4.99**
MCV (fL)	67	67	68	70*	67	68	68	70*
MCH (pg)	24	24	24	25*	24	24	24	25*
Reticulocytes [a]	ND/0	1	2	4	ND/0	ND/0	1	1
Heinz bodies	ND/0	ND/0	ND/0	1	ND/0	ND/0	1	1
Platelets	225	240	303**	282*	257	234	294	289
Haematology: week 12								
PCV (%)	41	41	37	39	44	46	39*	41
Hb (g/dL)	14.4	14.3	13.2	13.9	15.5	15.7	13.6*	14.4
RBC (10^6/µL)	6.27	6.09	5.62*	5.65*	6.77	6.67	5.81**	5.97*
MCV (fL)	65	67	66	69***	65	68	67	69*

Dose (mg/kg bw per day)	Males				Females			
	0	40	80	120	0	40	80	120
MCH (pg)	23	24	23	24**	35	34	35	35
Reticulocytes [a]	ND/0	1	4	4	ND/0	1	1	2
Heinz bodies	ND/0	ND/0	4	5	ND/0	ND/0	1	5
Platelets	199	237	283***	273**	202	237	269*	268*
Clinical chemistry: week 6								
ALT (IU/L)	29	18***	10***	9***	31	22	11**	8***
AP (IU/L)	79	95	84	87	87	95	100	87
AST (IU/L)	30	30	26	25	27	30	27	22
TBil (mg/dL)	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4
Albumin (g/dL)	2.8	2.9	3	3.1*	2.8	2.9	3	3.3*
Clinical chemistry: week 12								
ALT (IU/L)	31	20***	11***	7***	32	22**	16***	9***
AP (IU/L)	68	73	68	73	66	69	62	68
AST (IU/L)	28	25	25	28	24	21	23	21
T. Bil (mg/dL)	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.3
Albumin (g/dL)	3.1	3.2	3.2	3.6***	3.3	3.5	3.5	4.1***
Organ weights								
Ovaries (g)	-	-	-	-	0.77	0.90	0.92	0.99**
Ovaries (% bw)	-	-	-	-	0.0065	0.0070	0.0076	0.0085**
Kidneys (% bw)	0.40	0.41	0.46**	0.46**	0.39	0.41	0.46**	0.49**
Liver (% bw)	2.98	3.03	3.38	3.61**	2.87	3.01	3.33**	3.73**

ND/0 Not detected or zero

[a] Reticulocytes > 2% of erythrocytes

Significantly different to controls: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Source: Vandaele, 1990

Histopathological changes were seen in the liver (increased Kupffer cell pigmentation) in both sexes administered 80 and 120 mg/kg bw per day pyridate. An increased cellularity of the sternal bone marrow was observed in dogs at 120 mg/kg bw per day and is considered to represent a physiological response to changes in red cell parameters. All other histopathological findings were considered to be consistent with the spectrum of changes commonly encountered in dogs of this age at this laboratory. A slightly elevated incidence of thyroid parafollicular cell hyperplasia was observed in females at 120 mg/kg bw per day; the background incidence of this finding is reported to be 0–100% (mean 8.22%). Sciatic nerve myelin digestion chambers were seen in dogs in the control and treated dose groups. This finding is not reported in the laboratory's historical control data (consisting of 16 studies starting between February 1988 and October 1990), however the pattern of incidence in this study does not suggest any relationship to treatment.

Table 14. Histopathology in the 90-day study in dogs receiving pyridate by capsule

Dose	Males				Females			
mg/kg bw per day	0	40	80	120	0	40	80	120
Clinical signs								
Brain focal gliosis	ND	ND	ND	1	ND	ND	ND	ND
Epididymides: aspermia	ND	ND	ND	1	-	-	-	-
Kupffer cell pigmentation	3	1	5	5	2	2	5	5
Bronchopneumonia	ND	ND	ND	1	ND	ND	1	1
Sciatic nerve myelin digestion	1	ND	ND	2	2	1	ND	1
Bone marrow increased cellularity	ND	ND	ND	4	ND	ND	ND	4
Testes: immaturity	ND	ND	ND	1	-	-	-	-
Parafollicular cell hyperplasia	2	ND	1	2	1	1	1	3

Source: Vandaele, 1990

A NOAEL of 40 mg/kg bw/day was determined for this study, based on clinical signs, changes in haematological parameters indicative of mild anaemia, increased relative liver and kidney weight and Kupffer cell pigmentation at the next highest dose level of 80 mg/kg bw per day (Vandaele, 1990).

Study 3

In a one-year dose-escalation study groups of Beagle dogs (five/sex) were administered pyridate (batch No. 2759523, LH No. 23, 293A&B; purity 91.5%) in capsules at ascending dose levels of 0, 5→10→30 mg/kg bw per day (low dose), 20→60→80→100 mg/kg bw per day (mid dose) and 60→100→120→140→150 mg/kg bw per day (high dose) for 52 weeks. Clinical signs (including salivation, ataxia, mydriasis, dyspnoea, tremors and an inability to stand) were observed to a mild to moderate extent in dogs of the mid-dose group and were more marked in dogs of the high-dose group. With the exception of emesis, no clinical signs were observed below a dose level of 100 mg/kg bw per day. Body weight loss (equivalent to 16% of initial body weight) was seen in mid-dose and high-dose animals from week 35 onwards, corresponding with the increase in dose level to 60 and 100→120 mg/kg bw per day, respectively. No treatment-related effects were noted on food consumption in dogs of any dose group. Haematological parameters were unaffected by treatment with pyridate. Clinical chemistry revealed a reduction in serum globulin concentration in females at the highest dose level, which was statistically significant only at week 52. A tendency to reduced serum ALT activity was apparent in both sexes at the highest dose level; values do not attain statistical significance and are not considered to be of toxicological significance. Mean absolute and relative organ weights were unaffected by treatment. Treatment-related histopathology was limited to degenerative myelopathy of the sciatic nerve and increased splenic extramedullary haematopoiesis in one male dog of the highest dose group. A one-year exposure NOAEL was not determined due to the use of a dose-escalation experimental design (Bailey, 1989).

(b) Dermal application

Rat

A three-week dermal limit dose toxicity study was performed. Groups of five male and five female Sprague Dawley rats were dermally exposed (non-occlusive) for 21 days to 0 or 1000 mg/kg bw per day of pyridate technical (batch No. 2759523, CL 11.344/AR 22) over approximately 10% of the total body surface area. There were no observed systemic adverse effects that were attributable to the presence of pyridate. Slight skin reactions (scab formation, reddened skin) were observed at the dosing sites (Perry & Duffen, 1988).

(c) Exposure by inhalation

No study submitted

2.3. Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of pyridate has been evaluated in an 18 month carcinogenicity study in the mouse and in a 121-week study in the rat. The rat study included interim sacrifice cohorts at 52, 104 and 121 weeks.

Mouse

In a carcinogenicity study that was generally compliant with current test standards, groups of B6C3F1 mice (50/sex) were administered pyridate (batch No. 2759523, CL 11344/AR-22 #2659427; purity 91.5%) in the diet for 18 months. The initial dietary concentrations of pyridate used in this study were 0, 400, 800 and 1200 ppm; the highest concentration was increased to 1400 ppm on day 91 and was subsequently further increased to 1600 ppm on day 179. Immediately following this study, an additional study was undertaken in which groups of mice were administered pyridate at dietary concentrations of 0 or 7000 ppm for periods of up to 18 months. The dietary concentrations were calculated to be equal to mean achieved pyridate intakes of 0, 48, 98, 170 and 853 mg/kg bw per day in males, 0, 55, 115, 204 and 1045 mg/kg bw per day in females. Histopathological assessments were performed on all animals in the top-dose and control groups; investigations in the low- and mid-dose groups were limited to unscheduled deaths and gross lesions. Relevant study findings are summarized in Tables 14 and 15. A higher level of mortality was apparent in female mice administered pyridate at dietary concentrations of 1200→1400→1600 ppm and at 7000 ppm. Increased mortality was also observed in males at the highest dose level. There were no treatment-related clinical signs. Mean body weights were lower than controls in all groups of treated mice, but findings at the lower concentrations did not show a dose–response relationship. Mean body weights were considered to have been clearly reduced by treatment with pyridate in female mice at 1200→1400→1600 ppm and in both sexes at 7000 ppm. Food consumption was unaffected by treatment with pyridate. No treatment-related effects were observed on haematological parameters.

Table 15. Mortality and body weight in the carcinogenicity study in mice receiving pyridate in the diet

Dose (ppm)		0	0	400	800	1200→1400→1600	7000
Males	Mortality	2/50	2/50	2/50	1/50	0/50	3/50
	Initial body weight (g)	20.7	21.7	21.4	21.1	21.1	21.1
	Terminal body weight (g)	40.7	43.2	38.5	37.3	37.3	37.2
	(% control)	-	-	(95%)	(92%)	(92%)	(86%)
Females	Mortality	3/50	3/50	3/50	4/50	7/50	10/50
	Initial body weight (g)	17	17.1	17.6	16.4	16.9	17
	Terminal body weight (g)	41	45.1	39.1	38.7	35.6	34
	(% control)	-	-	(95%)	(94%)	(87%)	(75%)

Source: Lindamood, Coyne & Thompson, 1991

Mean relative liver weight was increased in both sexes administered pyridate at dietary concentrations of 1200→1400→1600 and 7000 ppm. Absolute adrenal weight was significantly reduced in males at 1200→1400→1600 ppm, however a similar finding was not apparent in males at 7000 ppm. A number of histopathological findings were observed with increased incidence in mice at the highest dose level including liver eosinophilic foci (in both sexes), liver haematopoietic cell proliferation (females), renal proteinaceous casts (females), alveolar epithelial hyperplasia (females), thymic necrosis (females) and suppurative inflammation (females). The incidence of hepatocellular carcinoma was slightly increased (not significant, $p > 0.05$) in males at 7000 ppm.

Table 16. Terminal findings in the carcinogenicity study in mice receiving pyridate in the diet

	Dose (ppm)	0	0	400	800	1200→1400 →1600	7000
Males	Adrenal weight (mg)	12.7	7.6	12.5	1.09	9.2*	11.4
	Adrenal weight (‰)	0.3	0.2	0.3	0.3	0.2	0.3
	Liver weight (‰)	45.9	49.2	43.1	49.3	51.1	55.7
	Adrenal: capsular hyperplasia	0/50	-	0/2	0/1	-	1/50
	Adrenal: focal hyperplasia	0/50	-	0/2	0/1	-	1/50
	Adrenal: focal hypertrophy	13/50	-	0/2	0/1	-	3/50
	Liver: basophilic focus	3/50	-	3/50	1/50	-	4/50
	Liver: eosinophilic focus	1/50	-	0/50	0/50	-	3/50
	Salivary gland: atrophy	3/50	-	0/2	0/1	-	5/50
	Adrenal: cortical adenoma	0/50	-	0/2	0/1	-	1/50
	Adrenal: phaeochromocytoma	0/50	-	0/2	0/1	-	1/50
	Liver: hepatocellular adenoma	7/50	-	12/50	10/50	-	5/50
	Liver: hepatocellular carcinoma	5/50	-	7/50	7/50	-	11/50
	Lung: malignant lymphoma	0/50	-	0/50	0/50	-	1/50
	Mediastinal lymph node: malignant	-	-	0/1	-	-	1/3
	Pancreas: haemangiosarcoma	0/50	-	0/2	0/2	-	1/50
Females	Adrenal weight (mg)	11.8	13.8	12.2	13.4	12.4	11.3
	Adrenal weight (‰)	0.3	0.3	0.3	0.4*	0.3	0.3
	Liver weight (‰)	45.4	38.6	43.7	47.5	50.0	55.1*
	Adrenal: fatty zone degeneration	0/50	-	0/3	0/3	-	1/50
	Kidney: cast proteins	11/50	-	15/50	9/49	-	19/50
	Kidney: tubular dilatation	0/50	-	0/50	1/49	-	3/50
	Liver: eosinophilic focus	0/50	-	0/50	0/49	-	3/50
	Liver: haematopoietic cell proliferation	1/50	-	2/50	1/49	-	6/50
	Lung: alveolar hyperplasia	1/50	-	2/49	1/49	-	4/50
	Lung: subacute inflammation	0/50	-	0/49	0/49	-	1/50
	Pancreas: suppurative inflammation	0/50	-	2/4	-	-	5/50
	Pancreas: necrosis	0/50	-	0/4	-	-	1/50
	Thymus: necrosis	0/49	-	0/3	0/3	0/3	3/48
	Ovary: suppurative inflammation	1/49	-	3/14	2/11	-	10/50
	Liver: hepatocellular adenoma	6/50	-	4/50	3/49	-	8/50
	Liver: hepatocellular carcinoma	7/50	-	0/50	1/49	-	2/50

*significantly different to controls, $p < 0.05$

Source: Lindamood, Coyne & Thompson, 1991

A NOAEL for toxicity of 800 ppm (equivalent to 98 mg/kg bw per day in males and 115 mg/kg bw per day in females) was determined for this study based on lower bodyweights and increased mortality in females at the dietary concentration of 1200→1400→1600 ppm, equivalent to 204 mg/kg bw per day. The NOAEL for carcinogenicity was 1200→1400→1600 ppm (equivalent to 170 mg/kg bw per day (Lindamood, Coyne & Thompson, 1991).

Rat

In a combined chronic toxicity and carcinogenicity study that was compliant with current test standards groups of Wistar rats (75/sex) were administered pyridate (batch No. 1001410, CL 11344; purity 90.3%) in the diet at concentrations of 0, 80, 400 or 2500 ppm (equivalent to 0, 4, 20 and 125 mg/kg bw per day) for periods of up to 121 weeks. Interim sacrifices were made following treatment for 52 weeks (10 rats/sex per group) and 104 weeks (15 rats/sex per group). All remaining rats were sacrificed following treatment for 121 weeks. The statistical methods used were appropriate. Relevant study findings are presented in Table 16. There was no treatment-related mortality and no clinical signs were observed that were attributable to treatment with pyridate. Mean body weights and food consumption were transiently decreased in rats at the highest dose level at the beginning of the study. Mean body weights of male rats administered 2500 ppm pyridate were generally slightly lower than controls from approximately six months of treatment onwards with values occasionally attaining statistical significance. A more marked effect of treatment was seen in females at 2500 ppm; mean body weights in this group were lower than controls from approximately 13 weeks of treatment and were significantly lower at the majority of time points. No consistent effects on body weight were observed at lower dietary concentrations. Food consumption was generally slightly lower in both sexes at 2500 ppm; findings were more notable in females. Haematology revealed effects on red blood cell parameters (reduced Hb concentration, erythrocyte count and PCV) consistent with mild anaemia in female rats at the highest dietary concentration of 2500 ppm. Total leucocyte counts were also reduced in females in this group. Clinical chemistry analyses showed transient decreases in serum AST, ALP and lactate dehydrogenase (LDH) activities in rats at 2500 ppm; these findings are not considered to be of clear toxicological significance. Serum LDH activity was significantly increased in top-dose males at week 121. T_3 uptake was significantly decreased at week 5 in males of the mid- and high-dose groups; similar effects were not seen at later time points. Mean relative kidney weight was significantly increased in males at the highest dose level at the 52-week interim sacrifice. Mean relative thyroid weights were significantly lower in males of all treated groups after one year of treatment, but without a clear dose–response relationship. No effects were observed on mean absolute or relative organ weights at the end of the study. Necropsy did not reveal any effects of treatment. Histopathological investigations did not indicate any effects attributable to treatment with pyridate.

Table 17. Findings in the carcinogenicity study in rats receiving pyridate in the diet

	Males				Females			
Dose (ppm)	**0**	**80**	**400**	**2500**	**0**	**80**	**400**	**2500**
Body weight (g) Day 0	81.2	81.5	81.1	81.2	72.6	72.8	72.2	72.7
Body weight (g) Day 182	426.9	433.2	418.8	409.4	239.6	237.1	235.8	220.4**
Body weight (g) Day 364	480.7	485.8	481.5	465.1	274.8	275.5	271.4	252.2**
Body weight (g) Day 686	485.5	494.4	470.3	446.0*	283.9	307.4	311.8*	250.1**
Body weight (g) Day 845	455.3	419.8	420.2	479.2	282.4	295.5	291.1	281.1
Food intake (g) Day 7	109.1	105.8	108.6	95.0***	92.2	89.7	93.3	85.5*
Haematology: week 79								
Haemoglobin (mM)	8.7	9.3	9.3	9.3	10.1	9.2**	10.0	9.0**
Packed cell volume (L/L)	0.458	0.489**	0.488	0.484	0.528	0.482**	0.529	0.485**
RBC (10^{12}/L)	7.2	7.5	7.4	7.3	7.6	6.9**	7.5	6.8**
WBC (10^9/L)	17.1	19.9	18	16.7	15.9	12.7	13.7	12**
Haematology: week 120								
Haemoglobin (mM)	9.2	9.5	8.9	9.3	9.6	8.6**	9.2	9.1
Packed cell volume (L/L)	0.478	0.491	0.468	0.466	0.505	0.441**	0.463**	0.468**
RBC (10^{12}/L)	7.2	7.5	7.2	7.5	7.1	6.2**	6.7	6.3**
WBC (10^9/L)	17.8	18.7	19.9	19.6	16.8	12.2**	16.3	13.4**

	Males				Females			
Dose (ppm)	0	80	400	2500	0	80	400	2500
Clinical chemistry: week 5								
AST (U/L)	59.2	59.8	60.7	57.8	56.3	51.7	51.8	49.9**
LDH (U/L)	123.6	142	131.7	94.3**	90.8	69**	78.1	90.5
T_3 uptake (%)	50.1	49.4	48.6**	47.5**	49.2	49.5	47.4	47.8
T_4 (nM)	72.2	68.0	70.7	65.8	47.0	53.4	47.2	43.0
Clinical chemistry: week 53								
ALP (U/L)	82.4	76.3	77.8	66.4**	66.5	67	60.8	47.9**
AST (U/L)	54.1	63.5	59.4	49.4**	65.8	57	64.8	48.5**
LDH (U/L)	132.5	146.1	125.8	129.5	128.9	83.6	100.8	72.6**
T_3 uptake (%)	51.9	50.7	54.1	53.1	54.3	55.4	56.3	52.3
T_4 (nM)	55.9	58.5	57.4	63.7	40.8	41.6	46.3	47.7
Clinical chemistry: week 121								
ALP (U/L)	98.2	104.8	97.0	95.9	88.5	72.1	91.6	91.1
AST (U/L)	69.0	66.7	69.9	69.4	99.7	94.4	103.9	88.8
LDH (U/L)	495.6	550.4	593.1	755.8**	502.5	434.2	488.1	495.6
T_3 uptake (%)	40.4	39.6	42.4	41.3	43.3	44.8	42.5	43.6
T_4 (nM)	31.2	23.9	27.7	33.4	27.8	30.1	28.3	27.3
Organ weights								
Kidney (‰) Week 52	5.3	5.6	5.8	6.0*	6.4	6.4	6.2	6.6
Thyroid (‰) Week 52	0.080	0.063*	0.053*	0.054*	0.080	0.075	0.072	0.089
Kidney (‰) Week 121	7.51	8.38	8.46	6.84	7.83	7.33	7.74	7.66
Thyroid (‰) Week 121	0.079	0.089	0.084	0.077	0.100	0.100	0.100	0.095

*significantly different to controls: $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Source: Til, Woutersen & Bosland, 1990

A NOAEL of 400 ppm (equivalent to 20 mg/kg bw per day) was determined for this study based on body weight effects in both sexes and changes in erythrocyte parameters in females at the highest dietary concentration of 2500 ppm, equivalent to 125 mg/kg bw per day (Til, Woutersen & Bosland, 1990).

2.4. Genotoxicity

Based on the weight of evidence, pyridate shows no genotoxicity in vitro or in vivo(see Table 18 for summary of results).

(a) In vitro studies

Pyridate does not induce reverse mutations via base pair substitutions or frame shifts in plate-incorporation and preincubation bacterial reverse mutation assays using DNA repair-deficient and wild-type *Salmonella typhimurium* and *Escherichia coli* strains. Based on an obsolete test methodology, pyridate does not induce unscheduled DNA synthesis in primary rat hepatocytes in the absence of an exogenous metabolic activation system. Pyridate does not induce chromosomal aberrations in Chinese Hamster Ovary cells in vitro. However this study was not performed in a manner that was consistent with current test standards. Based on weight of evidence pyridate shows no genotoxicity in vitro.

(b) In vivo studies

Pyridate does not induce micronuclei in Swiss random mice and does not induce unscheduled DNA synthesis in male Fisher 344 rat hepatocytes. Neither of these studies were compliant with current test standards. Based on an obsolete test method, pyridate does not induce somatic cell mutation in the embryos of C57B1/6J female mice bred with T-strain male mice. Based on the weight of evidence, pyridate shows no genotoxicity in vivo.

Table 18. Overview of genotoxicity testing with pyridate [a]

Test	Target	Concentration or dose tested	Purity (%)	Result	Reference
In vitro					
DNA damage in bacteria	*B. subtilis* H17 and M45	1–10 000 µg/plate (± S9)	92% (batch 2556520)	Negative	Hoorn, 1986c
Gene mutation in bacteria [a,b,e]	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	1–10 000 µg/plate (±S9) in DMSO; precipitation ≥ 39.06 µg/plate	92% (batch 2556520)	Negative	Hoorn, 1986a
Gene mutation in bacteria [a,b,e]	*E. coli* WP2 uvrA	1–10 000 µg/plate (±S9) in DMSO; precipitation at ≥ 39.06 µg/plate	92% (batch 2556520)	Negative	Hoorn, 1986b
Gene mutation in bacteria [a,b,f]	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	10–3160 µg/plate (±S9) in DMSO; cytotoxicity and precipitation at ≥ 3160 µg/plate	90.9% (batch H1009002)	Negative	Flügge, 2012
UDS assay [a,c,d,e]	Rat hepatocytes	3.91–500 nL/mL in DMSO; cytotoxicity at > 7.81 ng/mL	90.3%	Negative	Myhr & Brusick, 1981
Chromosomal aberration [a,b,e,g]	Chinese hamster ovary (CHO) cells	5–50 µg/mL (−S9) in DMSO; cytotoxicity at ≥ 50 µg/mL 10–100 µg/mL (+S9) in DMSO; cytotoxicity at > 50 µg/mL Turbidity at > 20 µg/mL	92% (batch 2556520)	Negative	Taalman, 1986a
In vivo					
Micronucleus test [a,b,e,h]	Male and female Swiss mice	400, 1300 and 4000 mg/kg bw; PO in corn oil; mortality at ≥ 400 mg/kg bw	92% (batch 2556520)	Negative	Taalman, 1986b
UDS assay [a,b,f,i]	Male F344 rats	40, 160 and 800 mg/kg bw; PO in corn oil; mortality at 800 mg/kg bw	91.5% (batch 2759523)	Negative	Curren, 1988
Spot test [a,c,d,e]	Female C57B1/6J and male T-strain mice	73, 242 and 725 mg/kg bw (destation days 9–11); PO, in corn oil	90.3%	Negative	Nguyen & Brusick, 1980

DMSO Dimethyl sulfoxide UDS Unscheduled DNA synthesis

S9 9000 × *g* supernatant fraction from rat liver homogenate;

[a] Dose ranging was used to establish the test assay dose range; assays were validated by the use of positive and solvent and/or negative controls

[b] Conducted in accordance with good laboratory practices

[c] Not conducted according to good laboratory practices

[d] No batch number supplied

[e] No certificate of analysis provided

[f] Certificate of analysis provided

[g] Insufficient cells scored; 200 cells per concentration were scored; current standards require that at least 300 well-spread metaphases should be scored per concentration and control to conclude a test chemical as clearly negative

[h] Insufficient cells scored; 1000 cells were scored; current standards require that at least 4000 immature erythrocytes per animal should be scored

[i] Insufficient cells scored; 50 cells/animal were scored; current standards require that at least 100 cells/animal should be scored

2.5. Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

Groups of Wistar (Cpb:Wu) rats (20/sex) were exposed to pyridate (batch 1001410; purity 90.3%) in the diet at concentrations of 0, 80, 400 and 2500 ppm (equivalent to 0, 5, 26 and 165 mg/kg bw per day respectively) prior to and throughout mating, gestation and lactation over three successive generations. Two litters were produced from each generation. In addition a four-week feeding study was conducted with weanling rats of the F3b generation. Blood samples collected from ten F1 rats/sex per group in week 61 were investigated for differential white blood cell count. Further blood samples taken from ten rats/sex per group of the F1 and F2 generations were investigated for chloride and potassium (F1) and LDH (F1 and F2). Histopathology was performed on control and high-dose parental animals of the F2 generation, and on the pituitaries of all F0 animals. Relevant study findings are summarised in Tables 18, 19 and 20. There was no treatment-related mortality and no clinical signs were observed. Reduced weight gain resulted in significantly lower mean body weights in F0 and F1 females administered 2500 ppm pyridate. Food consumption was variable but was significantly reduced in males at 2500 ppm, with some consistency. Haematology did not reveal any effects on leucocyte counts in F1 rats. No treatment-related effects were apparent on serum chloride or potassium concentrations. LDH levels in all pyridate-treated groups of F1 males were significantly lower than controls, however a dose–response relationship is not apparent at 80 or 400 ppm and the control value appears to be high. This finding is not considered to be of toxicological significance in the absence of similar effects in F2 males or in females of either generation.

Table 19. Parental findings in the three-generation reproductive toxicity study with pyridate

	Males				Females			
Dose (ppm)	**0**	**80**	**400**	**2500**	**0**	**80**	**400**	**2500**
F0 parents								
Body weight (g) Day 0	81.4	81.2	81.9	81.9	65.5	66.8	67.0	68.7
Body weight (g) Day 84	337.2	332.6	334.2	319.5	199.2	198.7	199	190.3
Body weight (g) Day 170	-	-	-	-	306.7	300.2	296.2	268.7**
Food intake (g/week) Day 7	106.9	105.0	101.8	94.4	83.8	84.2	88.5	81.2
Food intake (g/week) Day 84	120.1	113.0*	114.6*	110.1**	83.6	85.6	85.4	82.4
F1 parents								
Body weight (g) Day 0	83.0	81.9	75.2	69.0	76.5	73.9	69.9	63.5
Body weight (g) Day 84	374.8	374	349.5	339.5	220.3	212.9	211.2	203.4
Body weight (g) Day 170	-	-	-	-	334.9	318.7	324	301.1
Food intake (g/week) Day 7	116.9	110.1	105.9	93.6**	97.6	88.0	93.6	86.6
Food intake (g/week) Day 84	132.5	131.7	125.5	121.1	95.6	89.5	90.4	87.9
F2 parents								
Bodyweight (g) Day 0	43.3	45.1	44.7	43.4	40.9	42.0	42.0	40.4
Bodyweight (g) Day 84	322.0	311.5	318.5	319.2	198.9	196.2	191.2	200.9
Body weight (g) Day 170	-	-	-	-	300	291.6	286.5	292.2
Food intake (g/week) Day 7	77.5	60.1**	72.4	65.2**	63.6	52.4**	58.1	57.3
Food intake (g/week) Day 84	118.4	107.0	117.3	116.0	89.3	86.7	81.7	91.5

	Males				Females			
Dose (ppm)	**0**	**80**	**400**	**2500**	**0**	**80**	**400**	**2500**
F3 offspring								
Body weight (g) Day 0	41.4	39.2	39.0	38.2	41.2	40.7	37.3	37.7
Body weight (g) Day 28	180.7	177.6	174.3	168.1	132.4	128.5	127.6	127.4
Food intake (g/week) Day 7	55.6	50.1**	51.9**	50.1**	47.5	51.8**	51.7**	50.1**
Food intake (g/week) Day 28	139.1	139.4	135.5	139.8	102.6	99.8	99.2	101.9
Clinical chemistry								
F1 LDH (U/L)	344.3	225.9*	223.5*	175.3*	116.4	111.4	116.7	92.7
F2 LDH (U/L)	179.6	137.7	192.8	135.8	132.4	145.1	152.8	127.8

*significantly different to controls, $p < 0.05$ ** $p < 0.01$

Source: Til, Wouterson & Falke, 1982

A slight (but statistically significant) increase in mean relative kidney weight was observed at 2500 ppm in both sexes of all generations, at 400 ppm in F1 and F2 females and at 400 ppm in F2 males. A dose-related decrease in relative thyroid weight was observed in F1 males at 400 and 2500 ppm. Mean relative liver weight was significantly increased at 2500 ppm in F1 females and F2 males. Gross necropsy did not reveal any effects of treatment in parental animals of any generation. Marginal increases in the incidence of liver single cell necrosis and splenic extramedullary haematopoiesis were observed at 2500 ppm in F2 parental animals. Histopathology of the pituitaries of F1 parental animals did not reveal any effects of treatment. Gross necropsy did not reveal any effects of treatment in offspring of any generation. Histopathology of the pituitaries of F1 offspring did not reveal any effects of treatment.

Table 20. Organ weights and histopathology in the reproductive toxicity study with pyridate

	Males				Females			
Dose (ppm)	**0**	**80**	**400**	**2500**	**0**	**80**	**400**	**2500**
F1 parents								
Thyroid weight (g)	0.029	0.028	0.023	0.023	0.024	0.024	0.023	0.023
Thyroid weight (‰)	0.058	0.056	0.049	0.052	0.085	0.087	0.087	0.090
Kidney weight (g)	2.75	2.82	2.67	2.74	1.82	1.76	1.77	1.77
Kidney weight (‰)	5.49	5.61	5.59	5.98**	6.35	6.43	6.57	6.93**
Liver weight (g)	14.89	15.50	13.83	13.99	8.68	8.44	8.35	8.07
Liver weight (‰)	29.7	30.0	28.9	30.5	30.1	30.8	30.9	31.6
F2 parents								
Thyroid weight (g)	0.028	0.026	0.022	0.022	0.020	0.022	0.017	0.022
Thyroid weight (‰)	0.068	0.065	0.056**	0.053**	0.084	0.091	0.074	0.091
Kidney weight (g)	2.29	2.29	2.26	2.57	1.59	1.65	1.68	1.77
Kidney weight (‰)	5.53	5.78	5.76	6.31**	6.54	6.74	7.11**	7.29**
Liver weight (g)	12.48	11.92	11.63	12.80	7.80	7.96	7.71	8.65
Liver weight (‰)	30.1	30.0	29.4	31.3	31.9	32.5	32.6	35.6**

(continued on next page)

	Males				Females			
Dose (ppm)	0	80	400	2500	0	80	400	2500
F3								
Thyroid weight (g)	0.015	0.015	0.017	0.015	0.016	0.018	0.016	0.016
Thyroid weight (‰)	0.081	0.085	0.099	0.088	0.123	0.131	0.122	0.120
Kidney weight (g)	1.50	1.54	1.55	1.52	1.19	1.21	1.27	1.29
Kidney weight (‰)	8.27	8.67	8.88*	9.07**	8.96	9.18	9.76*	9.84**
Liver weight (g)	8.88	8.89	8.57	8.89	6.07	6.14	6.17	6.50
Liver weight (‰)	49.1	50.0	49.2	52.8*	45.7	46.3	47.5	49.3
Liver: single hepatocellular necrosis, minimal/slight	3/10	-	-	5/10	6/10	-	-	6/10
Liver: single cell necrosis, slight	0/10	-	-	1/10	0/10	-	-	0/10
Spleen: extramedullary haematopoiesis,								
minimal/slight	7/10	-	-	8/10	4/10	-	-	5/10
moderate	0/10	-	-	1/10	0/10	-	-	0/10

*significantly different to controls, $p < 0.05$ ** $p < 0.01$

Source: Til, Wouterson & Falke, 1982

No treatment-related effects were observed on the duration of gestation period, fertility and gestation indices or offspring sex ratio. Lactation index was slightly (but significantly) lower in F3a pups at 400 and 2500 ppm; however values across all generations are variable and there is no indication of a treatment-related effect in other generations. Mean pup weight at 2500 ppm was significantly lower in all generations at the first mating.

Table 21. Reproductive and offspring parameters in the reproductive toxicity study with pyridate

	Dose (ppm)	0	80	400	2500
F1a	Litter size (*N*)	9.8	10.9	11.1	10.3
	Viability index (%)	100	100	100	99
	Lactation index (%)	92	98**	98*	95
	Pup weight (g) Day 1	5.9	5.9	5.6	5.8
	Pup weight (g) Day 14	27.8	27.6	27.4	24.6**
	Pup weight (g) Day 21	40.9	40.9	41.2	36.2**
F1b	Litter size (*N*)	10.4	10.4	10.9	9.6
	Viability index (%)	100	95	90	100
	Lactation index (%)	81	94***	92**	91**
	Pup weight (g) Day 1	5.9	6.0	5..6	5.9
	Pup weight (g) Day 14	28.5	29.2	27.4	27.6
	Pup weight (g) Day 21	43.8	42.9	40.7	40.1
F2a	Litter size (*N*)	9.9	10.6	10.7	10.7
	Viability index (%)	90	100***	99***	99***
	Lactation index (%)	91	97*	97*	91
	Pup weight (g) Day 1	5.8	6.0	6.0	6.0
	Pup weight (g) Day 14	27.7	25.9	26.4	24.2**
	Pup weight (g) Day 21	40.2	36.5**	38.6	34.5**

	Dose (ppm)	0	80	400	2500
F2b	Litter size (*N*)	11.3	10.4	10.3	10.5
	Viability index (%)	100	100	96**	100
	Lactation index (%)	95	97	100**	99*
	Pup weight (g) Day 1	5.9	6.0	6.1	6.0
	Pup weight (g) Day 14	24.6	25.9	26.9**	25.7
	Pup weight (g) Day 21	38.3	38.8	39.7	38.1
F3a	Litter size (*N*)	10.4	10.5	10.4	10.5
	Viability index (%)	98	98	99	100
	Lactation index (%)	90	85	71***	76***
	Pup weight (g) Day 1	5.7	5.7	5.6	5.5
	Pup weight (g) Day 14	20.4	18.6	19.1	17.1**
	Pup weight (g) Day 21	30.9	27.7**	28.4	27.1**
F3b	Litter size (*N*)	10.3	10.4	10.3	10.1
	Viability index (%)	96	83***	91*	92
	Lactation index (%)	81	76	85	90**
	Pup weight (g) Day 1	5.8	5.9	6.0	6.0
	Pup weight (g) Day 14	25.5	24.2	24.5	24.7
	Pup weight (g) Day 21	36.0	36.5	34.4	34.6

*significantly different to controls, $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Source: Til, Wouterson & Falke, 1982

A parental NOAEL of 400 ppm (equivalent to 26 mg/kg bw per day) was determined for this study. In the absence of any effects on fertility or reproductive performance, a reproductive NOAEL of 2500 ppm (equivalent to 165 mg/kg bw per day) was determined for this study. An offspring NOAEL of 400 ppm (equal to 26 mg/kg bw per day) was determined, based on reduced pup weights at the highest dose level of 2500 ppm (equivalent to 165 mg/kg bw per day) (Til, Wouterson & Falke, 1982).

(b) Developmental toxicity

Rat

In a prenatal developmental toxicity study, groups of pregnant female Wistar Han rats were administered pyridate (batch 2420966; purity 92%) in aqueous CMC at gavage dose levels of 0 (35 rats), 55, 165, 400 and 495 mg/kg bw per day, (25 rats/group) on gestation days (GDs) 6–15. Deaths occurred at 400 mg/kg bw per day (five rats after the first dose) and at 495 mg/kg bw per day (16 deaths in total; 13 following the first dose). Relevant findings for the study, which was not compliant with current test standards, are shown in Table 21. Clinical signs (ventral body position, dyspnoea, sedation, somnolence, unresponsiveness, tonic and clonic muscle spasms, piloerection) were noted in rats treated with 400 and 495 mg/kg bw per day. The clinical signs were most pronounced on the first day of dosing and generally decreased in incidence and severity as the study progressed, with the exception of decedent animals. No signs of toxicity were observed after GD 11. Bodyweight gain was reduced at dose levels of greater than or equal to 165 mg/kg bw per day, but without a clear dose–response relationship. Food consumption was reduced at dose levels of greater than or equal to 400 mg/kg bw per day. The numbers of corpora lutea, implantations and the proportions of pre- and post-implantation loss were comparable in all groups. Mean pup weights were slightly decreased at ≥ 400 mg/kg bw per day; findings were associated with an increased incidence of incomplete fetal skeletal ossification. Skeletal abnormalities were also marginally increased at greater than or equal to 400 mg/kg bw per day, and included absent thoracic vertebral body 1, absent sternebra 5 and/or 6, wavy ribs, bipartite cervical vertebra 1, bipartite sternebra 1 and irregularly ossified sternebrae 3 and 4. There was no evidence of treatment-related malformations.

Table 22. Findings in the rat developmental toxicity study with pyridate

Dose (mg/kg bw per day)	0	55	165	400	495
Mated (*N*)	35	25	25	25	25
Mortality (*N*)	0	0	0	5	16
Non pregnant (*N*)	0	1	2	1	0
Litters (*N*)	35	24	23	19	9
Corrected weight gain (g) (% control)	9.7 -	9.3 (96%)	8.2 (85%)	8.6 (89%)	4.0 (41%)
Body weight gain (g) (% control)	22.2 -	21.0 (94%)	18.6 (84%)	19.2 (86%)	9.33 (42%)
Food consumption (g) GD 6–11	21.3	22.2	20.9	16.1	15.9
Food consumption (g) GD 11–16	23.6	23.4	23.5	21.9	19.3
Corpora lutea (*N*)	13.5	13.0	13.5	13.4	13.1
Implantations (*N*)	12.1	12.4	13.0	11.3	12.3
Pre-implantation loss (*N*)	1.3	0.6	0.5	2.1	0.8
Post-implantation loss (*N*)	1.0	0.6	1.1	0.6	0.8
Litter size (*N*)	11.2	11.8	12.0	10.7	11.6
Fetal weight (g)	4.8	4.9	4.8	4.6	4.4
Delayed ossification (%)	2	0.7	1.4	4.9	13.5

Significantly reduced ($p < 0.05$) food consumption occurred in the 400 and 495 mg/kg bw per day groups compared with the control group between GD 6 and 16

Significantly reduced ($p < 0.05$) body weights occurred in the 400 and 495 mg/kg bw per day groups compared with the control group between GD 10 and 21 with significantly reduced ($p < 0.05$) body weight gain compared with the control group in the 495 mg/kg bw per day group

Significantly reduced ($p < 0.05$) mean fetal body weight occurred in pups born to dams dosed at 400 or 495 mg/kg bw per day compared with those born to the control group dams;

There were no other statistically significant differences between the treated and control groups.

Source: Becker et al., 1986

A maternal NOAEL of 165 mg/kg bw per day was determined for this study, based on body weight effects and clinical signs at 400 and 495 mg/kg bw per day. A developmental NOAEL of 165 mg/kg bw per day was determined, based on reduced fetal weight and skeletal abnormalities seen at 400 and 495 mg/kg bw per day (Becker et al., 1986).

Rabbit

In a rabbit prenatal developmental toxicity study groups of 20 inseminated female New Zealand White rabbits were administered undiluted pyridate (batch 2659427; purity 93.1%) at gavage dose levels of 0 (water), 150, 300 and 600 mg/kg bw per day from GDs 7–19. The study was not compliant with current test standards. Relevant study findings are shown in Tables 22 and 23. There was no treatment-related mortality. The incidence of abortion was significantly increased at 600 mg/kg bw per day; one animal aborted at 300 mg/kg bw per day but this was not considered to be clearly related to treatment. An increased incidence of clinical signs (dried or absent faeces) was apparent in rabbits administered 600 mg/kg bw per day pyridate. Body weight gain and food consumption was reduced over GDs 7 to 20 at 600 mg/kg bw per day. A reduced mean maternal body weight was observed over GDs 17 to 29 at 600 mg/kg bw per day.

Table 23. Maternal findings in the rabbit developmental toxicity study with pyridate

Dose (mg/kg bw per day)	Controls	150	300	600
Inseminated (*N*)	20	20	20	20
Pregnant (*N*)	19	19	18	18
Aborted (*N*)	0	0	1	4**
Deaths (*N*)	1	2	0	1
Premature delivery (*N*)	0	0	0	1
Total resorption (*N*)	0	0	2	0
Litters examined (*N*)	18	17	15	12
Dried faeces (days/rabbits)	0/0	0/0	7/1	39/9
No faeces (days/rabbits)	0/0	0/0	0/0	12/3
Body weight (kg) GD 0	3.75	3.72	3.72	3.69
Body weight (kg) GD 7	3.92	3.98	3.98	3.86
Body weight (kg) GD 20	4.16	4.09	4.06	3.68**
Body weight (kg) GD 29	4.18	4.18	4.17	3.78**
Body weight change (kg) GD 0–7	+0.17	+0.15	+0.18	+0.17
Body weight change (kg) GD 7–20	+0.23	+0.21	+0.15	−0.17**
Body weight change (kg) GD 20–29	+0.02	+0.08	+0.11	+0.02
Food consumption (g) GD 7–20	165.1	161.4	157.5	92.2**
Food consumption (g) GD 20–29	110	125	120.1	80.0
Corpora lutea (*N*)	9.9	10.3	9.6	9.0
Implantations (*N*)	8.4	7.9	6.4	7.8
Litter size (*N*)	7.7	7.2	5.9	7.2
Early resorption (*N*)	0.6	0.4	0.4	0.2
Late resorption (*N*)	0.2	0.4	0.0	0.2

*significantly different to controls, $p < 0.05$ ** $p < 0.01$

Source: Hoberman, 1987

No treatment-related effects were observed on reproduction parameters: numbers of corpora lutea and implantations, litter size, number of live fetuses, resorptions, sex ratio and fetal viability. Mean pup weight was slightly decreased at 600 mg/kg bw per day. At 300 mg/kg bw per day, a single incidence of inward-rotated right paw was detected. This incidence was slightly above the historical control range, but is not considered to be related to treatment due to the single incidence and the absence of similar effects at the highest dose level. Single instances of fused kidneys, diaphragmatic hernia and absent adrenals was present at 300 mg/kg bw per day. However these effects are not considered to be treatment related due the absence of similar effects at the highest dose level.

Table 24. Fetal findings in the rabbit developmental toxicity study with pyridate

Dose (mg/kg bw per day)	Control	150	300	600	Background[b]
Fetal body weight (g)	42.7	44.3	45.6	39.3	-
Head domed	-	-	-	1 (8.3)[a]	4 (0.7) (0–8.3)
Meningocoele lumbar-sacral	-	-	1 (6.7)[a]	-	2 (0.35) (0–9.1)
Meningocoele cervical	-	-	1 (6.7)	-	2 (0.35) (0–9.1)
Right paw rotated inward	-	-	1 (6.7)[a]	-	1 (0.18) (0–5.9)
Hydrocephalus	-	-	-	1 (8.3)[a]	5 (0.97) (0–8.3)
Hydronephrosis	-	-	-	1 (8.3)	No data
Kidneys fused	-	-	1 (6.7)[a]	-	No data

Dose (mg/kg bw per day)	Control	150	300	600	Background[b]
Diaphragmatic hernia	-	-	1 (6.7)	-	No data
Adrenals absent	-	-	1 (6.7)[b]	-	No data
Anterior fontanelle, enlarged	-	-	-	1 (8.3)	9 (1.74) (0–8.3)
Vertebrae thoracic hemivertebra; Centrum bifid Centra asymmetric	-	-	-	1 (8.3)	9 (1.74) (0–20) 2 (0.39) (0–33.3) 1 (0.19) (0–33.3)
Sternebra 1: incomplete ossification	-	-	-	1 (8.3)	1 (0.19) (0–6.25)
Pelvis pubes incomplete ossification	1 (5.6)	-	-	1 (8.3)	5 (0–13.3) (not ossified)

[a] Findings in the same individual

[b] Historical control data based on 570 litters from studies performed in 1983–1986; % (range in %) (% litter inncidence)

Source: Hoberman, 1987

A maternal NOAEL of 300 mg/kg bw per day was determined for this study based on clinical signs, reduced body weight gain, reduced body weight and reduced food consumption at 600 mg/kg bw per day. A developmental NOAEL of 300 mg/kg bw per day was determined for this study due to the effects observed at 600 mg/kg bw per day (Hoberman, 1987).

2.6. Special studies

(a) Acute neurotoxicity

Rat

An acute single-dose oral gavage neurotoxicity screening study was conducted using Sprague Dawley Crl:CD(SD) rats. The study was conducted in two phases: a dose-ranging phase and a phase designed to establish the time to peak effect for observed neurological effects. In the dose-ranging phase pyridate was administered by gavage at doses of 0, 500 and 1000 mg/kg bw. In the time-to-peak-effect phase pyridate was administered by gavage at doses of 0, 62.5, 177 and 500 mg/kg bw. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight gains, food consumption, full functional observational battery (FOB), motor activity, gross necropsy findings, organ weights and histopathologic examinations.

Pyridate-related mortality occurred on day 1 in both experimental phases and consisted of one male and two females found dead at 500 mg/kg bw, three females found dead at 1000 mg/kg bw, and two males and two females euthanized in a moribund condition at 1000 mg/kg bw. Decreased activity, inco-ordination, weakness, abnormal breathing (abnormal sounds, irregular rate, increased respiratory rate, laboured or shallow breathing), lying on side, non-sustained convulsions, tremors, and locomotory stereotypy were noted prior to death. Transient pyridate-related body weight loss and/or decreases in body weight gain compared to the controls (up to 50%) were observed in the dose-ranging phase at doses ≥ 500 mg/kg bw. Correlating decreases in mean food consumption (up to 73%) were noted in 500 mg/kg males and females and at 1000 mg/kg in males. Recovery in body weight and food consumption was noted by days 7 and 4, respectively. There were no pyridate-associated changes in absolute body weight or body weight gain in pyridate-treated animals in the time-to-peak-effect experimental phase. However, transient (up to 36%) decreases in mean food consumption occurred on days 1 to 2 in 500 mg/kg males and females. Recovery was noted by day 3.

Pyridate-related clinical signs were observed in both experimental phases and consisted of decreased activity, weakness, laboured breathing, incoordination, shallow breathing, salivation, eye discharge, lying on side, increased respiratory rate, and locomotory stereotypy at 500 mg/kg and/or 1000 mg/kg bw; also unsustained convulsions, tremors, abnormal breathing sounds, lying on side, shallow breathing, and irregular respiratory rate at 1000 mg/kg bw. The clinical signs were noted beginning approximately one hour post dose with recovery by the following day. A few sporadic clinical signs were noted on day 8 and included incoordination in two of three surviving 1000 mg/kg animals and in one 500 mg/kg female.

Pyridate-related changes in FOB parameters were observed in 500 mg/kg bw females, and to a lesser extent males, on day 1 compared to controls. The effects seen in one or both sexes consisted of changes in body posture (flattened, possibly limbs be spread out), slightly abnormal gait (ataxia or hind-limbs exhibits exaggerated or overcompensated movements, drags, or are splayed), slightly to totally impaired mobility, decreased rearing, decreased righting ability (animal does not right itself or does so in greater than five seconds), decreased body temperature, laboured respiration, piloerection, low to somewhat low arousal, stereotypy (head weaving), bizarre behaviour (backward walking), decreased startle response (slight to no reaction), decreased tail-pinch (little to no reaction), no pupil response, and shallow respiration, involuntary clonic motor movements (repetitive movements of the mouth and jaw), and decreased motor activity (up to –81%).

There were no pyridate-associated organ weight changes, gross or microscopic findings in the time-to-peak-effects experimental phase (including no pyridate-associated microscopic changes in nervous system tissues).

A NOAEL of 177 mg/kg bw was identified in the study, based on the presence of mortality at doses greater than or equal to 500 mg/kg bw (Diehl, 2016).

(b) Safety pharmacology

Mouse

In a safety pharmacology study, groups of male KFM NMRI mice were administered a single gavage dose of pyridate (batch No. 2759523; purity 91.5%) at dose levels of 0, 1000, 3000, 5000 and 8000 mg/kg bw. Decreased activity and dyspnoea were observed at dose levels of ≥ 1000 mg/kg bw; hunched posture was additionally observed at dose levels of ≥ 3000 mg/kg bw. Treatment with pyridate had no effects on hexobarbitone sleeping time, convulsions induced by pentetrazole, convulsions induced by strychnine, locomotor activity, electroshock or tremorine antagonism in mice. These tests were validated by the use of positive and negative controls as appropriate (Janiak & Braunhofer, 1989).

Rat

Groups of KFM Han Wistar rats were administered a single gavage dose of pyridate (batch No. 2759523; purity 91.5%) at dose levels of 0, 250, 500, 1000 and 2300 mg/kg bw. One animal dosed with pyridate at 2300 mg/kg bw was found dead. No effects on the general behaviour of the rats were observed (Janiak & Braunhofer, 1989).

Rabbit

The effects of intravenously injected pyridate on blood pressure, heart and respiratory rates was evaluated in a dose escalation study in anesthetized rabbits. No effects of pyridate following dosing of up to 2.7 g/animal of pyridate (batch No. 2759523; purity 91.5%) were observed. At this dose one of the rabbits died due to pulmonary embolism from the solid form of the test material (Janiak & Braunhofer, 1989).

(c) Studies on metabolites

Pyridafol

A number of studies have been performed with pyridafol (CL-9673, SAN 1367H), identified as the major metabolite of pyridate in rat studies. In an acute oral toxicity study, pyridafol was administered to groups of male and female Wistar rats at dose levels of 600, 1000, 2000, 3000 and 5000 mg/kg bw. Signs of toxicity were observed at dose levels of ≥ 600 mg/kg bw (sedation, dyspnoea, ataxia, piloerection, hunched posture, lateral recumbency), ≥ 1000 mg/kg bw (additionally rales and ventral body position), ≥ 3000 mg/kg bw (additionally rolling movements and distended abdomen) and at 5000 mg/kg bw (coma). Necropsy revealed reddened intestines or red intestinal foci at dose levels of ≥ 1000 mg/kg bw. Acute oral median lethal dose (LD_{50}) values of 1511 and 1420 mg/kg bw were calculated for males and females, respectively (Ullmann, 1987).

No evidence of skin sensitization was seen in a 9-induction Buehler test performed using topical induction and challenge applications of 50% pyridafol in PEG 400/water (Ullman & Kups, 1988b).

No evidence of mutagenicity was seen in an Ames test performed in *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 using concentrations of up to 10 000 µg/plate in either the presence or absence of metabolic activation (Hoorn, 1987a).

No evidence of mutagenicity was seen in an Ames test performed in in *E. coli* strain WP2*uvrA* using concentrations of up to 10 000 µg/plate in either the presence or absence of metabolic activation (Hoorn, 1987b).

CL-9673 N-glucoside

The metabolism of the *N*-glucoside conjugate of [^{14}C]-radiolabelled CL-9673 was investigated in the Wistar rat (three/sex) following a single gavage dose of 1 mg/kg bw. The majority of the administered radioactivity was excreted within the first 24 h after dosing (32–53% in urine and 45–65% in the faeces), with a higher level of urinary excretion in females. Levels of radioactivity in blood and tissues were very low and were mostly below the limit of quantification. CL-9673 was identified as the major radiolabelled component in urine, representing 37–47% of the 0–24 h urinary radioactivity. A glucuronide conjugate of CL-9673 was also identified in urine. A minor radioactive component was tentatively identified as a hydroxylated derivative of CL-9673 (van Dijk, 1992).

In an acute oral toxicity study in male and female Wistar rats performed at dose levels of 1000 and 2000 mg/kg bw, no deaths occurred. Signs of toxicity were limited to sedation, hunched posture and piloerection (males) and rales (both sexes) at 2000 mg/kg bw. The acute oral LD_{50} of CL-9673 *N*-glucoside was therefore found to be > 2000 mg/kg bw (Ullman et al., 1990).

3. Observations in humans

No adverse effects are reported in workers involved in the manufacture of pyridate (Fuqiang, 2012).

Comments

Biochemical aspects

The toxicokinetics and metabolism of ^{14}C-radiolabelled pyridate have been investigated in the rat and, to a lesser extent, in the dog. Studies in rats demonstrated rapid and extensive oral absorption of radioactivity following dosing at up to 200 mg/kg bw. Based on the urinary excretion of radioactivity, more than 80% of the oral dose was absorbed within 24 h of administration. This was confirmed in studies using bile duct-cannulated rats. Following oral dosing, plasma radioactivity levels reached a maximum within 1–2 h and decreased rapidly thereafter. Following single or repeated oral dosing, peak plasma radioactivity concentrations were dose-proportional at doses up to 200 mg/kg bw and no important sex differences were noted. Following oral dosing with radiolabelled pyridate at up to 200 mg/kg bw the highest levels of radioactivity were found in the GI tract, plasma, liver and kidneys, reflecting both the route of administration and the major routes of excretion. Low levels of radioactivity were found in other tissues with the levels in fat and brain being an order of magnitude below plasma levels. Following oral dosing the elimination of radioactivity was rapid and essentially complete 96 h after dosing. Oral dosing at 600 mg/kg bw resulted in disproportionately high plasma and tissue radioactivity levels due to the saturation of urinary excretion.

Pyridate is almost completely metabolized in rats following oral dosing. Pyridate is initially hydrolysed to pyridafol (CL-9673, SAN 1367H) with liberation of the *n*-octylthiocarbonyl side-chain. Pyridafol is further metabolized by conjugation with either glucuronic acid or sulfate (excreted in the urine), or by hydroxylation of the phenyl group (excreted in the urine and faeces). The major metabolites in rat urine are pyridafol (14–22% of detected radioactivity) and the phenyl-hydroxylated metabolite of pyridafol (22–39% of detected radioactivity).

In contrast to the rat, the *O*- and *N*-glucuronide conjugates of pyridafol were the major metabolites found in the urine of orally-dosed dogs (70% of detected radioactivity) with free pyridafol accounting for 18–23% of the total radioactivity in urine.

Toxicological data

The acute oral toxicity LD_{50} for pyridate in rats was greater than 2000 mg/kg bw (Ullman & Sacher, 1984; Ullman, Sacher & Vogel, 1988; Pels Rijcken, 1996a, b, c). However, deaths occurred following oral gavage dosing at 500 mg/kg bw in the rat acute neurotoxicity study (Diehl, 2016)and following the first oral gavage dose of 400 mg/kg bw in the rat developmental study (Becker et al., 1986). This likely correlates with the saturation of urinary excretion in rats. The acute dermal LD_{50} in rabbits was greater than 2000 mg/kg bw (Ullman, Sacher & Chevalier, 1984) and the acute inhalation LC_{50} in rats was greater than 4.37 mg/L (Ullman, Zbinden & Chevalier, 1983). Pyridate was mildly irritating to the skin of rabbits (Kynoch & Liggett, 1976a; Shults, Brock & Laveglia, 1995a). Pyridate was transiently irritating to the conjunctiva in rabbits (Shults, Brock & Laveglia, 1995b) and induced skin sensitization in guinea pigs, based on both Buehler and maximization tests (Kynoch, 1976; Ullman & Kups, 1988a).

Repeated dose toxicity studies in mice, rats and dogs demonstrated the effects of pyridate on mortality, the nervous system (clinical signs consistent with neurotoxicity), reduced weight gain, changes in erythrocyte parameters, increased organ (spleen, liver and kidney) weights, the presence of debris and mineralized deposits in lymph nodes, haemosiderin deposition in the spleen and Kupffer cell pigmentation.

A 28-day toxicity study in rats used pyridate at dietary concentrations of 0, 1000, 3000 and 10 000 ppm (equivalent to 0, 100, 300 and 1000 mg/kg bw per day). The NOAEL was 1000 ppm (equivalent to 100 mg/kg bw per day) based on reduced body weight gain and food consumption in both sexes at 10 000 ppm (equivalent to 1000 mg/kg bw per day) (Til et al., 1979).

A 90-day toxicity study (with a 28-day recovery period) was performed in rats using gavage dose levels of 0, 62.5, 177 and 500 mg/kg bw per day with an additional study cohort initially dosed at 500 mg/kg bw per day, incrementing to 600 mg/kg bw per day after two weeks of treatment. The NOAEL was 62.5 mg/kg bw per day based on mortality, clinical signs and histopathological findings (mineralized deposits in the mesenteric lymph nodes, haemosiderin deposition in the spleen) seen at 177 mg/kg bw per day (Henck et al., 1987).

A 90-day toxicity study in dogs was performed using gelatine capsule dosing at levels of 0, 20, 60 and 200 mg/kg bw per day. The NOAEL was 20 mg/kg bw per day based on the presence of clinical signs (consistent with neurotoxicity) and mortality at 60 mg/kg bw per day (Tomkins, 1987).

A carcinogenicity study in mice was performed at dietary concentrations of 0, 400, 800 and 1200 ppm (incremented to 1400 ppm on day 91 and 1600 ppm on day 179). Additional dose cohorts of 0 ppm and 7000 ppm were also evaluated. The pyridate intakes were equal to 0, 48, 98, 170 and 853 mg/kg bw per day in males, 0, 55, 115, 204 and 1045 mg/kg bw per day in females. The NOAEL for toxicity was 800 ppm (equal to 98 mg/kg bw per day) based on lower body weights in both sexes and increased mortality in females at the dietary concentration of 1200→1400→1600 ppm (equal to 170 mg/kg bw per day). The NOAEL for carcinogenicity was 1200→1400→1600 ppm (equal to 170 mg/kg bw per day (Lindamood, Coyne & Thompson, 1991).

A combined chronic toxicity/carcinogenicity study was performed in rats with dietary exposure at concentrations of 0, 80, 400 or 2500 ppm (equivalent to 0, 4, 20, and 125 mg/kg bw per day). The NOAEL for toxicity was 400 ppm (equivalent to 20 mg/kg bw per day) based on body weight effects in both sexes and changes in erythrocyte parameters in females at 2500 ppm (equivalent to 125 mg/kg bw per day (Til, Woutersen & Bosland, 1990). The NOAEL for carcinogenicity was 2500 ppm (equivalent to 125 mg/kg bw per day).

The Meeting concluded that pyridate is not carcinogenic in mice or rats.

Pyridate has been tested for genotoxicity in a battery of studies in vitro and in vivo. Many of these studies were non-compliant with current test standards. However no clear evidence of genotoxicity was found.

The Meeting concluded that pyridate is unlikely to be genotoxic.

Due to the absence of carcinogenic effect in rats and mice and the lack of genotoxicity the Meeting concluded that pyridate is unlikely to pose a carcinogenic risk to humans.

In a three-generation toxicity study in the rat using dietary concentrations of 0, 80, 400 and 2500 ppm (equivalent to 0, 5, 26 and 165 mg/kg bw per day) no effects on fertility or reproductive

capacity were observed. The reproductive NOAEL was 2500 ppm (equivalent to 165 mg/kg bw per day), the highest dose tested. The parental NOAEL was 400 ppm (equivalent to 26 mg/kg bw per day) based on the increased relative kidney weights seen at dietary concentrations of 2500 ppm (equivalent to 165 mg/kg bw per day). The offspring NOAEL was 400 ppm (equivalent to 26 mg/kg bw per day) based on reduced pup weights at the highest dose level of 2500 ppm (equivalent to 165 mg/kg bw per day) (Til, Wouterson & Falke, 1982).

In a rat developmental study gavage dose levels of 0, 55, 165, 400 and 495 mg/kg bw per day were used. The maternal NOAEL was 165 mg/kg bw per day based on reduced body weight gain and mortality at the LOAEL of 400 mg/kg bw per day (correlated with saturation of urinary excretion). The embryo/fetal NOAEL was 165 mg/kg body weight based on reduced fetal weight and associated reductions in skeletal ossification seen at the LOAEL of 400 mg/kg bw per day (Becker et al., 1986).

In a developmental study performed in rabbits at gavage dose levels of 0, 150, 300 and 600 mg/kg bw per day the maternal NOAEL was 300 mg/kg bw per day based on abortions, reduced body weight gain, reduced body weight and reduced food consumption following dosing at 600 mg/kg bw per day. The embryo/fetal NOAEL was 300 mg/kg bw per day based on reduced fetal body weights following dosing at 600 mg/kg bw per day (Hoberman, 1987).

The Meeting concluded that pyridate is not teratogenic

An acute neurotoxicity study was conducted in rats using gavage doses of 0, 62.5, 177 and 500 mg/kg bw. Clinical signs and mortality occurred following dosing at 500 mg/kg bw (correlating with saturation of urinary excretion). Surviving animals dosed at 500 mg/kg body weight displayed transient behavioural effects which were considered secondary to generalized toxicity. There were no pyridate-associated microscopic anatomic pathology changes in the nervous system. The NOAEL was 177 mg/kg bw due to mortality following dosing at 500 mg/kg bw (Diehl, 2016). Clinical signs consistent with neurotoxicity occurred in the 90-day repeat-dose toxicology study in dogs.

The Meeting concluded that pyridate is not acutely neurotoxic in the rat, but shows clinical signs of neurotoxicity in a 90-day study of toxicity in dogs.

No studies on immunotoxic effect were submitted.

Toxicological data on metabolites and/or degradates

Pyridafol (CL-9673, SAN 1367H), a plant metabolite and seen at significant levels in the rat, had an acute oral LD_{50} of 1511 mg/kg bw in the rat (Ullman, 1987). It was not a skin sensitizer in a Buehler assay in albino guinea pigs (Ullman & Kups, 1988b). Pyridafol was not mutagenic in two bacterial reverse mutation assays (Hoorn, 1987a, b). Given that pyridafol is a major rat metabolite, its toxicity is considered covered by the parent compound.

Pyridafol-*N*-glucoside, a plant metabolite, had an acute oral LD_{50} greater than 2000 mg/kg bw in rats (Ullman et al., 1990). In a metabolism study in the rat, pyridafol-*N*-glucoside was rapidly excreted in urine as pyridafol, with minor metabolites like the glucuronic acid conjugate of pyridafol and a hydroxylated derivative of pyridafol (Van Dijk, 1992). Given that pyridafol-*N*-glucoside is converted to pyridafol in mammals, its toxicity is considered covered by pyridafol, and thus by the parent compound.

Microbiological data

The available data indicate that pyridazines require an N^3,N^6-diphenylpyridazine-3,6-diamine chemical structural skeleton for antimicrobial activity (Javed, Sapra & Alam, 2018). Pyridate lacks the required chemical structural skeleton and is unlikely to show antimicrobial activity relevant to humans.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No adverse effects have been reported in exposed users of pyridate-based products.

The Meeting concluded that the existing database on pyridate was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for pyridate of 0–0.2 mg/kg bw based on the NOAEL of 400 ppm (equivalent to 20 mg/kg bw per day) in the two-year study in rat, where body weight effects in both sexes, and changes in erythrocyte parameters in females occurred following dosing at 2500 ppm (equivalent to 125 mg/kg bw per day). A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) for pyridate of 2 mg/kg bw based on the NOAEL of 177 mg/kg bw in the rat acute neurotoxicity study where clinical signs and mortality occurred following dosing at 500 mg/kg bw. This was supported by the maternal NOAEL value set on account of mortality, the observed adverse effect in this case following the first dose of 165 mg/kg bw per day in the rat developmental study. A safety factor of 100 was applied. A higher safety factor was not regarded as being necessary since human food exposure is unlikely to result in saturation of renal excretion.

Levels relevant to risk assessment of pyridate

Species	Study	Effect	NOAEL	LOAEL
Mouse	18-month study of carcinogenicity[b]	Toxicity	800 ppm (equivalent to 98 mg/kg bw per day)	1200→1400→1600 ppm (equivalent to 204 mg/kg bw per day)
		Carcinogenicity	1200→1400→1600 ppm (equivalent to 204 mg/kg bw per day)	-
Rat	28-day study of toxicity[b]	Toxicity	1000 ppm (equivalent to 100 mg/kg bw per day)	3000 ppm (equivalent to 300 mg/kg bw per day)
	90-day study of toxicity[a]	Toxicity	62.5 mg/kg bw per day	177 mg/kg bw per day
	Two-year study of carcinogenicity[b]	Toxicity	400 ppm (equivalent to 20 mg/kg bw per day)	2500 ppm (equivalent to 125 mg/kg bw per day)
		Carcinogenicity	2500 ppm (equivalent to 125 mg/kg bw per day)[d]	-
	Multigeneration study of reproduction[b]	Reproductive toxicity	2500 ppm (equivalent to 165 mg/kg bw per day)[d]	-
		Parental toxicity	400 ppm (equivalent to 26 mg/kg bw per day)	2500 ppm (equivalent to 125 mg/kg bw per day)
		Offspring toxicity	400 ppm (equivalent to 26 mg/kg bw per day)	2500 ppm (equivalent to 125 mg/kg bw per day)
	Developmental toxicity study[a]	Maternal toxicity	165 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	165 mg/kg bw per day	400 mg/kg bw per day
	Acute neurotoxicity study[a]	Toxicity	177 mg/kg bw per day	500 mg/kg bw per day
Rabbit	Developmental toxicity study[a]	Maternal toxicity	300 mg/kg bw per day	600 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day	600 mg/kg bw per day
Dog	90-day study of toxicity[c]	Toxicity	20 mg/kg bw per day	60 mg/kg bw per day

[a] Oral gavage administration [b] Dietary administration [c] Capsule administration [d] Highest dose tested

Acceptable daily intake (ADI) applies to pyridate, pyridafol and pyridafol-N-glucoside, expressed as pyridate

0–0.2 mg/kg bw

Acute reference dose (ARfD) applies to pyridate, pyridafol and pyridafol-N-glucoside, expressed as pyridate

2 mg/kg bw

Critical end-points for setting guidance values for exposure to pyridate

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Extensive (> 80% based on urinary and bile); saturation of oral absorption occurred at 200 mg/kg bw in dogs (but not in rats)
Dermal absorption	No data
Distribution	Widely distributed; highest amounts in the GI tract, plasma, liver and kidney
Potential for accumulation	None
Rate and extent of excretion	> 90% within 96 h, > 80% via urine; saturation of urinary excretion in rats occurred at doses > 200 mg/kg bw
Metabolism in animals	Extensively metabolized, hydrolysis to pyridafol (main metabolite); pyridafol glucuronic acid and sulfate conjugates
Toxicologically significant compounds in animals and plants	Pyridate and pyridafol (CL-9673, SAN 1367H)
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw **Note:** mortalities occurred in other rat studies atdoses ≥ 400 mg/kg bw (doses likely resulting in saturation of urinary excretion)
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.37 mg/L
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Mildly irritating
Guinea pig, dermal sensitization	Sensitizing (guinea pig maximization and Buehler tests)
Short-term studies of toxicity	
Target/critical effect	Mortality, clinical (neurotoxic) signs, reduced body weight, reduced body weight gain
Lowest relevant oral NOAEL	20 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	> 1000 mg/kg bw per day
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight gain (rat)
Lowest relevant oral NOAEL	20 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in rats and mice[a]
Genotoxicity	Unlikely to be genotoxic[a]
Reproductive toxicity	
Target/critical effect	Parental effects: increased relative kidney weight Offspring effects: reduced pup weight Reproductive effects: none
Lowest relevant parental NOAEL	26 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	26 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	165 mg/kg bw per day (highest dose tested) (rat)

Developmental toxicity	
Target/critical effect	Rat (maternal effects) mortality, reduced bodyweight gain; (developmental effects) skeletal variations Rabbit (maternal effects) reduced bodyweight gain; (developmental effects) abortion, reduced fetal weight
Lowest relevant maternal NOAEL	165 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	165 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	500 mg/kg bw (highest dose tested)
Subchronic neurotoxicity NOAEL	20 mg/kg bw per day (90-day toxicity study in dog)
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data

Studies on toxicologically relevant metabolites

Pyridafol (CL 9673, SAN 1367H)	Pyridafol is well absorbed and rapidly excreted in the urine. The metabolic pathways are identical for pyridate and pyridafol Acute oral LD_{50}: 1511/1420 mg/kg bw (males/females) Buehler test (9-induction): negative Bacterial reverse mutation test: negative
Pyridafol-N-glucoside	ADME (single oral dose): 32–53.4% absorbed, rapidly eliminated (32%–53.4% via urine; 45–65% via faeces) low residual radioactivity in blood and tissue, similar further metabolic steps as pyridate Acute oral LD_{50} > 2000 mg/kg bw
Human data	No adverse effects reported in humans
Microbiological data	Unlikely to have antimicrobial effects

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.2 mg/kg bw[a]	Two-year study of toxicity (rat)	100
ARfD	2 mg/kg bw[a]	Acute neurotoxicity (rat)	100

[a] Applies to pyridate, pyridafol and pyridafol-*N*-glucoside, expressed as pyridate

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PYRIFLUQUINAZON

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Explanation

Pyrifluquinazon is the ISO-approved common name for 1-acetyl-3-[(pyridin-3-ylmethyl)amino]-6-[1,2,2,2-tetrafluoro-l-(trifluoromethyl)ethyl]-3.4-dihydro-lH-quinazolin-2-one (IUPAC), which has the Chemical Abstracts Service number 337458-27-2.

Pyrifluquinazon is an insecticide for use on a wide range of crops including plums, potatoes, tree nuts and tea. Its mode of insecticidal action is by modification of the ins ect feeding behaviour. It acts through interaction with transient receptor potential vanilloid (TRPV) channel complexes of the chordotonal stretch receptor neurons.

Pyrifluquinazon has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

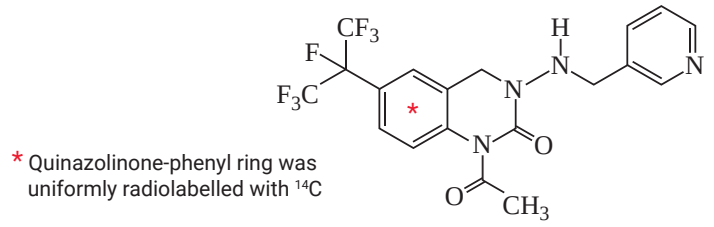
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with the relevant national or international test guidelines, unless otherwise specified. A literature search did not identify any toxicological information additional to that submitted for the current assessment.

Evaluation for acceptable intake

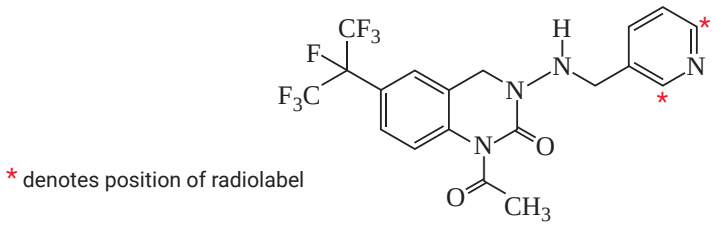
1. Biochemical aspects

Absorption, distribution, metabolism and excretion (ADME) studies were conducted in rats using pyrifluquinazon radiolabelled with ^{14}C in the quinazolinone-phenyl ring or in the pyridine ring (Fig. 1).

Figure 1. Position of ^{14}C label in pyrifluquinazon used in rat ADME studies.



(Redrawn from Yoshizane, 2006a)



(Redrawn from Yoshizane, 2006b)

1.1 Absorption, distribution and excretion

(a) Oral route

Rat

In an ADME study various experiments were conducted in Fischer F344/DuCrlcrlj rats to investigate the toxicokinetics and metabolism of pyrifluquinazon (Table 1). Groups of rats (four per sex and per dose) received a single gavage administration of either a low dose of 1 mg/kg body weight (bw) or a high dose (100 mg/kg bw) of ^{14}C-pyrifluquinazon (radiochemical purity > 96%, radiolabel in the quinazolinone-phenyl ring), dissolved in an aqueous vehicle (0.5% w/v) sodium carboxymethyl cellulose (CMC) containing 0.1% w/v Tween 80. An overview of the study design is presented in Table 1. Animals were fasted for 18 hours prior to dosing.

Table 1. Summary of experiments performed to investigate the toxicokinetics and metabolism of ^{14}C-quinazolinone-phenyl-labelled pyrifluquinazon in rats

Group	Dose (mg/kg bw)	No.	Sex	Sampling time (hours post dosing)
A	1	4	M	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
B	1	4	F	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
C	100	4	M	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
D	100	4	F	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
E	1	4	M	Organs/tissues: 3
F	1	4	M	Organs/tissues: 24
G	1	4	M	Expired air: 24 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
H	1	4	F	Organs/tissues: 3
I	1	4	F	Organs/tissues: 24
J	1	4	F	Expired air: 24 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
K	100	4	M	Organs/tissues: 9
L	100	4	M	Organs/tissues: 24
M	100	4	M	Expired air: 24 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
N	100	4	F	Organs/tissues: 9
O	100	4	F	Organs/tissues: 24
P	100	4	F	Expired air: 24 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168

Source: Yoshizane (2006a)

Metabolite profiles in excreta were identified using thin layer chromatography (TLC) and radiometric-high performance liquid chromatography (HPLC). These data are presented in Section 1.2 Biotransformation.

Total recovery of radioactivity was 95–97%. The toxicokinetic parameters for males and females given a 1 or 100 mg/kg bw dose of pyrifluquinazon are presented in Table 2. There were no significant differences between males and females.

Table 2. Kinetic parameters following single oral administration of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon to male and female rats

	Dose administered (mg/kg bw)							
	1				100			
	Blood		Plasma		Blood		Plasma	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Males								
T_{max} (h)	1		1		12		12	
C_{max} (µg equiv./g)	0.518		0.414		30.6		23.6	
$t_{½}$ (day) T_{max}–72 h	0.64	0.03	0.63	0.01	0.75	0.07	0.94	0.10
$t_{½}$ (day) 72–168 h	4.78	0.70	2.44	0.07	1.63	0.33	1.40	0.08
AUC (µg equiv. h/g)	12.05	0.53	8.89	0.51	1153.7	235.2	1060.5	215.1
Females								
T_{max} (h)	3		3		9		9	
C_{max} (µg equiv./g)	0.397		0.337		31.1		26.4	
$t_{½}$ (day) T_{max}–72 h	0.85	0.06	0.68	0.01	0.90	0.17	1.08	0.21
$t_{½}$ (day) 72–168 h	4.6	0.5	2.9	0.3	1 .7	0.4	1.4	0.04
AUC (µg equiv. h/g)	12.5	0.7	8.2	0.2	1321	410	1220	352

SD Standard deviation C_{max} maximum concentration T_{max} time to reach maximum concentration
$t_{½}$ Half life AUC Area under the the concentration–time curve Source Yoshizane (2006a)

Radioactivity in blood and plasma following administration of a dose of 1 and 100 mg/kg bw reached their maximum at 1–3 and 9–12 hours post dose, respectively. After reaching peak concentrations, radioactivity in blood and plasma was eliminated in an apparently biphasic manner. In both male and female, blood and plasma radioactivity concentrations and areas under the concentration–time curve (AUCs) for the 100 mg/kg bw groups were almost 100 times higher than those for the 1 mg/kg bw groups, indicating that the bioavailability of pyrifluquinazon was similar for both dose rates.

In distribution studies, the rats were euthanized at 3, 24 and 168 hours post dose in the 1 mg/kg bw groups and at 9, 24 and 168 hours post dose in the 100 mg/kg bw groups. Time points were based on the blood and plasma radioactivity profiles. After the 1 mg/kg bw dose, peak organ and tissue radioactivity concentrations were found at 3 h post dose, with highest concentrations found in the gastrointestinal tract (GIT, 1.1–1.3 µg equiv./g), liver (3.3–3.6 µg equiv./g), adrenal (3.2–3.6 µg equiv./g) and kidney (2.0–2.1 µg equiv./g). After the 100 mg/kg bw dose, peak organ and tissue radioactivity concentrations were found at 9 h post dose, with highest concentrations found in the GIT (191–200 µg equiv./g), liver (156–170 µg equiv./g), adrenal (110–111 µg equiv./g) and kidney (103–111 µg equiv./g). At 24 and 168 h after dosing radioactivity concentrations in all organs and tissues were markedly decreased.

Excretion of radioactivity over 168 h was predominantly in faeces (75–76% at 1 mg/kg bw; 78–81% at 100 mg/kg bw). In urine 20–21 and 15–16% of radioactivity was excreted after a 1 and 100 mg/kg bw dose, respectively. After 48 hours, 92–94% and 72–81% of the administered dose was excreted following the low and high dose, respectively (Yoshizane, 2006a).

In a second toxicokinetics study, various experiments were conducted in Fischer F344/DuCrlcrlj rats to investigate the toxicokinetics and metabolism of ^{14}C(2,6-pyridine-labelled)-pyrifluquinazon. Groups of rats (four per sex and per dose) received a single gavage administration of either a low dose (1 mg/kg bw) or a high dose (100 mg/kg bw, females with blood sampling only) of ^{14}C-pyrifluquinazon (radiochemical purity > 98%, radiolabel in the pyridine ring), dissolved in an aqueous vehicle (0.5% w/v sodium carboxymethyl cellulose (CMC) containing 0.1% w/v Tween 80). An overview of the study designs is presented in Table 3. Animals were fasted for 16 h prior to dosing. Data on identification of metabolite profiles are presented in Section 1.2.

Table 3. Summary of experiments performed to investigate the toxicokinetics and metabolism of ^{14}C-pyridine-labelled pyrifluquinazon in rats

Group	Dose (mg/kg bw)	No.	Sex	Sampling time (hours post dosing)
A	1	4	M	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
B	1	4	F	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
C	100	4	M	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
D	100	4	F	Blood:1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
E	1	4	M	Organs/tissues: 3
F	1	4	M	Organs/tissues: 24
G	1	4	M	Expired air: 24, 48, 72, 96, 120, 144, 168 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
H	1	4	F	Organs/tissues: 3
I	1	4	Fe	Organs/tissues: 24
J	1	4	F	Expired air: 24, 48, 72, 96, 120, 144, 168 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168
K	100	4	M	Organs/tissues: 9
L	100	4	M	Organs/tissues: 24
M	100	4	M	Expired air: 24, 48, 72, 96, 120, 144, 168 Urine: 24, 48, 72, 96, 120, 144, 168 Faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168

Source: Yoshizane (2006b)

Total recovery of radioactivity was 91–94%. The toxicokinetic parameters for males and females given a 1 or 100 mg/kg bw dose of ^{14}C-pyridine-labelled pyrifluquinazon are presented in Table 4. There were no significant differences between males and females. On this basis, high-dose females were omitted from the remainder of the studies with ^{14}C-pyridine-labelled pyrifluquinazon.

Table 4. Pharmacokinetics of pyrifluquinazon equivalents in rats following administration of ^{14}C-pyridine-labelled pyrifluquinazon

	Dose administered (mg/kg bw)							
	1				100			
	Blood		Plasma		Blood		Plasma	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Males								
T_{max} (h)	1		1		9		9	
C_{max} (µg equiv./g)	0.376		0.183		18.1		10.4	
$t_{½}$ (day) T_{max}–72 h	2.57	0.29	0.95	0.10	2.01	0.17	0.90	0.10
$t_{½}$ (day) 72–168 h	6.26	1.58	3.85	0.93	11.54	2.58	3.42	1.04
AUC (µg equiv. h/g)	21.4	1.45	3.68	0.28	1419	133	389	48.4

	Dose administered (mg/kg bw)							
	1				100			
	Blood		Plasma		Blood		Plasma	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Females								
T_{max} (h)	1		1		3		3	
C_{max} (µg equiv./g)	0.353		0.171		16.9		11.2	
$t_{½}$ (day) T_{max}–72 h	3.18	0.57	0.98	0.02	1.94	0.13	0.96	0.09
$t_{½}$ (day) 72–168 h	6.60	3.02	4.39	0.61	9.60	1.80	3.65	1.69
AUC (µg equiv. h/g)	19.6	0.70	3.82	0.02	1344	232	433	69

AUC: estimated area under the concentration–time curve

C_{max} maximum concentration; T_{max} time to reach maximum concentration $t_{½}$ Half life

Source: Yoshizane, 2006b

Radioactivity in blood and plasma following administration of a dose of 1 and 100 mg/kg bw reached their maximum at 1 and 3–9 h post dose, respectively. After reaching peak concentrations, radioactivity in blood and plasma was eliminated in an apparently biphasic manner. In both male and female, blood and plasma radioactivity concentrations and AUCs for the 100 mg/kg bw groups were about 50–100 times higher than those for the 1 mg/kg bw groups, indicating that the bioavailability of pyrifluquinazon was roughly similar at both dose rates.

For distribution studies, the rats were euthanized at 3, 24 and 168 hours post dose in the 1 mg/kg bw groups and at 9, 24 and 168 hours post dose in the 100 mg/kg bw groups. The time points were based on blood and plasma radioactivity profiles. After the 1 mg/kg bw dose, peak organ and tissue radioactivity concentrations were found 3 h post dose, with highest concentrations found in liver (6.9–9.3 µg equiv./g), GIT (0.8–0.9 µg equiv./g), adrenal (1.6–2.4 µg equiv./g) and kidney (1.7–1.9 µg equiv./g) . After the 100 mg/kg bw dose in males, peak organ and tissue radioactivity concentrations were found at 9 h post dose, with highest concentrations found in liver (437 µg equiv./g), GI tract (130 µg equiv./g), adrenal (94 µg equiv./g) and kidney (240 µg equiv./g). Organ and tissue radioactivity levels were not measured in high-dose females. At 24 and 168 h after dosing lower radioactivity concentrations in all organs and tissues were observed, although significant levels of radioactivity were still measurable at 168 h.

Excretion of radioactivity over 168 h was similar in faeces (24–28% at 1 mg/kg bw in males and females; 39% at 100 mg/kg bw, measured in males only) and urine (29–31% at 1 mg/kg bw in males and females; 33% at 100 mg/kg bw dose, measured in males only). The proportion of administered radioactivity excreted in expired air was 4–7%. After 24 h, 35–41% at 1 mg/kg bw (males and females) and 46% at 100 mg/kg bw (males only) of the administered dose was excreted following the low and high dosing. After 24 h excretion continued slowly up to 168 h, when in total 59–76% was excreted, while at both low and high doses, 18–31% still remained in the carcass. As the pyridine moiety of pyrifluquinazon may be metabolized to nicotinic acid, nicotinamide and related molecules the Meeting hypothesized that the residual activity in the carcass at 168 h represents residues of the compound that are incorporated into organs and tissues (Yoshizane, 2006b).

In a third ADME study, biliary excretion of pyrifluquinazon was studied in male Fischer rats. A group of three male bile duct-cannulated rats received a single oral administration of 1 mg/kg bw of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon (radiochemical purity 97%) suspended in aqueous 0.5% (w/v) sodium CMC containing 0.1% (w/v) Tween 80. Excreted bile, urine and faeces were collected until 72 h post dose after which the rats were euthanized and the GI contents were collected. Radioactivity and metabolites in the samples were determined. Data on identification of metabolite profiles are presented in Section 1.2.

Total recovery of administered radioactivity was 82%. Excretion of radioactivity into bile, urine and faeces was 34.50%, 11.84% and 4.74%, respectively. Of the administered radioactivity 14% was found in GI contents and 17% in the carcass after 72 h. Based on this, the oral absorption of pyrifluquinazon after 72 h was calculated to be 63%. (Yoshizane, 2006c).

(b) Dermal route

In vivo

The dermal absorption, distribution and excretion of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon (batch no. CP-2842-2; radiochemical purity 98.4%) was studied in vivo in male Sprague Dawley rats (24 per dose group). Pyrifluquinazon was applied to the skin at a concentration of 0.001, 0.01 or 0.1 mg/cm^2. Urine and faeces were sampled over the following intervals: 0–0.5, 0.5–1, 1–2, 2–4, 4–10, and 10–24 hours post dose. Four rats per dose and per time point were euthanized at 0.5, 1, 2, 4, 10 and 24 h. The exposed skin area was washed, and the following tissues and organs collected for measurement of radioactivity: adrenal glands, blood, brain, carcass, eyes, fat (brown), fat (reproductive), heart, kidneys, liver, lungs, muscle, pancreas, skin (test site), spleen, thyroid, testes. Tape stripping of the exposed skin area was not performed.

The mean total recoveries of radioactivity ranged from 88 to 98%. At 24 h after dosing the urine, faeces, skin wash, skin (test site) contained, respectively, 0.70, 2.62, 74.30 and 12.00% of the dose following a 0.001 mg/cm^2 application; 0.37, 1.27, 77.60 and 9.92 % of the dose following a 0.01 g/cm^2 application. Following a 0.1 mg/cm^2 application the comparable values were 0.05, 0.16, 92.60 and 3.47%. The total amount from all collected tissues, including the residual carcass, was 15.20, 11.70 and 3.72%, respectively, following 0.001, 0.01 and 0.1 mg/cm^2 applications, . This indicates that the fraction of the dose absorbed decreases with increased concentrations of pyrifluquinazon. Based on the radioactivity levels in urine, faeces, skin (test site), and other tissues and residual carcass, the total dermal absorption was 18.7, 13.4 and 3.93% of the administered dose following a dermal dose of 0.001 mg/cm^2, 0.001 mg/cm^2 and 0.001 mg/cm^2, respectively. Mean plasma maximum concentration (C_{max}) values increased approximately eight-fold between the 0.001 and 0.01 mg/cm^2 groups, and only approximately two-fold between the 0.01 and 0.1 mg/cm^2 groups, indicating that dermal absorption became saturated between the mid- and high-dose levels. In organs and tissues, highest radioactivity levels were found in the liver and kidney (Musick, LiuPerez & Masterson, 2010).

1.2 Biotransformation

(a) In vivo

Rats

Study 1

The metabolism of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon was studied in groups of male and female Fischer F344/DuCrlcrlj rats treated with low (1 mg/kg bw) or high (100 mg/kg bw) oral doses. The study design and toxicokinetics are described in Section 1.1. Pyrifluquinazon and its metabolites were identified and quantified by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in pooled samples of plasma from high-dose animals obtained 9 h post dosing, and urine and faeces collected over the 0–48 h following a 1 mg/kg bw dose, and collected over the 0–72 h following a 100 mg/kg bw dose. Identification of major metabolites was confirmed by liquid chromatography–mass spectrometry (LC-MS). The amounts of metabolites are expressed as a percentage of applied radioactivity.

[Quinazolinone-phenyl ring-^{14}C] pyrifluquinazon was extensively metabolized in the rat. No significant qualitative differences in metabolite profile between male and female were identified. Unchanged pyrifluquinazon was not observed in urine after a low or high dose, or in faeces following a low dose. Significant amounts of pyrifluquinazon (6–11%) were found in faeces of the high-dose rats. In the faeces of male and female rats, the main metabolites were:

- pyrifluquinazon-aminoquinazolinone-*N*-Ac-4-OH (IV-212) conjugate; 14–17% at low dose, 10–12% at high dose,
- pyrifluquinazon-1H-imino (IV-02); 11–15% at low dose, 12–17% at high dose,
- pyrifluquinazon-1H-4-OH (IV-27) glucuronide; 5–8% at low dose, 6–8% at high dose,
- pyrifluquinazon-8-OH-quinazolinedione (IV-211); 4–5% at low dose, 1–2% at high dose.

In addition, at the high-dose, significant levels (6–8%) of pyrifluquinazon-1H (IV-01) were found.

In urine only significant levels of IV-211 or its glucuronide (5–6% at low dose, 3–4% at high dose) and pyrifluquinazon anthranilic acid (IV-303) or its glucuronide were found (7% at low dose, 5–6% at high dose), with minor levels (< 4%) of pyrifluquinazon-lH-imino oxide (IV-04).

In plasma, the main metabolites were:

- pyrifluquinazon-1H (IV-01)
- pyrifluquinazon-1H-imino (IV-02)
- pyrifluquinazon-quinazolinedione (IV-203) and
- pyrifluquinazon-aminoquinazolinone-*N*-Ac (IV-208).

There were also minor levels of

- pyrifluquinazon-1H-oxide (IV-03)
- pyrifluquinazon-1H-imino-oxide (IV-04)
- pyrifluquinazon-aminoquinazolinone (IV-204)
- pyrifluquinazon-quinazolinone (IV-206)
- pyrifluquinazon-anthranilic acid (IV-303)

(Yoshizane, 2006a).

Study 2

In a second ADME study, the metabolism of [pyridine ring-2,6-^{14}C] pyrifluquinazon (radiochemical purity > 96%) was studied in groups of four male and four female Fischer F344/DuCrlcrlj rats treated with low (1 mg/kg bw, both sexes) or high (100 mg/kg bw, males only) oral doses. The study design and toxicokinetics are described in Section 1.1. Pyrifluquinazon and its metabolites were identified and quantified by TLC and HPLC in pooled samples of blood and liver obtained at 0–3, 3–24 and 24–168 h, brain and heart obtained at 0–168 h after dosing. Excretion of dosed radioactivity into urine and faeces was almost complete within 120 and 72 h post dose, respectively after a 1 mg/kg bw dose, and 120 and 48 h after a 100 mg/kg bw dose (see Section 1.1). Identification of some metabolites was confirmed by nuclear magnetic resonance (NMR) spectrometry.

[Pyridine ring-2,6-^{14}C] pyrifluquinazon was extensively metabolized in the rat. No significant qualitative differences in metabolite profile between male and female were identified. Unchanged pyrifluquinazon was observed, at low levels (2%), only in faeces of high-dose males. In faeces of male and female rats, the main metabolites were:

- pyrifluquinazon-1-H-imino (IV-02); 8–9% in both sexes at low dose,
  10% in males at high dose;
- pyrifluquinazon-1H-4-OH (IV-27) glucuronide;
  2–3 % in both sexes at low dose; 6% in males at high dose;
- pyrifluquinazon-1H (IV-01); 1–2 % in both sexes at low dose; 4% in males at high dose).

In urine only significant levels of pyrifluquinazon-methylnicotinamide (IV-405; 18–21% in both sexes at low dose; 21% in males at high dose) were found, with lower levels of nicotinic acid (IV-403; 2–3% in both sexes at low dose; 1% in males at high dose) and pyrifluquinazon-1H-imino oxide (IV-04; 3% in both sexes at low dose; 4% in males at high dose).

Metabolites in blood, liver, brain and heart were almost solely niacin (vitamin B_3), composed of nicotinamide (IV-404) and nicotinic acid (IV-403) (Yoshizane, 2006b).

Study 3

In a third ADME study, measuring biliary excretion, the metabolism of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon (radiochemical purity > 97%) was studied in groups of three bile duct-cannulated male Fischer F344/DuCrlcrlj rats treated with a 1 mg/kg bw oral dose. The study design and toxicokinetics are described in Section 1.1. Pyrifluquinazon and its metabolites were identified and quantified by TLC and HPLC in bile, urine and faeces obtained over 0–72 h, and GI contents obtained 72 h after dosing. Identification of some metabolites was confirmed by LC-MS.

[Quinazolinone-phenyl ring-^{14}C] pyrifluquinazon was extensively metabolized in the rat. Unchanged pyrifluquinazon was observed only in the GI tract.

In bile the main metabolites were pyrifluquinazon-1H-4-OH (IV-27) and its glucuronide (9%), pyrifluquinazon-8-OH-quinazolinedione (IV-211)-glucuronide (8%) and pyrifluquinazon-aminoquinazolinone-*N*-Ac-4-OH (IV-212; 7%) with lower levels of pyrifluquinazon-anthranilic acid (IV-303; 1.5%). Metabolites IV-02 and IV-04 were identified at levels < 1%.

In urine levels of identified metabolites were < 2% of the administered dose.

In faeces low levels of IV-02 (1%) and IV-01 and IV-203 (both < 1%) were identified. In urine IV-04 (2%) and IV-303 (< 1%) were found. In GI contents parent compound (5%) and IV-01 (4%) were the main residues, with low levels of IV-02 (2%) and IV-203 (1%). The presence of metabolites in the GI tract of bile duct-cannulated rats suggests that microbial or chemical degradation might occur in the intestine (Yoshizane, 2006c).

(b) In vitro

A comparative in vitro metabolism study with [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon (radiochemical purity 98%) was performed using liver microsomes from male rats, male dogs and a female human, and nasal mucosa microsomes from male rats, male and female dogs. Nasal mucosa microsomes were also prepared from the control group and the high-dose group (5 mg/kg bw per day) of the Kosaka (2008) study in dogs. Oxidative metabolism was examined by adding β-NADPH (final concentration 1.0 mM) to a microsomal mixture containing 0.2 µM ^{14}C-pyrifluquinazon. After 60 minutes the reaction was stopped. The metabolites were identified and quantified by TLC-radioluminography.

^{14}C-pyrifluquinazon was rapidly metabolized by the liver and nasal microsomes under examination. No parent compound was detected. The metabolites that were identified with all microsome preparations were IV-01, IV-02, IV-03, IV-04, IV-27 and IV-206. IV-303 was detected only with human liver microsomes and rat nasal microsomes. The metabolites that were formed by microsomes obtained from different species, sexes and tissues, although qualitatively similar, showed quantitative differences in metabolite levels (see Table 5). In particular with human liver microsomes, 70% of the metabolites were not identified (Yoshizane, 2008).

Table 5. In vitro metabolism of [quinazolinone-phenyl ring-^{14}C] pyrifluquinazon

	Percentage of applied radioactivity							
	Liver microsomes			Nasal mucosa microsomes				
	Rat	Dog	Human	Rat	Dog (control group)[a]		Dog (dose group)[b]	
Metabolite	Male	Male	Female	Male	Male	Female	Male	Female
Pyrifluquinazon	ND	ND	ND	ND	ND	ND	ND	ND
IV-01	30.6	31.7	1.07	26.9	41.0	59.1	70.2	69.7
IV-02	16.7	22.5	8.94	9.15	13.7	17.1	4.70	7.24
IV-03	2.34	4.48	2.51	3.97	0.70	0.99	ND	0.39
IV-04	1.45	6.20	5.48	1.29	3.03	2.76	ND	0.43
IV-15	ND	ND	ND	ND	ND	ND	ND	ND
IV-27	4.18	3.36	ND	3.16	3.00	3.65	4.95	4.31
IV-206	3.39	3.20	4.26	4.25	2.05	1.34	ND	1.09
IV-211	ND	ND	ND	ND	ND	ND	ND	ND
IV-212	ND	ND	ND	ND	ND	ND	ND	ND
IV-303	ND	ND	4.68	2.61	ND	ND	ND	ND
Others[c]	38.9	28.6	70.0	46.4	36.0	14.5	19.9	16.5
Unextractable	2.50	1.70	3.01	2.31	0.53	0.55	0.25	0.38
Total	100	100	100	100	100	100	100	100

ND Not detected

Source: Yoshizane, 2008

[a] Control group (undosed) on the "One-year oral toxicity study in dog followed by six month recovery period"

[b] High-dose group (5 mg/kg per day) on the "One-year oral toxicity study in dog followed by six month recovery period"

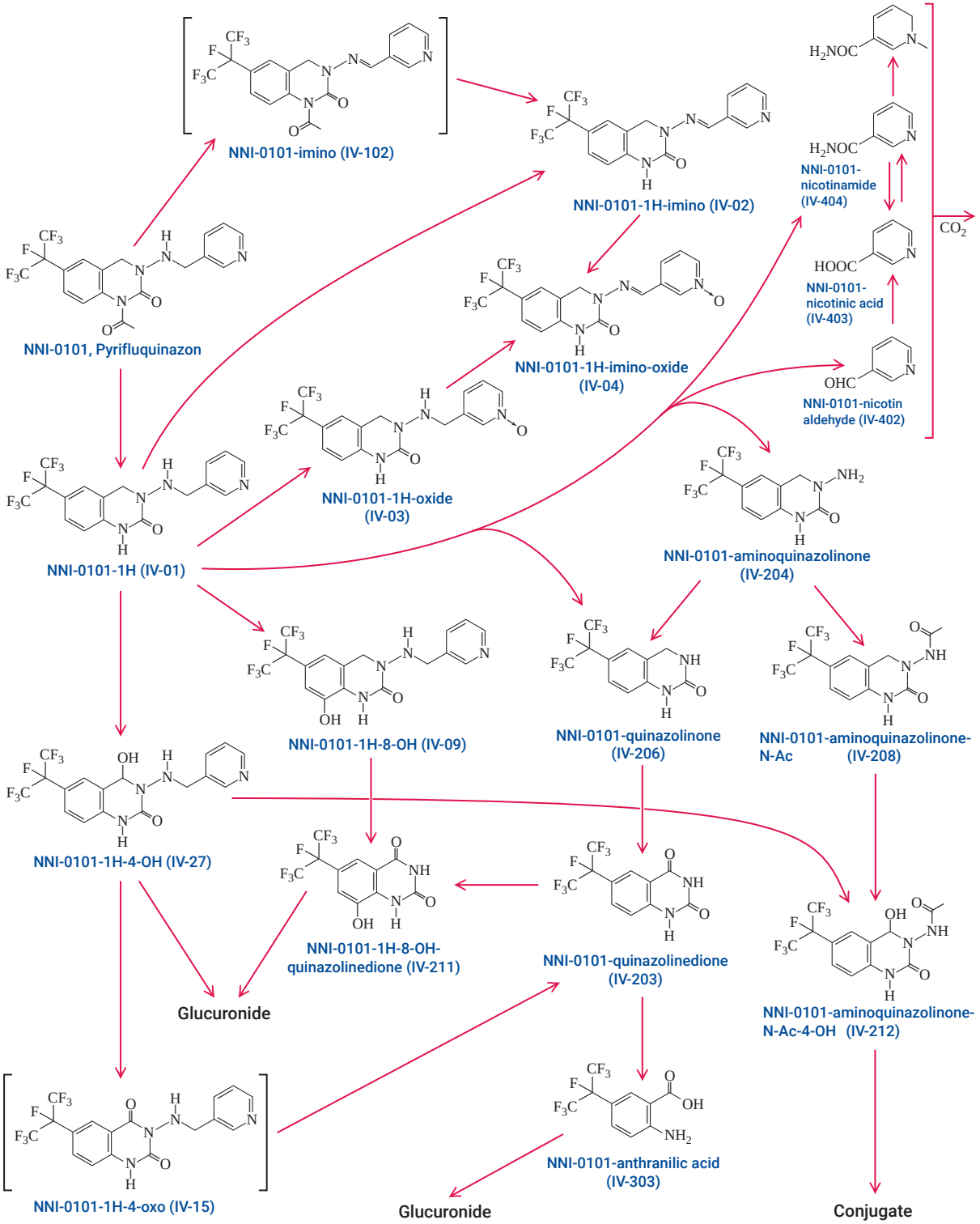
[c] Sum of minor metabolites

In conclusion, the data from the ADME studies in the rat indicate that pyrifluquinazon is relatively rapidly and well absorbed; (about 63% after a 1 mg/kg bw dose, that is, the percentage of the administered dose recovered from urine, bile and carcass in a study with bile duct-cannulated rats) from the GI tract following a single gavage administration of 1 or 100 mg/kg bw. Repeated dose studies of toxicokinetics were not performed. Following a 1 mg/kg bw dose, 34.50% of the administered dose was excreted in the bile collected 72 hours post dose. Studies with 1 and 100 mg/kg bw doses of pyrifluquinazon showed that the C_{max} was not entirely proportional to the administered dose, increasing by only about 60-fold at 100 mg/kg bw compared to the 1 mg/kg bw dose. However, in both sexes the AUC values in blood and plasma calculated up to 168 h post dose were almost proportional to dose. The time to peak plasma concentrations (T_{max}) was 1 h for males and 1–3 h for females after a low dose (1 mg/kg bw), and 9–12 h for males, 9 h for females after a high dose. Oral and dermal absorption were calculated to be 63% and 18%, respectively. Pyrifluquinazon was widely distributed, with highest levels of radioactivity in the liver, lung, kidney, small and large intestine, and the adrenals, with lower levels (around three- to ten-fold lower) in the brain, eyes, heart, thymus, spleen, pancreas, pituitary, stomach, salivary gland, urinary bladder, testes, prostate, uterus, and the ovaries. The major route of excretion was the faeces (75–80%), with lower amounts in the urine (15–20%). For both sexes, elimination was biphasic and almost complete 48 h post dose for the 1 mg/kg bw dose (ca 80%), and 144 h for the 100 mg/kg bw dose (ca 90%).

Numerous metabolites were generated from the parent compound. Pyrifluquinazon represented only a minor component of the excreted material. In addition, results from an in vitro comparative metabolism study show that the identified metabolic pathways for pyrifluquinazon in humans and dogs were qualitatively the same as those in rats. There were no significant differences between the sexes in toxicokinetic parameters or metabolism.

The proposed metabolic pathways are shown on the following page in Fig. 2.

Figure 2. Proposed metabolic pathways for pyrifluquinazon in rats (structures in parentheses are proposed intermediates). Note that pyrifluquinazon is indicated by the sponsor code NNI-0101.



Source: Redrawn from Yoshizane (2006a, 2006b, 2006c)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with pyrifluquinazon are summarized in Table 6.

Table 6. Summary of rat acute toxicity studies with pyrifluquinazon

Strain of rat	Sex	Route	Vehicle	Purity (%)	LD_{50} or LC_{50}	Reference
Fischer 344/ DuCrj	F	Oral	0.5% (w/v) carboxymethyl cellulose sodium salt	98.0	300–2000 mg/kg bw	Horiuchi, 2006a[a]
Wistar	F	Oral	0.5% (w/v) carboxymethyl cellulose	97.0	550–2000 mg/kg bw	Amanuma, 2013[b]
Sprague Dawley (Slc:SD)	M/F	Dermal	Moistened with distilled water	98.0	> 2000 mg/kg bw	Horiuchi 2006b[c]
Fischer 344	M/F	Inhalation	–	98.0	1.2–2.4 mg/L	Janssen, 2005[d]

LC_{50} median lethal concentration LD_{50} median lethal dose;

[a] All animals in the 2000 mg/kg bw group and two of six animals at 300 mg/kg died. In the low-dose group, body weight loss was observed in the first week following treatment, which subsequently recovered in the second week. At 300 mg/kg bw moribundity, prone position, lateral position, crouching position, decrease and loss of locomotor activity, abnormal gait, bradypnoea, hypothermia, piloerection, soiled fur, lacrimation and incontinence of urine were observed. At 2000 mg/kg bw moribundity, decrease and loss of locomotor activity, abnormal gait, bradypnoea, hypothermia, piloerection and lacrimation were observed. No treatment-related effects were observed at necropsy. Batch no. 3FZ0013G.

[b] Rats received a gavage dose of 175, 550 and 2000 mg/kg bw, respectively. In the high-dose group 6/7 (86%) rats died. No mortality was observed in the mid- and low-dose groups. Clinical signs included abnormal gait, decrease/loss of locomotor activity, stained fur, lacrimation, stained urine, lower body temperature, prone/lateral position, crouching position and tremor in the high-dose group. High-dose animals failed to gain weight after dosing, apart from the animal that survived to the end of the observation period. In the high-dose group various abnormalities were observed at necropsy, including stained fur, dark coloured liver, urinary retention, discolouration of the lungs and atrophic thymus. Batch no. 511801.

[c] Rats were dermally treated with pyrifluquinazon at 2000 mg/kg bw. No mortality and no clinical signs were observed. Body weights of the rats were unaffected and no abnormalities were detected at necropsy. Batch no. 3FZ0013G.

[d] Rats were exposed to a four-hour, nose-only aerosol atmosphere at actual concentrations of 1.2, 2.4 and 4.3 mg/L (maximum attainable concentration). The mass median aerodynamic diameter (MMAD) was 2.3, 2.0 and 2.1 µm for the low-, mid- and high-dose, respectively. No mortality was observed in the low-dose group. All animals in the mid- and high-dose group were found in a moribund condition on day 2 after treatment and euthanized. Clinical signs observed in all dose groups included: clonic spasms, dark eyes, hunched posture, lethargy, pale skin, lean body, piloerection, ptosis, slow breathing, chromodacryorrhoea, hypothermia, laboured respiration, ventro-lateral recumbency and/or uncoordinated movements. In the low-dose group, body weight loss was observed in the first week following treatment, which subsequently recovered in the second week. Batch no. 3FZ0013G.

(b) Dermal irritation

In an acute dermal irritation study, the intact skin of three male Japanese White rabbits was exposed for four hours, under semi-occlusion, to 0.5 g pyrifluquinazon (batch no. 3FZ0013G; purity 98.0%) moistened with water. Dermal irritation was scored according to the Draize method 0.5–1, 24, 48 and 72 hours after patch removal.

No skin irritation was observed at any time point (Horiuchi (2006c).

(c) Ocular irritation

In an acute eye irritation study, 0.1 mL (73 mg) of pyrifluquinazon (batch no. 3FZ0013G; purity 98.0%) was instilled into the conjunctival sac of the right eye of each of six male Japanese White rabbits. Three animals were used for the unwashed group and the other three for the washed group. The untreated eye

served as a control. The eyes were examined macroscopically according to the Draize method for signs of irritation 1, 24, 48 and 72 h post instillation.

No change was observed in the cornea and iris of any of the animals treated with pyrifluquinazon. In the conjunctivae, slight congestion (redness score 1) was observed in all animals of the non-washed group one hour after instillation. This reaction remained until 24 hs after instillation in two animals, but disappeared by 48 h after instillation. In the washed group, a similar reaction was observed but disappeared by 24 h after instillation in two animals.

Slight discharge (score 1) in the conjunctivae was observed one hour after instillation in one animal of the non-washed group, but this reaction disappeared by 24 h after instillation. In the washed group, the discharge was not observed during the experimental period.

It was concluded that pyrifluquinazon is not irritating to the eye (Horiuchi, 2006d).

(d) Dermal sensitization

In a dermal sensitization study using the Guinea pig maximization test, pyrifluquinazon (batch no. 3FZ0013G; purity 98.0%) was tested on 20 female Hartley guinea-pigs. The vehicle control group consisted of six animals. As a positive control 2,4-dinitrochlorobenzene (DNCB) was used. In a preliminary study the concentrations of pyrifluquinazon for intradermal induction, topical induction and topical challenge were determined as 5, 50 and 50%, respectively. In the test phase, skin reactions to the challenge were observed 24 and 48 h after removal of the challenge patch. In the control group no skin reaction was observed following challenge by vehicle or test substance. In the pyrifluquinazon group no skin reaction was observed following challenge by vehicle, while skin reactions (grades 1 and 2) were observed following challenge by pyrifluquinazon in four animals at 24 hs, and in two animals after 48 h (grades 1 and 2). The sensitization rate was calculated as 20% and pyrifluquinazon was considered to be mildly sensitizing. The DNCB group gave 100% positive results (Amanuma, 2006a).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a 90-day dietary toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered to groups of ten male and ten female Crj:CD-1(ICR) mice at 0, 60, 750 or 1500 ppm (equal to 0, 7.6, 102 and 206 mg/kg bw per day for males, 0, 9.1, 119 and 202 mg/kg bw per day for females). The animals were checked daily for mortality and clinical signs of toxicity. Body weight and feed consumption was measured weekly. At termination, blood samples were taken for haematology and blood biochemistry. All mice underwent complete necropsy. Brain, pituitary, kidneys, adrenals, spleen, heart, lung, liver, testes, epididymides, thymus, thyroid with parathyroid, ovaries and uterus were weighed. An extensive range of organs and tissues was examined microscopically from all animals in the control and 1500 ppm groups. In addition, thyroids, liver, spleen (males and females), adrenals (males only), testes, epididymides, ovaries and all gross lesions from animals in the 60 and 750 ppm groups were examined microscopically.

No mortality or clinical signs were observed. The major findings in this study are presented in Tables 7 and 8. No treatment-related effect on body weight was observed. An 8% increase in body weight of males at 60 ppm was considered incidental. At 1500 ppm a statistically significant decrease in feed consumption was observed in males at week 1 (19%), and in females almost all weeks throughout the treatment period (20%). A decrease in food efficiency was also observed in males at week 1. Haematological and clinical chemistry findings are presented in Table 7. There were significant decreases in haematocrit (Ht, 7–9%), haemoglobin (Hb, 7–9%) and erythrocyte count (EC, 9–10%) in both males and females at 1500 ppm and in Ht (5%) and Hb (5%) in females at 750 ppm. Males at 1500 ppm showed a significant increase in reticulocyte count (34%) and significant decreases in total leukocyte count (WBC, 48%). Differential leukocyte count at 1500 ppm showed significant decreases in lymphocyte (L, 56%) and eosinophil (E, 50%) counts. Males at 750 ppm showed significant decreases in total leukocyte count (46%) and lymphocyte counts (54%). A significant increase in blood urea nitrogen (BUN) was observed in males at 750 ppm, but not at 1500 ppm, therefore this effect was considered not toxologically relevant.

At 1500 ppm significant increases were observed in aspartate transaminase (AST, 186–625%), alanine transaminase (ALT, 317–772%) in both sexes and in alkaline phosphatase (ALP, 98%) in males. Increases in AST (155%) and ALT (190%) were also observed in males at 750 ppm. In both sexes γ-glutamyl transpeptidase (GGTP) was also significantly increased at 1500 ppm.

In males and females at 1500 ppm significant reductions in total bilirubin (T.Bil, 110–150%), and in glucose (17–25%) were found. Females at 1500 ppm showed a significant increase in inorganic phosphorus (21%) and a reduction in triglycerides (TG; 51%). In males at 750 and 1500 ppm significant reductions in total protein (7 and 8%, respectively), albumin (9 and 8%, respectively), globulin (6 and 8%, respectively) and calcium (6% at both doses) were found.

Table 7. Haematological and clinical chemistry findings of the 13-week dietary toxicity study in mice

	Males				Females			
Diet concentration (ppm)	**0**	**60**	**750**	**1500**	**0**	**60**	**750**	**1500**
Haematology								
Haematocrit (%)	41.9	41.8	40.8	38.9*	43.5	42.6	41.2*	39.8**
Haemoglobin (mmol/L)	13.2	13.1	13.0	12.3*	13.9	13.6	13.2*	12.7**
RBC (10^6/μL)	8.33	8.14	8.06	7.61**	8.63	8.22	8.17	7.77**
Retics (10^9/L)	229.5	225.9	257.7	306.8**	230.0	270.8	259.4	295.3
WBC (10^3/μL)	3.45	2.65	1.86**	1.80**	2.25	2.56	1.86	1.58
Clinical chemistry								
ALP (U/L)	147	174	179	291**	305	226	243	314
AST (U/L)	44	50	112*	319**	64	61	60	183*
ALT (U/L)	29	34	84*	253**	30	23	30	125**
GGTP (U/L)	0	0	0	1**	0	0	0	1**
BUN (mg/dL)	28.1	35.5	40.0**	32.5	29.0	25.7	27.6	24.5
TP (g/dL)	4.81	4.70	4.46*	4.41*	4.69	4.78	4.52	4.55
Albumin (g/dL)	2.90	2.81	2.65*	2.66	2.98	3.02	2.83	2.83
Globulin (g/dL)	1.91	1.90	1.80*	1.75**	1.71	1.76	1.69	1.72
Glucose (mg/dL)	209	213	196	157*	192	185	197	160**
TG (mg/dL)	90	114	105	67	71	96	87	35**
T.Bil (mg/dL)	0.10	0.09	0.13	0.21**	0.08	0.09	0.10	0.20**
Ca (mg/dL)	8.7	8.5	8.2**	8.2**	8.5	8.5	8.2	8.2
Inorganic P (mg/dL)	5.7	5.8	5.7	6.2	5.2	5.4	5.4	6.3*

* $0.01 < p < 0.05$ ** $p < 0.01$

Source: Kuwahara (2005)

Organ weight and histopathology findings are presented in Table 8. In both sexes at 1500 ppm significant increases in the absolute and relative weights of the thyroid (abs. 27–48%, rel. 36–50%), liver (abs. 40–48%, rel 47–58%) and spleen (abs. 27–79%, rel. 34–93%) were found. To a lesser extent weights of liver in both sexes (abs. 26–31%, rel. 21–29%) and thyroid in males (abs. 33%, rel. 25%) were also significantly increased at 750 ppm. In addition, in males at 1500 ppm adrenal weights were significant increased (abs. 41%, rel. 56%). Weights of epididymides were significantly decreased at 750 ppm (abs. 13%, rel. 16%) and 1500 ppm (abs. 18%, rel. 16%). Histopathology showed significant increases in both sexes in incidence of follicular cell hypertrophy in the thyroid and centrilobular hepatocellular hypertrophy at 750 and 1500 ppm, and focal hepatocellular necrosis and cell infiltration in the liver at 1500 ppm. Males at 1500 ppm showed significant increases in the incidence of congestion and increased extramedullary haematopoiesis in the spleen, interstitial cell hyperplasia in the testis, and diffuse cortical cell vacuolation and subcapsular cell hyperplasia in the adrenal. Females at 1500 ppm showed a significant increase in incidence of atrophy in the ovary. No treatment-related changes were observed for any parameters in either sex in the 60 ppm groups.

Table 8. Effects on organ weight and histopathology in the 13-week dietary toxicity study in mice

	Males				Females			
Diet concentration (ppm)	**0**	**60**	**750**	**1500**	**0**	**60**	**750**	**1500**
Thyroid (mg)	3.3	3.0	4.4**	4.9**	3.7	3.5	4.2	4.7*
Thyroid (% bw)	0.008	0.007	0.010*	0.012**	0.011	0.010	0.012	0.015*
Liver (g)	2.15	2.33	2.71**	3.02**	1.57	1.74	2.06**	2.33**
Liver (% bw)	5.06	5.02	6.12*	7.43**	4.68	5.09	6.06**	7.38**
Spleen (mg)	117	108	136	210**	117	121	145	149*
Spleen (% bw)	0.28	0.24	0.31	0.54**	0.35	0.35	0.43	0.47**
Adrenals (mg)	3.9	4.1	4.8	5.5**	9.2	9.8	9.3	9.9
Adrenals (% bw)	0.009	0.009	0.011	0.014**	0.028	0.030	0.028	0.031
Epididymidis (mg)	105	100	91*	86**				
Epididymidis (% bw)	0.25	0.22	0.21*	0.21*				
Histopathology	**(Incidence: number animals affected/number of animals observed)**							
Spleen								
Congestion/hyperaemia	0/10	0/10	0/10	9/10**	0/10	0/10	0/10	0/10
Haematopoiesis, extramedullary, increased	0/10	0/10	0/10	10/10**	0/10	0/10	0/10	0/10
Liver								
Necrosis, hepatocyte, focal	0/10	0/10	0/10	8/10**	0/10	0/10	0/10	4/10*
Hypertrophy, hepatocyte, centrilobular	0/10	0/10	10/10**	10/10**	0/10	0/10	6/10**	10/10**
Cellular infiltration	0/10	0/10	0/10	5/10*	0/10	0/10	0/10	8/10**
Ovary								
Atrophy					0/10	0/10	0/10	7/10**
Testis								
Hyperplasia, interstitial cell	0/10	0/10	0/10	9/10**				
Thyroid								
Hypertrophy, follicular cell	0/10	0/10	5/10*	10/10**	0/10	0/10	6/10**	9/10**
Adrenal								
Vacuolation, cortical cell, diffuse	0/10	0/10	2/10	8/10**	0/10	0/10	0/10	0/10
Hyperplasia, subcapsular cell	0/10	1/10	3/10	5/10*	0/10	0/10	0/10	0/10

* $0.01 < p < 0.05$ ** $p < 0.01$

Source: Kuwahara, 2005

The no-observed-adverse-effect level (NOAEL) was 60 ppm (equal to 7.6 mg/kg bw per day), based on marginally decreased Hb and Ht in females, increases in AST, ALT and thyroid weight, and reductions in total protein, albumin, globulin and calcium, and decreased total leukocyte count (46%) and lymphocyte counts and epididymidis weights in males, and increased liver weights and incidences of follicular cell hypertrophy in the thyroid and centrilobular hepatocellular hypertrophy in both sexes at 750 ppm (equal to 102 mg/kg bw per day) (Kuwahara, 2005).

Rat

In a 90-day dietary toxicity study, pyrifluquinazon (Batch no. 2FZ0009P; purity 96.3%) was administered to groups of ten male and ten female Fischer (F344/DuCrj) rats at 0, 50, 100, 500 or 2500 ppm (equal to 0, 2.9, 5.7, 29 and 155 mg/kg bw per day for males, 0, 3.2, 6.4, 33 and 159 mg/kg bw per day for females). The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Body weights were measured weekly and feed consumption twice per week. Ophthalmology was performed on all animals prior to treatment and on control and high-dose animals at week 13. The animals were subjected to a functional observation test at week 11. At week 13 blood and urine samples were taken for haematology, clinical chemistry and urine analysis. At termination, all rats were necropsied and brain, pituitary, kidneys, adrenals, spleen, heart, lung, liver, testes, epididymides, thymus, thyroid with parathyroid, ovaries and uterus weighed. An extensive range of organs and tissues was examined microscopically from all animals in the control and 2500 ppm groups. In addition, pituitary, thyroid, liver, pancreas, kidneys, spleen, adrenals, testes, epididymides, ovaries, uterus (horns and cervix), vagina, eyes (females only) and all gross lesions from animals in the 50, 100 and 500 ppm groups were examined microscopically.

No deaths were observed. Most of the animals in the 2500 ppm group showed bilateral red adhesive substance in the periocular region. Detailed clinical examination showed no treatment-related effects. At 2500 ppm a decrease in motor activity (35%) and forelimb grip strength (22%) was observed in females, whereas motor activity was increased in males (109%). In the 2500 ppm group, body weight gain was markedly decreased at the early stage of the treatment, specifically at weeks 1–3 in males and at weeks 1 and 2 in females. Mean body weights in males were significantly lower than in controls at week 1–10 (up to 11% at weeks 3–4) and in females throughout the treatment period (7–10%). Feed consumption was decreased in males during weeks 1–4 (up to 17%) and in females during weeks 1–5 (up to 18%). No effects on body weight and feed consumption were observed in the other dose groups.

The key findings of the short-term study are presented in Table 9. Haematology revealed decreases in Ht (4–5%) and Hb (6–8%), mean corpuscular haemoglobin (MCH, 3–5%), and mean corpuscular haemoglobin concentration (MCHC, 2–4%) and an increase in reticulocytes (50–171%) in males and females at 2500 ppm. In addition, a decrease in mean corpuscular volume (MCV) was observed in males (3%), and a decrease in RBC count in females (5%). Males also showed a decrease in WBC (22%) based on a decreased lymphocyte count (25%). In the 500 ppm group, a slight increase in the reticulocyte count was observed in males (11%) and in females (21%, not statistically significant).

Clinical chemistry revealed elevations of AST (159% in females), ALT (239%) and GGTP activities, total bilirubin (60%) and total cholesterol (191%), and slight decreases in total protein (8%) and albumin (10%) in females at 2500 ppm. Small increases in AST, ALP and GGTP and a decrease in cholesterol (32%) were found in males at 2500 ppm. Decreases in calcium (4-6%), sodium (2%) and chloride (2-4%) were observed in both sexes at 2500 ppm.

Organ weight measurements revealed marked increases in absolute and relative weights of liver, heart and thyroid in both sexes at 2500 ppm. In high-dose females there were also increases in absolute and relative weights of, kidneys and spleen, and decreases in absolute and relative weights of, pituitary, adrenals, thymus, uterus and ovaries were observed. Weights of, kidneys, pituitary, adrenals and spleen were moderately increased in high-dose males. Whereas males showed increases in pituitary and adrenals weights, females showed decreases in weights of these organs. In addition, there were slight to moderate decreases in absolute brain weight and increases in relative lung weight in both sexes Remarkably, no change in testis weights were found, although histopathological examination showed atrophy in all high-dose males.

At 500 ppm increases in absolute and relative liver weight were observed in both sexes (rel. 9–14%) and in relative kidney weight in males.

Necropsy revealed darkened colour of the liver in both sexes, with accentuated lobular pattern in males and hypertrophy of the thyroid in both sexes at 2500 ppm.

Histopathological examination of high-dose animals revealed centrilobular hepatocellular hypertrophy, periportal hepatocellular fatty change, and bile duct hyperplasia in both sexes and hepatocellular single cell necrosis in females. In both sexes the following changes were observed:

follicular cell hypertrophy and an increased number of follicles in the thyroid, glomerular mesangium thickening and tubular basophilic change in the kidney, hypertrophy of anterior basophilic cells in the pituitary, and hypertrophy of zona fasciculata cells in the adrenal. Furthermore, haematopoiesis in the bone marrow (more evident in females) and extramedullary haematopoiesis were increased in both sexes while congestion/hyperaemia was found in the spleen of females. Acinar single cell necrosis in both sexes and decreased zymogen granules (males) were seen in the pancreas. Microscopy also revealed retinal atrophy in the eye, general atrophy of the ovary and uterus, and dominant mucus-containing epithelial cells in the vagina in females and tubular atrophy in the testis and intraductal degenerating cells (exfoliated spermatogenic cells) in the epididymis of males.

No treatment-related changes in either sex were noted in the 500, 100 and 50 ppm groups.

Table 9. Key findings of the 90-day feeding study in rats

Diet concentration (ppm)	**Males**					**Females**				
	0	**50**	**100**	**500**	**2500**	**0**	**50**	**100**	**500**	**2500**
Bodyweight	191	173	166**	176	164**	90	88	88	89	69**
Week 4	216	222	218	219	193**	142	142	142	144	128**
Week 8	277	283	276	277	258**	168	164	165	169	157**
Week 13	313	322	312	309	302	182	178	177	182	164**
Haematology										
Ht (%)	47.2	46.5	46.8	46.9	45.1*	45.7	45.8	45.4	46.0	43.6**
Hb (g/dL)	15.7	15.4*	15.6	15.5	14.8**	15.7	15.7	15.6	15.8	14.4**
RBC (10^6/µL)	9.72	9.63	9.72	9.82	9.58	8.97	8.96	8.93	9.14	8.54*
MCV (fl)	48.5	48.3	48.2	47.7**	47.0**	50.9	51.1	50.9	50.4*	51.1
MCH (pg)	16.2	16.0	16.0	15.8**	15.4**	17.5	17.5	17.5	17.3*	16.9**
MCHC (g/dL)	33.3	33.2	33.2	33.0*	32.8 **	34.4	34.3	34.5	34.3	33.0**
Retic's (10^9/L)	160.2	171.9	175.5	177.3*	239.0**	134.2	139.7	144.4	162.1	363.8**
PT (s)	19.8	18.4	18.6	18.8	19.4	16.3	16.4	16.1	16.3	15.0**
WBC (10^3/µL)	7.31	6.65	6.87	6.61	5.73 **	5.53	4.90	4.57*	4.67	5.54
Lymphocytes (10^3/µL)	5.63	5.01	5.19	5.09	4.21 **	4.14	3.91	3.62	3.75	4.23
Blood chemistry										
ALP (U/L)	454	451	452	458	528**	337	330	339	319	369
AST (U/L)	77	77	82	70	87*	69	70	69	66	179**
ALT (U/L)	49	47	49	38**	53	33	34	32	33	112**
GGTP (U/L)	0	0	0	1	1**	0	0	0	1	4**
BUN (mg/dL)	17.8	17.3	17.6	16.4*	16.3*	18.6	18.7	17.7	17.5	33.2
TP (g/dL)	6.34	6.29	6.30	6.31	6.41	6.10	6.04	5.98	6.13	5.59**
Alb (g/dL)	4.38	4.32	4.31	4.35	4.47	4.00	4.01	4.00	4.10	3.58**
A:G ratio	2.23	2.19	2.17	2.23	2.31	1.91	1.98	2.03*	2.03*	1.81
Gluc (mg/dL)	129	128	130	130	132	110	117	112	123**	117
T. cholesterol (mg/dL)	50	51	53	51	34**	64	67	68	77**	186**
TG (mg/dL)	50	57	56	45	5**	14	15	14	16	15
T.Bil (mg/dL)	0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.05	0.05	0.08**
Ca (mg/dL)	10.6	10.5	10.5	10.4	10.2**	10.1	10.0	10.0	9.9	9.5**
Na (mequiv./L)	145.5	145.5	145.0	145.5	143.3**	145.5	145.4	145.6	144.9	143.0**
Cl (mequiv./L)	108.1	108.0	107.8	108.4	105.5**	110.5	110.4	110.7	109.9	105.9**

Diet concentration (ppm)	Males					Females				
	0	50	100	500	2500	0	50	100	500	2500
Organ weights										
Brain (g)	1.91	1.91	1.90	1.91	1.80**	1.82	1.81	1.79	1.81	1.64**
Pituitary (mg)	7.4	7.8	7.4	8.0	8.5**	11.6	11.0	11.4	11.7	6.5**
Pituitary (% bw)	0.0025	0.0025	0.0025	0.0027	0.0030**	0.0068	0.0066	0.0069	0.0068	0.0044**
Thyroid (mg)	19.5	18.9	17.9	20.6	30.1 **	13.3	13.0	13.4	14.2	21.5**
Thyroid (% bw)	0.0066	0.0062	0.0060	0.0070	0.0106**	0.0078	0.0078	0.0081	0.0083	0.0146**
Heart (mg)	888	890	878	898	970*	561	546	550	568	638**
Heart (% bw)	0.30	0.29	0.29	0.31	0.34**	0.33	0.32	0.33	0.33	0.43**
Lung (mg)	1001	1005	1002	1011	1061	786	766	738	797	746
Lung (% bw)	0.34	0.33	0.34	0.34	0.37**	0.46	0.46	0.45	0.47	0.51**
Thymus (mg)	173	172	172	180	154	159	153	156	158	116**
Thymus (% bw)	0.058	0.056	0.058	0.061	0.055	0.094	0.092	0.094	0.093	0.078**
Liver (g)	6.53	6.86	6.70	7.04*	9.62**	3.70	3.63	3.70	4.25*	6.66**
Liver (% bw)	2.20	2.24	2.26	2.40**	3.39**	2.18	2.17	2.23	2.49**	4.52**
Kidneys (g)	1.81	1.88	1.85	1.89	2.03**	1.13	1.10	1.10	1.15	1.45**
Kidneys (% bw)	0.61	0.62	0.62	0.65*	0.71**	0.66	0.66	0.67	0.67	0.99**
Spleen (mg)	601	619	604	609	664*	412	394	396	421	496
Spleen (% bw)	0.20	0.20	0.20	0.21	0.23**	0.24	0.24	0.24	0.25	0.34**
Adrenals (mg)	38.5	38.8	39.0	38.1	49.5**	46.2	42.6	42.5	45.0	34.9**
Adrenals (% bw)	0.013	0.013	0.013	0.013	0.017**	0.027	0.026	0.026	0.026	0.024**
Epididymis (mg)	911	886	901	884	619 **	-	-	-	-	-
Epididymis (% bw)	0.31	0.29	0.31	0.30	0.22**					
Ovaries (mg)	-	-	-	-	-	58.3	56.1	55.1	60.5	34.6**
Ovaries (% bw)						0.034	0.034	0.033	0.035	0.023**
Uterus (mg)	-	-	-	-	-	520	516	592	719	161**
Uterus (% bw)						0.31	0.31	0.36	0.42	0.11**
Histopathology										
Rats examined	10	10	10	10	10	10	10	10	10	10
Bone marrow										
Haematopoiesis, increased	0	-	-	-	3	0	-	-	-	2
Spleen										
Congestion/ hyperaemia	0	0	0	0	0	0	0	0	0	9**
Haematopoiesis extra-medullary, increased	0	0	0	0	9**	0	0	0	0	10**

Diet concentration (ppm)	Males					Females				
	0	50	100	500	2500	0	50	100	500	2500
Liver										
Fatty change, hepatocyte, periportal	0	0	0	0	10**	0	0	0	0	6**
Necrosis, hepatocyte, single cell	0	0	0	0	0	0	0	0	0	10**
Hypertrophy, hepatocyte, centrilobular	0	0	0	0	10**	0	0	0	0	10**
Hyperplasia, bile duct	0	0	0	0	8**	0	0	0	0	7**
Pancreas										
Necrosis, acinar cell, single cell	0	0	0	0	6**	0	0	0	0	8**
Decreased zymogen granules	0	0	0	0	10**	0	-	-	-	0
Kidney										
Tubular basophilic change	0	0	0	0	5**	0	0	0	0	10**
Thickening, glomerular mesangium	0	0	0	0	3	0	0	0	0	10**
Testis										
Atrophy, seminiferous tubule	0	0	0	1	10**	-	-	-	-	-
Epididymides										
Degenerated cell, ductal lumen	0	0	0	0	10**	-	-	-	-	-
Ovary	-	-	-	-	-					
Atrophy						0	0	0	0	10**
Uterus	-	-	-	-	-					
Atrophy						0	0	0	0	10**
Vagina	-	-	-	-	-					
Mucus-containing cell, epithelium						0	0	0	0	10**
Pituitary										
Hypertrophy, basophilic cell	0	0	0	0	10**	0	0	0	0	8**
Thyroid										
Hypertrophy, follicular cell	0	0	0	0	10**	0	0	0	1	10**
Increased number of follicles	0	0	0	0	10**	0	0	0	0	9**

Diet concentration (ppm)	Males					Females				
	0	50	100	500	2500	0	50	100	500	2500
Adrenal										
Hypertrophy, zona fasciculata cell	0	0	0	0	10**	0	0	0	0	10**
Eye										
Atrophy, retina	0	-	-	-	0	0	0	0	0	10**

Level of statistical significance: * $0.01 < p < 0.05$, ** $p < 0.01$ in comparison to control

Source: Nakashima (2004)

The NOAEL was 500 ppm (equal to 29 mg/kg bw per day), based on effects on body weight, and various changes in haematology, clinical chemistry, organ weights and histopathological parameters at 2500 ppm (equal to 155 mg/kg bw per day) (Nakashima, 2004).

Dog

Study 1

In a 90-day oral toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered by gelatine capsule to groups of four male and four female beagle dogs at a dose of 0, 2, 5 or 30 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Ophthalmoscopy was performed prior to treatment and during the last week of treatment. Body weights were measured pretreatment and weekly during treatment. Feed consumption was recorded daily. Blood and urine were sampled prior to treatment, during weeks 7 and 13 of treatment for haematological and clinical biochemistry examinations and urine analysis. After termination, all dogs were macroscopically examined, and weights of brain, pituitary, thyroids with parathyroids, heart, thymus, liver with gallbladder, kidneys, spleen, adrenals, testes, epididymides, prostate, ovaries and uterus were determined. Microscopic examination was performed on a wide range of organs and tissues of all animals.

No mortality or treatment-related effects on clinical signs, feed consumption, ophthalmology, urinalysis, haematological examination, and necropsy were observed. Effects on body weight, clinical chemistry, haematology and histopathology are presented in Table 10.

Body weight gains were decreased compared to controls in both sexes at 5 and 30 mg/kg bw per day, more markedly so in males than in females. The shortfall in females at 5 mg/kg bw per day was largely due to a single animal that showed a body weight loss over the course of the study. When body weight data from this animal were excluded, the body weight gain in this group was 0.7 kg, compared to an increase in controls of 1.1 kg. Ht and RBC were slightly reduced in high-dose males, but this was evident prior to treatment, hence was not an effect of pyrifluquinazon. Increased ALP levels were seen in males at mid dose (40–63%) and high dose (147–216%) and in females at the high dose (445–703%). In both sexes ALT was slightly increased at the high dose (55–85%). Albumin was reduced in high-dose animals of both sexes (10–15%) and a decrease in calcium (6%) was noted in high-dose males. High-dose animals of both sexes showed increases in absolute (39–52%) and relative (49–56%) liver weights. Females also displayed trends suggesting an increase in absolute (53%) and relative (56%) thyroid weights. Histopathology revealed diffuse hepatocellular hypertrophy in the liver in all high-dose males and females. Follicular cell hypertrophy in the thyroid was observed in one male and one female at the high dose and one female at the mid dose.

The NOAEL for males and females was 2 mg/kg bw per day, based on reduced body weight gain in both sexes at 5 mg/kg bw per day (Takeuchi, 2005).

Table 10. Key results of the 13-week oral study in dogs

	Males				Females			
Dose (mg/kg bw per day)	**0**	**2**	**5**	**30**	**0**	**2**	**5**	**30**
Body weight gain week 0–13 (kg)	1.5	1.2	0.9	0.8	1.1	0.9	0.5	0.8
Haematology								
Haematiocrit (%), week 13	48.2	46.8	48.7	44.4*	49.4	48.8	49.2	48.4
RBC (10^6/µL), week 13	7.32	7.25	7.37	6.85*	7.43	7.58	7.45	7.41
Blood chemistry								
ALP (U/L)								
week 7	371	328	517*	915**	222	325	392	1210**
week 13	261	249	426*	824**	167	245	315	1342**
ALT (U/L)								
week 13	27	34	34	50**	33	24	39	51*
Albumin (g/dL)								
week 7	3.26	3.10*	3.06*	2.83**	3.17	3.26	3.21	3.02
week 13	3.26	3.10	3.09	2.77**	3.31	3.28	3.17	2.97**
A:G ratio								
week 7	1.25	1.16	1.18	0.98**	1.28	1.25	1.35	1.14
week 13	1.16	1.10	1.08	0.90**	1.25	1.22	1.26	1.03**
Calcium (mg/dL)								
week 7	10.5	10.4	10.3	9.9**	10.4	10.3	10.4	10.1
week 13	10.4	10.2	10.2	9.8*	10.4	10.2	10.3	10.0
Organ weights								
Liver (g)	260	263	272	361**	230	240	240	350**
Liver (% bw)	2.37	2.46	2.60	3.52**	2.43	2.60	2.71	3.78**
Histopathology (incidence)								
Liver: hypertrophy, hepatocyte, diffuse	0/4	0/4	0/4	4/4*	0/4	0/4	0/4	4/4*
Thyroid: follicular cell hypertrophy	0/4	0/4	0/4	1/4	0/4	0/4	1/4	1/4
Prostate: atrophy	0/4	0/4	0/4	2/4				

Level of statistical significance: * $p < 0.05$ different from control ** $p < 0.01$ different from control

Source: Takeuchi (2005)

Study 2

In a one-year oral toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered by gelatine capsule to groups of four male and four female beagle dogs at a dose of 0, 1.5, 5 or 15 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Ophthalmoscopy was performed prior to treatment and during week 52 of treatment. Body weights were measured pretreatment and weekly from week 1–13 of treatment and once every four weeks thereafter. Feed consumption was recorded daily. Blood and urine were sampled prior to treatment, during weeks 13, 26 and 52 of treatment for haematological and clinical biochemistry examinations and urine analysis. After termination all dogs were macroscopically examined and weights of brain, pituitary, thyroids with parathyroids, heart, liver with gallbladder, kidneys, spleen, adrenals, testes, epididymides, prostate, ovaries and uterus were determined. Microscopic examination was performed on a wide range of organs and tissues of all animals.

No animals died during the study. There were no treatment-related effects on clinical signs, feed consumption, ophthalmology, urinalysis, and necropsy. Body weight and body weight gain in males at 15 mg/kg bw per day were lower than those of the control, but almost the same as those of the low-dose, 1.5 mg/kg bw per day group. Body weights and body weight gains at 5 mg/kg bw per day were higher than those of the control. Therefore, the effect of treatment on body weight was unclear. Platelet counts were increased in high-dose males at week 26 but not at other time points, with no clear dose–response relationship and not in females. Therefore this finding was considered incidental. Males and females at 15 mg/kg bw per day showed a significant shortening of activated partial thromboplastin time at weeks 26 and 52. However, as the effects were minimal (up to 1 second), and as prothrombin time was not affected, this finding was considered not toxicologically relevant. Treatment-related effects on clinical chemistry, organ weights and histopathology are presented in Table 11.

Clinical chemistry showed a significant increase in ALP (70–201%) in high-dose males and females. Albumin was reduced in males at mid dose (up to 9%) and high dose (up to 7%) at 52 weeks. Other effects on clinical chemistry parameters were not dose-dependent or found in isolation and were therefore considered incidental.

High-dose males showed increases in absolute (20%, not statistically significant) and relative (29%) liver weights and absolute (33%, not statistically significant) and relative (42%) thyroid weights. Smaller (not statistically significant) increases in liver and thyroid weights were observed in high-dose females. Histopathology showed centrilobular hepatocellular hypertrophy in the liver of all high-dose males and females. In addition, at the high dose mononuclear cell infiltration of the lumina propria of the olfactory epithelium was observed in three males and all females, while one female showed a severe lesion associated with alteration/necrosis of the olfactory epithelium. At the mid dose, mononuclear cell infiltration in the lumina propria of the olfactory epithelium was observed in two males and two females. Among these dogs, one male showed severe mononuclear cell infiltration associated with alteration/necrosis of the olfactory epithelium. At the low dose mononuclear cell infiltration of the lumina propria of the olfactory epithelium was observed in one male and three females. The Meeting considered this to be an early indicator of alteration/necrosis of the olfactory epithelium observed at the mid and high doses. No mononuclear cell infiltration of the lumina propria of the olfactory epithelium was observed in any of the controls.

The lowest-observed-adverse-effect level (LOAEL) was 1.5 mg/kg bw per day based on slight to moderate mononuclear cell infiltration of the lumina propria of the olfactory epithelium (Kuwahara, 2006a).

Table 11. Key results of the one-year oral study in dogs

		Males				Females			
Dose (mg/kg per day)		**0**	**1.5**	**5.0**	**15**	**0**	**1.5**	**5.0**	**15**
Blood biochemistry									
ALP (U/L)	Pre-test	368	366	372	307	324	302	318	345
	Week 13	289	269	398	490*	208	261	395	542*
	Week 26	215	205	357	589*	180	266	422	542**
	Week 52	210	204	390	683	205	258	474	660*
Albumin (g/dL)	Pre-test	3.00	2.95	2.95	3.05	3.05	3.12	2.95	3.07
	Week 13	3.21	3.16	3.00	2.98	3.12	3.16	2.99	3.04
	Week 26	3.08	3.12	2.94	2.89	3.12	3.14	2.97	2.97
	Week 52	3.03	3.02	2.76**	2.83*	3.01	3.02	2.80	2.87
Organ weights									
Thyroid (mg)		667	763	740	885	708	836	761	881
Thyroid (% bw)		0.0059	0.0071	0.0063	0.0084*	0.0071	0.0079	0.0071	0.0083
Liver (g)		288	312	312	346	263	297	305	321
Liver (% bw)		2.51	2.92	2.67	3.25**	2.61	2.82	2.83	3.02

	Males				Females			
Dose (mg/kg per day)	**0**	**1.5**	**5.0**	**15**	**0**	**1.5**	**5.0**	**15**
Histopathology (incidence)								
Liver centrilobular hepatocyte hypertrophy	0/4	0/4	0/4	4/4*	0/4	0/4	0/4	4/4*
Nasal cavity mononuclear cell infiltration (respiratory epithelium)								
slight	0/4	0/4	1/4	0/4	0/4	0/4	1/4	0/4
mononuclear cell infiltration (olfactory region)								
slight	0/4	0/4	1/4	1/4	0/4	2/4	1/4	1/4
moderate	0/4	1/4	0/4	2/4	0/4	1/4	1/4	2/4
severe	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4
combined	**0/4**	**1/4**	**2/4**	**3/4**	**0/4**	**3/4**	**2/4**	**4/4***
olfactory epithelium degeneration/ necrosis	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
moderate	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
severe	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4

Level of statistical significance: * $p < 0.05$ ** $p < 0.01$

Source: Kuwahara, (2006a).

Study 3

In an additional one-year oral toxicity study, performed to further study the effect of pyrifluquinazon on the nasal cavity, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered by gelatine capsule to groups of four male and four female beagle dogs at doses of 0, 0.15, 0.5 or 5 mg/kg bw per day. An additional group of four female beagle dogs was treated with 5 mg/kg bw per day for one year, followed by a recovery period of 26 weeks. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Ophthalmoscopy was performed prior to treatment and during week 52 of treatment and at recovery week 26. Body weights were measured pretreatment and weekly thereafter. Feed consumption was recorded daily. Blood and urine were sampled prior to treatment, during weeks 13, 26, 39 and 52 of treatment and at recovery week 26 for haematological and clinical biochemistry examinations and urine analysis. After termination all dogs were macroscopically examined, and weights of heart, spleen, thymus, liver with gallbladder, kidneys, testes, epididymides, prostate, ovaries, uterus, brain, pituitary, thyroids with parathyroids and adrenals were determined. Microscopic examination was performed on a wide range of organs and tissues of all animals.

No animals died during the study. There were no treatment-related effects on clinical signs, feed consumption, ophthalmology, urinalysis, and necropsy. Occasional statistically significant findings on haematology, clinical chemistry were small, not observed at other time points and/or not dose-dependent and were considered not to be treatment-related. After 52 weeks the body weight gains in males of the 5 mg/kg bw per day group (1.9 kg) were lower than those in the control group (3.2 kg). At this dose, body weight gains were statistically different from controls after 26 and 39 weeks of treatment. It is noted however that no effects on body weight gain were observed at doses up to 15 mg/kg bw per day in the previous one-year study in dogs (Kuwahara, 2006a).

Treatment-related effects on histopathology are presented in Table 12. Histopathological examination showed mononuclear cell infiltration in the olfactory mucosa of nasal cavity in two males (degree of lesion "slight") and one female (degree of lesion "moderate") in the 5 mg/kg bw per day group at the end of treatment. This finding was similar to the lesion reported in the one-year study by Kuwahara (2006), suggesting that the lesions are treatment-related. No other lesions were detected in the nasal cavity. In addition, there were no changes in white blood cell numbers or differential leukocyte counts, or any blood biochemical readings. These results suggest that mononuclear cell infiltration was a focal lesion limited to the olfactory mucosa. No lesions in the nasal cavity were detected in females of

the 5 mg/kg per day group following the 26-week recovery period, perhaps suggesting that mononuclear cell infiltration in the olfactory mucosa is reversible after cessation of treatment, but the number of animals tested was too small to provide a definitive conclusion.

The NOAEL was 0.5 mg/kg bw per day, based on mononuclear cell infiltration in the nasal cavity in both sexes at 5.0 mg/kg bw per day (Shibuya, 2008).

Table 12. Key results of one-year oral study in dogs to follow up nasal cavity effects

	Males				Females			
Dose (mg/kg per day)	**0**	**0.15**	**0.5**	**5.0**	**0**	**0.15**	**0.5**	**5.0**
Bodyweight (kg)								
Week 52	12.7	11.8	12.8	11.5	10.1	10.3	11.8	10.2
Recovery week 26							11.8	
Histopathology (incidence)								
Nasal cavity mononuclear cell infiltration (olfactory region)	0/4	0/4	0/4	2/4	0/4	0/4	0/4	1/4

Level of statistical significance: * $p < 0.05$ ** $p < 0.01$

Source: Shibuya (2008)

(b) Dermal application

In a 28-day dermal toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered to groups of ten male and ten female Sprague Dawley (Crl:CD(SD)) rats at 0, 40, 200 or 1000 mg/kg bw per day, for five days per week, six hours per day. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Body weights and feed consumption were measured prior to treatment, weekly throughout the study and on the day of the scheduled necropsy. The rats were subjected to ophthalmoscopy prior to treatment and near the end of the treatment period. Blood was sampled on the day of necropsy for haematological and clinical biochemistry examinations and urine was sampled for analysis on days 22–23 of treatment. All the rats were macroscopically examined, and weights of brain (as cerebrum, cerebellum, medulla oblongata), pituitary, thyroid (including parathyroid), adrenal, thymus, spleen, heart, liver, kidney, testis/ovary, epididymis/uterus, prostate, seminal vesicle were determined. An extensive range of organs and tissues of all control and high-dose rats was microscopically examined.

There were no treatment-related effects on any of the parameters tested. The application site showed no macroscopic or microscopic abnormalities.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Ishida, 2010).

(c) Exposure by inhalation

In a four-week inhalation toxicity study, groups of ten male and ten female Sprague Dawley (Crl:CD(SD)) rats were exposed to pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%), nose-only, by inhalation at actual mean analytical concentrations of 0, 0.042, 0.157 or 0.56 mg/L air for six hours per day, five days per week. The average mass median aerodynamic diameter was 2.0–3.3 μm (geometric standard deviation 2.1–2.2). The rats were checked daily for mortality and clinical signs. Detailed physical examinations were performed prior to treatment and weekly during treatment. Ophthalmoscopy was performed before treatment on all animals and in week 4 in control and high-dose animals. Body weights and feed consumption were determined prior to treatment, weekly during treatment and at termination (body weight only). At day 25 of treatment blood was sampled following overnight starvation, for haematology and clinical chemistry. As high-dose females were in poor condition following starvation prior to blood collection for clinical pathology, blood from this group was collected at necropsy without starvation. Urine was collected on day 29 for urine analysis. All the animals underwent necropsy, and the weights of adrenals, heart, spleen, brain, liver, kidneys, thymus, ovaries, uterus, testes with

epididymides, seminal vesicles and lungs determined. Histopathological examinations were performed on a wide range of organs and tissues from the control and high-dose animals, on heart and respiratory tract tissues from all animals and on one rat that was found dead.

One high-dose female was euthanized on day 25 due to sluggish behaviour, coloured tears and generally moribund appearance. Macroscopic findings for this animal included dark lungs with several dark foci, dark uterus and a softened brain. Microscopic findings for this female included inflammatory cell foci and centrilobular hypertrophy in the liver, lymphoid atrophy of the spleen, thymic cysts, inflammatory cell foci in the lung and inflammatory cells/alveolar macrophages in the terminal airway. Agonal congestion/haemorrhage was present in the lung and uterus. No microscopic findings were noted in the brain. The deterioration in condition was considered due to effects of the test article in combination with the starvation prior to clinical pathology blood sampling.

Treatment-related clinical signs that were observed after exposure, predominantly in high-dose rats included splayed gait, ataxia, palpebral closure, breathing irregularities, piloerection and lethargy. In the first week of treatment intermediate dose rats showed piloerection during first hour after exposure. No ophthalmoscopic abnormalities were observed. Body weights were lower than controls in high-dose males and females (by up to 7%) from day 8 onwards. Feed consumption was lower (7–10%) throughout the study for both sexes at the high dose.

High-dose males also had a statistically lower mean cell volume (5%) and higher prothrombin time (11%). Clinical chemistry revealed no toxicologically relevant effects of treatment. Samples taken for haematology and clinical chemistry from high-dose females were not considered comparable as they were taken at terminal necropsy rather than at day 25; these data were not, therefore, given further regard. Urine volume in high-dose males and females was higher than in control animals (46–267%). Individual data indicated that the urine of all high-dose males contained calcium oxalate crystals. In high-dose animals, increases in relative weights of liver (21–35%) and lung (7–17%) were observed. Relative liver weight was also increased in mid-dose males (21%). Centrilobular hypertrophy was observed in all high-dose animals. In the lung, the level of terminal airway inflammatory cells/alveolar macrophages was higher in mid- and high-dose males and in high-dose females.

The no-observed-adverse-effect concentration (NOAEC) was 0.042 mg/L, based on clinical signs, increased relative liver weights and some histopathological findings in the lungs at 0.157 mg/L which can be considered a marginal lowest-observed-adverse-effect concentration (LOAEC) (Shaw, 2009).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In an 18-month dietary carcinogenicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered to groups of 52 male and 52 female ICR (Crj:CD-1) mice at 0, 60, 250 or 1000 ppm (equal to 0, 6.3, 27 and 122 mg/kg bw per day for males, 0, 5.8, 25 and 120 mg/kg bw per day for females). The mice were checked daily for mortality and clinical signs and weekly for palpable masses. Body weights and feed consumption were recorded weekly during the first three months and every four weeks thereafter. Blood was sampled for haematological examinations at termination. Blood smears were prepared after 52 weeks, at termination and from all animals killed in extremis. Animals found dead or euthanized before, or at the end of, the treatment period underwent necropsy. Organs (adrenals, brain, heart, liver with gallbladder, kidneys, thyroids with parathyroids, spleen, ovaries, uterus, testes and epididymides) from ten animals per sex and per dose were weighed. A wide range of tissues was examined microscopically from all control and high-dose animals and from all low- and mid-dose animals found dead during the treatment period. In addition, the thyroid, liver, nasal cavity from both sexes, testis, epididymides, seminal vesicle, coagulating gland, and adrenals from males, and the mammary gland, pancreas, and uterine horn from females, and all gross lesions from all animals killed at termination in the 60 and 250 ppm groups, were examined.

There was no effect of treatment on survival rate. There were higher incidences of tactile hair loss and loss of fur in males at the mid and high doses, although the substance-dependency of effects at the mid dose was equivocal. In addition high-dose males showed an increased incidence of wetted fur and high-dose females showed an increased incidence of fur loss. Body weight gain was decreased, starting from 2–4 weeks and throughout treatment thereafter in high-dose males (11% at termination) and females (26% at termination) compared to controls. Body weight was not affected at week 1. In males at 250 ppm, mean body weight was low from week 8–56 (by up to 8%). Feed consumption was not affected. Haematology showed no treatment-related effects. Necropsy revealed increased incidences of dark coloured liver (7/32) and liver with coarse surface (5/32) in high-dose males compared to controls (0/34 and 0/34 respectively). Examination of the testes of animals killed in extremis or found dead, or animals examined at termination, revealed increased incidences of spots (14/52) and masses (6/52) in high-dose males compared to controls (2/51 and 0/51, respectively). Significant increases in the incidence of pelvic dilatation of the kidney, and soiled fur in abdominal and external genital region were noted in high-dose males killed in extremis or found dead.

Absolute brain weights were significantly reduced in males at 250 ppm (6%) and 1000 ppm (7%), while relative brain weight was increased (25%) in high-dose females. The toxicological significance of this finding is not clear. In addition, at the high dose significant increases in weights of heart (16–26% rel. in both sexes), thyroids (25–27% rel. in both sexes), liver (30–34% abs., 51–68% rel. in both sexes) kidneys (40% rel. in females) and epididymides (27%, abs.) were found.

The effects of treatment on histopathology are presented in Table 13. At the high dose, increases in the incidence of hepatocytic focal and single cell necrosis (both sexes) and centrilobular hepatocyte hypertrophy (males), thyroid follicular cell hypertrophy (both sexes), intracytoplasmic eosinophilic bodies in mucosal epithelial (respiratory and olfactory) cells (both sexes) and in olfactory epithelial cells (males), diffuse atrophy of acinar cells in the pancreas (females), endometrial hyperplasia of the uterine horn and mammary gland hyperplasia were observed. In mid- and high-dose males increased incidence of subcapsular cell hyperplasia in the adrenals was observed.

In the 1000 ppm group, a statistically significant and treatment-related increase in the incidence of benign interstitial cell tumours in the testis was observed in 12/52 males (23%), compared with 0/51 in the control group. Such tumours are rare in mice and occurred in the test laboratory's historical control data at only 0.39%. High-dose males also showed a significant increase in the incidence of interstitial cell hyperplasia at terminal kill. A significant increase in incidence of this lesion was also observed in the high-dose group (1500 ppm) in the previously conducted 90-day repeated dose, oral toxicity study by Kuwahara (2005). The study's authors suggested it is possible that the antiandrogenic activity of the test substance (postulated on the basis of the reproductive study in rats) causes atrophy of the seminiferous tubule which stimulates reactive proliferation of interstitial cells, elevating testosterone levels and creating an increase in the incidence of interstitial cell tumours. There were no increases in the incidence of other neoplastic lesions.

Table 13. Effects of pyrifluquinazon treatment on histopathology in mice

	Males				Females			
Diet concentration (ppm)	**0**	**60**	**250**	**1000**	**0**	**60**	**250**	**1000**
Nasal cavity: (*N* =)	51	52	52	52	52	52	52	52
Intracytoplasmic eosinophilic body (IEB), respiratory epithelial cell	2	7	3	19**	12	12	6	23*
IEB, olfactory epithelial cell	5	3	7	28**	11	8	7	18
Liver: (*N* =)	51	52	52	52	52	52	52	52
Fatty change, hepatocyte, centrilobular	8	9	1*	1*	1	1	0	0
Necrosis, hepatocyte, focal	1	0	1	18**	0	4	4	0
Necrosis, hepatocyte, single cell	9	7	8	26**	2	2	3	32**
Hypertrophy, hepatocyte, centrilobular	0	0	0	42**	0	0	0	46**
Microgranuloma	3	1	1	0	5	5	1	0*
Pancreas: (*N* =)	51	19	20	52	52	52	52	52
Atrophy, acinar cell, diffuse	0	0	0	0	0	0	0	8**
Testis: (*N* =)	51	52	52	52	0	0	0	0
Hyperplasia, interstitial cell	0	1	0	9**	-	-	-	-
Uterine horn: (*N* =)	0	0	0	0	52	52	52	52
Hyperplasia, endometrium	-	-	-	-	23	24	31	33*
Adenomyosis	-	-	-	-	8	6	6	1*
Mammary gland: (*N* =)	0	0	0	0	52	52	52	52
Hyperplasia, glandular epithelial cell	-	-	-	-	5	6	11	19**
Thyroid: (*N* =)	51	52	52	52	52	52	52	52
Hypertrophy, follicular cell	0	0	3	11**	0	0	0	22**
Adrenal: (*N* =)	51	52	52	52	52	14	16	52
Hyperplasia, subcapsular cell	18	21	28*	32**	37	6	6	39
Neoplastic findings (all animals examined)								
Testis: (*N* =)	51	52	52	52	0	0	0	0
Interstitial cell tumour	0	0	0	12**	-	-	-	-

* $p < 0.05$ different from control by Fisher's Exact Test ** $p < 0.01$ different from control by Fisher's Exact Test
\- No finding

Source: Kuwahara (2006b)

The NOAEL was 60 ppm (equal to 6.3 mg/kg bw per day) based on an increased incidence of adrenal subcapsular cell hyperplasia in males at 250 ppm (equal to 27 mg/kg bw per day). At 1000 ppm (equal to 122 mg/kg bw per day) an increase in the incidences of interstitial cell hyperplasia and benign tumours in the testis was observed. There were no increases in the incidence of other neoplastic lesions. The NOAEL for carcinogenicity was 250 ppm (equal to 27 mg/kg bw per day) (Kuwahara, 2006b).

Rat

In a one-year toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered to groups of 20 male and 20 female Fischer (F344/DuCrj) rats in the diet at 0, 100, 350 or 1300 ppm (equal to 0, 4.1, 14 and 56.5 mg/kg bw per day for males, 0, 5.0, 18, and 66 mg/kg bw per day for females). All rats were checked daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weights and feed consumption were recorded weekly during the first three

months and every four weeks thereafter. Blood and urine from ten animals per sex and per dose were sampled for haematological and clinical chemistry examinations and urine analysis at weeks 13, 26 and at termination in week 52. Ophthalmic examinations were conducted prior to test substance exposure and during study week 52. A functional observation battery (FOB) was performed on ten animals/sex per group at week 49. Smears from femur bone marrow were prepared from ten animals per dose and per sex at termination. All animals were necropsied. A number of organs (adrenals, brain, heart, liver, kidneys, thyroids (with parathyroid), spleen, ovaries, uterus, testes and epididymides) were weighed. A wide range of tissues was examined microscopically from all control and high-dose animals and all animals found dead from the other dose group. In addition, gross lesions from all animals and the pituitary, testis, and epididymis from males, and the heart, spleen, eye, adrenal, and pancreas from females of the 100 and 350 ppm groups were examined microscopically.

Mortality was not affected by treatment. The results on clinical observations, haematology and clinical chemistry are presented in Table 14. No effect of treatment on clinical signs, detailed clinical examination and ophthalmoscopy were observed. Slight increases in body weight gain up to week 13 and slight reductions in bodyweight gain (up to 6%) from week 44 onward were observed in high-dose females. Feed consumption was increased in high-dose males throughout treatment (9% over the entire treatment period) and in females during the first 13 weeks of treatment. Detailed clinical examination showed a statistically significant increase in rearing in females at 1300 ppm intermittently from week 3 to week 28. A statistically significant decrease in forelimb and hind limb grip strength was seen in the FOB test in females at 1300 ppm.

For males treated at 1300 ppm haematological examinations showed statistically significant decreases in haemoglobin concentration, mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCHC) throughout the treatment period, and a significant decrease in hematocrit at weeks 26 and 52, and increases in reticulocyte count and bone marrow nucleated cell count. The study's author considered these effects to be adaptive responses to microcystic hypochromic anaemia caused by pyrifluquinazon. Females treated at 1300 ppm and males treated at 350 ppm showed small decreases in MCV and MCHC and small increases in red blood cell count throughout all examination periods. The concomitant increases or increasing trends in reticulocyte count and bone marrow nucleated cell count (BMNC) suggested that these effects, though small, were treatment-related. The slight haematological changes observed at 100 and 350 ppm were considered not toxicologically significant.

Clinical chemistry showed significant decreases or decreasing trends in triglyceride and chloride in both sexes at 1300 ppm, total cholesterol decrease in males and females, an increase in γ-glutamyl transpeptidase (GGTP), and a significant decrease in calcium. The decrease in calcium in females and the decrease in chloride in both sexes at 1300 ppm, and in females at 350 ppm, was considered indicative of kidney effects, as these changes were accompanied by increases in kidney weight. Other statistically significant changes were not considered treatment-related or toxicologically relevant as they were transient, not consistent, not dose-related or not adverse.

High-dose males urinalysis showed statistically significant decreases in ketones at weeks 13 and 26, increase in urine volume at weeks 26 and 51, and increase in protein levels at week 51. The effects were considered treatment-related and toxicologically relevant since they were accompanied by an increase in kidney weight and because in a 90-day study in rats similar effects and histopathological changes in the kidney were observed at 2500 ppm (Nakashima, 2004). Increases in pH observed at week 13 in females at 350 ppm, and increase in specific gravity, observed in females at 1300 ppm at week 26 were considered incidental.

Necropsy showed no treatment-related effects.

The results for organ weights and histopathology are presented in Table 15. At 1300 ppm, animals of both sexes showed significant increases in the absolute and relative weights of the heart, liver and kidneys, and a significant decrease in the absolute weight of the brain. Males at 1300 ppm showed a significant increase in the absolute weight of the adrenals and increasing trends in the absolute and relative weights of the thyroids, along with a significant increase in the relative weight of epididymides. High-dose females showed significant increases in the absolute and relative weights of the thyroids and the relative weight of the spleen, and significant decreases in the absolute and relative weights of the uterus. Significant increases in the relative weights of the liver and kidneys in animals

of both sexes and increases in the relative weight of the heart, and the absolute weights of the liver and kidneys, along with increasing trends in the absolute and relative weights of the thyroids in females, were also seen at 350 ppm. Histopathologically, at 1300 ppm animals of both sexes showed significant increases in the incidence of centrilobular hepatocellular hypertrophy and follicular cell hypertrophy in the thyroid. Males showed significant increases in the incidence of atrophy of seminiferous tubules and interstitial cell hyperplasia in the testis, degenerated cells in ductal lumen of the epididymis, increase in basophilic cells, with hydropic degeneration in the pituitary. Females showed significant increases in the incidence of periportal hepatocellular fatty change, single-cell necrosis of hepatocytes and bile duct hyperplasia, increase of interstitium in the pancreas, hypertrophy of the zona fasciculata cells in the adrenals, and retinal atrophy in the eye.

At 350 ppm males showed a significant increase in the incidence of increased basophilic cells , with hydropic degeneration in the pituitary.

Liver weight increases at 100 and 350 ppm were considered not toxicologically relevant in the absence of changes in clinical chemistry parameters and histopathological changes in the liver.

Weight changes observed in other organs (adrenals, epididymis, heart, kidneys, spleen and uterus) were considered to be related to treatment although there were no corresponding histopathological changes. Changes at 100 ppm were considered incidental and unrelated to the treatment as there was no dose–response relationship.

The NOAEL was 100 ppm, equal to 4.1 mg/kg bw per day, based on organ weight changes in both sexes and indications of kidney effects in females at 350 ppm, equal to 14 mg/kg bw per day (Kuwahara, 2006c).

Table 14. Key results for clinical observations, haematology and clinical chemistry in rats

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Clinical observations								
Grip strength, hind (kg)	0.38	0.44	0.41	0.43	0.33	0.32	0.32	0.27**
Grip strength, fore (kg)	1.34	1.32	1.30	1.33	1.08	1.08	1.06	0.87**
Haematology (week 52)								
Haematocrit (%)	45.0	44.6	44.9	43.6**	43.6	44.0	44.2	43.1
Haemoglobin (g/dL)	15.4	15.3	15.4	15.0**	15.1	15.4	15.4	15.1
RBC (10^6/µL)	9.36	9.57	9.63**	9.46	8.39	8.60	8.67**	8.73**
Mean cell volume (fL)	48.1	46.7**	46.7**	46.1**	52.0	51.1**	50.9**	49.4**
Mean cell Hb (pg)	16.5	16.0**	16.0**	15.8**	18.0	17.8	17.8*	17.3**
Reticulocytes (10^9/L)	161	172	174	189**	153.3	143.3	138.7	171.5
BMNC (10^5/µL)	13.7	14.3	16.2*	16.6**	13.1	13.9	13.9	14.7
Clinical biochemistry (week 13)								
GGTP (U/L)	1	1	1	0*	1	1	1	2**
Total cholesterol (mg/dL)	55	55	58	51	68	75**	80**	70
Triglycerides (mg/dL)	74	71	63	27**	18	21	19	12*
Calcium (mg/dL)	10.2	10.1	10.1	10.1	10.1	10.0	9.9	9.8*
Chloride (m equiv./L)	106.9	106.7	106.4	105.4	108.0	107.9	107.8	105.7**
Clinical Biochemistry (week 26)								
GGTP (U/L)	1	1	1	1	1	1	1	4**
Total cholesterol (mg/dL)	62	63	61	50**	79	87*	90**	76
Triglycerides (mg/dL)	69	58	48	17**	28	34	37	12**
Calcium (mg/dL)	10.0	10.1	10.0	9.9	10.0	9.9	9.8	9.6*
Chloride (m equiv./L)	107.7	106.9	106.8	105.8**	108.0	107.4	106.7	105.7*

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Clinical biochemistry (week 52)								
GGTP (U/L)	4	6	3	3*	2	1	1	4**
Total cholesterol (mg/dL)	78	82	73	70*	96	111**	118**	85
Triglycerides (mg/dL)	64	59	50*	28**	38	61	81*	13
Calcium (mg/dL)	10.3	10.4	10.3	10.1	9.7	10.0	9.8	9.3**
Chloride (m equiv./L)	108.4	107.2	107.7	106.3**	107.9	107.0	106.2**	106.0**

BMNC Bone marrow nucleated cell count GGTP γ-Glutamyl transpeptidase

* $p < 0.05$ different from control by Dunnett's test ** $p < 0.01$ different from control by Dunnett's test

Source: Kuwahara, 2006c

Table 15. Key results for organ weights and histopathology in rats

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Organ weights – *Terminal sacrifice (week 52)*								
Liver (g)	8.07	9.14**	8.77	11.0**	4.10	4.34	4.71*	5.22**
Liver (% bw)	2.00	2.16**	2.20**	2.70**	2.00	2.16*	2.36**	2.76**
Kidney (g)	2.23	2.41*	2.39	2.67**	1.26	1.28	1.35*	1.37**
Kidney (% bw)	0.55	0.57	0.60**	0.66**	0.62	0.64	0.68**	0.72**
Thyroid (mg)	21.1	21.4	18.5	24.6	1.11	11.9	13.0	15.2**
Thyroid (% bw)	5.2	5.1	4.6	6.0	5.4	5.9	6.5	8.0**
Heart (g)	1.02	1.07	1.04	1.14**	618	641	658	668*
Heart (% bw)	0.25	0.25	0.26	0.28**	0.30	0.32	0.33**	0.35**
Spleen (% bw)	0.17	0.17	0.17	0.18	0.20	0.20	0.21	0.23**
Adrenals (g)	38.5	39.0	38.2	44.2*	42.6	43.3	43.2	41.9
Epididymides (% bw)	0.23	0.22	0.22	0.21*	-	-	-	-
Uterus (g)	-	-	-	-	1137	874*	929	649**
Uterus (% bw)	-	-	-	-	0.56	0.43*	0.47	0.34**
Histopathology								
Liver (*N*)	20	20	20	20	20	20	20	20
Fatty change, hepatocyte, periportal	0	0	0	0	0	0	0	16**
Necrosis, hepatocyte, single cell	0	0	0	0	0	0	0	7**
Hypertrophy, hepatocyte, centrilobular	0	0	0	12**	0	0	0	20**
Hyperplasia, bile duct	20	20	20	20	3	3	8	20**

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Pancreas (*N*)	20	0	1	20	20	20	20	20
Increased interstitium	0	-	0	0	0	0	0	18**
Testis (*N*)	20	20	20	20	0	0	0	0
Atrophy, seminiferous tubule	6	5	7	20**	-	-	-	-
Hyperplasia, interstitial cell	0	1	3	15**	-	-	-	-
Epididymis (*N*)	20	20	20	20	0	0	0	0
Degenerated cell, ductal lumen	0	0	0	20**	-	-	-	-
Pituitary (*N*)	20	20	20	20	20	0	4	20
Basophilic cell with hydropic degeneration, increased	0	0	10**	16**	0	-	0	0
Thyroid (*N*)	20	20	20	20	20	20	20	20
Hypertrophy, follicular cell	0	0	0	20**	0	0	0	20**
Adrenal (*N*)	20	0	1	20	20	20	20	20
Hypertrophy, zona fasciculata cell	0	-	0	0	0	0	0	20**
Eye (*N*)	20	1	2	20	20	20	20	20
Atrophy, retinal	3	1	1	1	3	0	2	16**

* $p < 0.05$ different from control by Dunnett's test ** $p < 0.01$ different from control by Dunnett's test

N Number examined - No finding

Source: Kuwahara, 2006c

In a two-year carcinogenicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered in the diet to groups of 50 male and 50 female Fischer (F344/DuCrj) rats at 0, 100, 350 or 1300 ppm (equal to 0, 3.5, 13 and 49 mg/kg bw per day for males, 0, 4.5, 16 and 60 mg/kg bw per day for females). All rats were checked daily for mortality and clinical signs. A detailed physical examination including palpation for masses and observations in an open field was performed weekly. Body weights and feed consumption were recorded weekly during the first three months and every four weeks thereafter. Blood from all animals was sampled for haematological examination at weeks 52, 78 and 104. All animals were necropsied. Organs were weighed (adrenals, brain, heart, liver, kidneys, thyroids with parathyroids, spleen, ovaries, uterus, testes and epididymides) from ten animals per sex and per dose. A wide range of tissues was examined microscopically from all control and high-dose animals, and all animals found dead from the other dose group. In addition, gross lesions from all animals and the liver, kidney, thyroid, adrenal, eye and mammary gland from both sexes were similarly examined. In all males the pituitary, testis, epididymis, prostate, seminal vesicle, coagulating gland, skeletal muscle (M. triceps surae, unilateral) and nasal cavity were also examined microscopically, whilst the same was done for the pancreas, ovary, uterine horn, uterine cervix, and vagina from all females.

The results of the two-year study are presented in Table 16. Mortality was not affected by treatment. During clinical examination, as well as at necropsy, an increased incidence in opacity of the eye was observed in both sexes at 1300 ppm. Other significant changes observed were considered incidental as their occurrence decreased with dose, they were not dose-dependent and/or were only observed in one sex. In males of the 1300 ppm group, body weights were significantly lower than controls from week 64 to the end of the treatment period, and in females of this group from week 36 to the end of treatment period. Body weights at termination of treatment in males and females were 81% and 80% of the respective control values. High-dose females had a slightly higher body weight (up to 6%) than controls during weeks 1–16. At 350 ppm body weight was

significantly lower (up to 6%) than the controls in males from week 68 to the end of the treatment period, and in females of this group (up to 10%) from week 36 to the end of the treatment period. Feed consumption was increased during the first part of the study in males and females, but was lower than controls from week 72 in males and from week 36 in females. Taken over the entire study no changes were observed. Haematology revealed a significant decrease in lymphocyte count in high-dose males (2620/μL) compared to controls (3210/μL).

At 1300 ppm in males and females increases in absolute and relative weights of the liver and kidneys (in females, relative weights only), and a small decrease in absolute weight of the brain and increase in relative weight of the brain and heart were noted. In addition females showed an increase in the relative thyroid weight. Males at 350 and 1300 ppm showed significant decreases in absolute and relative weights of the epididymides. Also, in males at 350 ppm increases in the relative weights of the liver were noted. Apart from the heart and brain, all changes in organ weight were accompanied by histopathological changes. The (small) changes in relative weights of brain and heart may have been secondary to the low body weight at the high dose.

At necropsy, eye opacity was noted in both sexes at 1300 ppm. Males at 350 and 1300 ppm showed a significant increase in the incidence of masses in the testis and softening of the epididymis. Females at 1300 ppm showed a significant increase in the incidence of luminal dilatation of the uterus and a decreased incidence of mammary gland hypertrophy, which the study's author considered possibly related to the estrogenic effect of pyrifluquinazon. As histopathological findings were observed that plausibly corresponded to these macroscopic lesions, they were considered to be treatment-related.

At 1300 ppm histopathology showed increases in the incidence of cataract and retinal atrophy and changes in the kidney, thyroid and adrenals in both sexes, bile duct hyperplasia and luminal dilatation and hyperplasia of the endometrial gland of the uterine horn, mammary gland atrophy, luminal dilatation of the endometrial gland of the uterine cervix, atrophy of the ovaries in females and striated muscle fibre atrophy, rhinitis in the nasal cavity, atrophy in the epididymis, seminal vesicle, coagulating gland and prostate in males. In addition, at 350 ppm histopathology showed cataract, atrophy in the epididymis, seminal vesicle, coagulating gland and prostate in males, and retinal atrophy, bile duct hyperplasia and luminal dilatation of the endometrial gland of the uterine horn in females.

There was a significant increase in the incidence of Leydig cell tumours in the testis at 350 and 1300 ppm (82%, 76%, 98% and 96% at 0, 100, 350 and 1300 ppm, respectively). It was postulated by the study's author that the antiandrogenic activity of pyrifluquinazon caused atrophy of the seminiferous tubule with subsequent reactive proliferation of interstitial cells, elevating testosterone levels and thus increasing the incidence of Leydig cell tumours. A significant decrease in the incidence of seminiferous tubule atrophy in high-dose males was attributed to the small number of testes with an observable seminiferous tubule as a result of the increase in Leydig cell tumours. In addition, the increased incidence of atrophy in the epididymis, seminal vesicle, coagulating gland and prostate, were considered by the study's author to possibly be secondary to the Leydig cell tumours or any antiandrogenic activity of pyrifluquinazon.

There were no increases in the incidence of other neoplastic lesions. Significantly decreased incidence of mononuclear cell leukemia was observed in mid- and high-dose females, of pituitary adenoma in high-dose females and of thyroid C-cell adenoma observed in high-dose males and females.

The NOAEL was 100 ppm, equal to 3.5 mg/kg bw per day, based on histopathological changes in the eyes in both sexes, organ weight changes and macroscopic and histopathological changes, including an equivocal increase in Leydig cell tumours, set against a high background incidence, in the male reproductive organs and bile duct hyperplasia and uterine horn dilatation in females at 350 ppm, (equal to 14 mg/kg bw per day). Apart from the Leydig cell tumours, there were no increases in the incidence of other neoplastic lesions. Although the high background incidence in Leydig cell tumours in this study makes it difficult to assess with confidence any carcinogenic effect of pyrifluquinazon treatment in the testis, the Meeting noted that an increased incidence in this tumour type was also observed in the 18 month study in mice. Therefore the NOAEL for carcinogenicity was 3.5 mg/kg bw per day (Kuwahara, 2006d).

Table 16. Key results of the carcinogenicity study in rats

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Mortality	15/50	8/50	9/50	9/50	12/50	7/50	6/50	5/50
Clinical observations								
Eye/eyelid: opacity	4/50	4/50	9/50	34/50**	5/50	2/50	6/50	47/50**
Organ weights – *week 104*								
Brain (g)	2.09	2.08	2.09	2.01**	1.87	1.87	1.86	1.77**
(% bw)	0.48	0.53	0.52	0.60**	0.76	0.78	0.81	0.91**
Heart (% bw)	0.27	0.29	0.28	0.33**	0.32	0.31	0.34	0.39**
Liver (g)	10.19	9.92	11.02	12.40**	6.42	6.23	6.01	6.22
(% bw)	2.35	2.53	2.74**	3.78**	2.61	2.58	2.61	3.18**
Kidneys (g)	2.54	2.59	2.58	2.82*	1.64	1.57	1.61	1.63
(% bw)	0.59	0.67	0.64	0.85**	0.67	0.65	0.70	0.83**
Spleen (mg)	975	980	1193	3116	1626	2015	526	446**
Epididymides (mg)	598	512	392*	270**	-	-	-	-
(% bw)	0.14	0.13	0.10*	0.08**	-	-	-	-
***N*ecropsy**								
Testis (*N*)	50	50	50	49				
Masses	36	38	47*	47**	-	-	-	-
Epididymides (*N*)	50	50	50	49				
Softening	34	36	43*	45**	-	-	-	-
Uterus (*N*)					50	50	50	50
Lunimal dilatations	-	-	-	-	7	8	12	21*
Eye (*N*)	50	50	50	50	50	50	50	50
Opacity	4	4	9	34**	4	2	6	47**
Histopathology; *non-neoplastic findings*								
Eye (*N*)	50	50	50	50	50	50	50	50
Cataract	3	5	12*	45**	5	3	11	49**
Atrophy, retina	3	6	9	42**	7	8	31**	49**
Muscle (*N*)	50	50	50	50	50	7	6	50
Atrophy, striated muscle	0	5	6	44**	1	0	0	1
Nasal cavity (*N*)	50	50	50	50	50	7	6	50
Rhinitis	15	9	18	28**	3	0	0	1
Liver (*N*)	50	50	50	50	50	50	50	50
Fatty change, hepatocyte, diffuse	0	0	0	3	0	0	1	43**
Hypertrophy, hepatocyte, centrilobular	0	0	0	40**	0	0	0	50**
Hyperplasia, bile duct	49	50	50	49	18	26	35**	46**
Thyroid (*N*)	50	50	50	50	50	50	50	50
Increased small sized follicles	0	0	0	43**	0	0	0	47**
Hypertrophy, follicular cell	0	0	0	27**	0	0	0	42**

	Males				Females			
Diet concentration (ppm)	**0**	**100**	**350**	**1300**	**0**	**100**	**350**	**1300**
Pancreas (*N*)	50	8	9	50	50	50	50	50
Vacuolation, acinar cell	0	1	0	1	0	0	1	21**
Infiltration, fat	0	0	0	0	0	0	0	19**
reduc zymogen granules	0	0	1	1	3	2	8	36**
Atrophy, acinar cell, focal	30	2	4	24	8	10	14	26**
Foci of cellular alteration	0	0	0	0	0	0	0	23**
Kidney (*N*)	50	50	50	50	50	50	50	50
Oedema, renal papilla	23	17	22	12*	19	18	18	8*
Tubular basophilic change	29	29	28	13**	7	5	17*	14
Chronic nephropathy	11	8	12	31**	3	0	1	10*
Adrenals (*N*)	50	50	50	50	50	50	50	50
Hypertrophy, cortical cell, focal	3	0	2	3	7	3	3	0**
Hypertrophy, zona fasciculata/reticularis	0	0	0	13**	0	0	0	50**
Hyperplasia, cortical cell, focal	13	10	9	2**	22	19	18	1**
Mammary gland (*N*)	48	50	48	50	49	50	49	50
Atrophy	2	3	3	4	5	4	7	30**
Testis (*N*)	50	50	50	49	0	0	0	0
Atrophy, seminiferous tubule	16	26*	10	5**	-	-	-	-
Epididymis (*N*)	50	50	50	49	0	0	0	0
Atrophy	5	6	30**	44**	-	-	-	-
Seminal vesicle (*N*)	50	50	50	49	0	0	0	0
Atrophy	0	3	17**	44**	-	-	-	-
Coagulating gland (*N*)	50	50	50	49	0	0	0	0
Atrophy	0	3	17**	44**	-	-	-	-
Prostate (*N*)	50	50	50	48	0	0	0	0
Atrophy	0	0	6*	41**	-	-	-	-
Ovary (*N*)					50	50	50	50
Atrophy	-	-	-	-	6	2	1	23**
Uterine horn (*N*)					50	50	50	50
Luminal dilatation, endometrial	-	-	-	-	3	8	13**	31**
Hyperplasia, endometrial gland	-	-	-	-	1	3	4	10**
Uterine cervix (*N*)					50	50	50	50
Atrophy	-	-	-	-	0	0	0	10**
Histopathology; *neoplastic findings*								
Testis - Leydig cell tumour	41/50	38/50	49/50**	47/49*	-	-	-	-

* $p < 0.05$ different from control by Fisher's Exact Test ** $p < 0.01$ different from control by Fisher's Exact Test

N Number examined - No finding

Source: Kuwahara (2006d)

2.4 Genotoxicity

Pyrifluquinazon was tested for genotoxicity in three in vitro assays and two in vivo assays. All tests gave negative results (Table 17).

Table 17. Overview of genotoxicity tests with pyrifluquinazon

End-point	Test system	Concentrations/ doses tested	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	1.22–1250 µg/plate in DMSO (± S9)	98.0	Negative [a]	Inagaki (2005)
Mammalian cell gene mutation	Mouse lymphoma L5178Y cells/HPRT)	10–80 µg/ml in DMSO, (−S9) 50–180 µg/mL in DMSO (+S9)	97.45	Negative [b]	Gilby (2015)
Chromosomal aberration	Chinese hamster lung cells (CHL/IU)	10–230 µg/plate in DMSO, (−S9, 6 h) 30–270 µg/plate in DMSO, (+S9, 6 h) 5–100 µg/plate in DMSO, (–S9, 1.5 and 3 cell cycles)	98.0	Negative for aberrations Positive for polyploidy [c]	Inagaki (2006)
In vivo					
Mouse micronucleus	Male CD-1 mouse, bone marrow	125, 250 and 500 mg/kg bw in 1% aqueous CMC by gavage as single doses	99.0	Negative [d]	Mason (2005)
Unscheduled DNA synthesis assay	Male Wistar rat, hepatocytes	50 and 100 mg/kg bw in 1% aqueous CMC by gavage as single doses	99.24	Negative [e]	Dony (2016)

CHO Chinese hamster ovary

DMSO Dimethyl sulfoxide

HPRT Hypoxanthine–guanine phosphoribosyltransferase

S9 9000 × *g* supernatant fraction from rat liver homogenate

[a] Batch no. 3FZ0013G. In a preincubation assay at concentrations up to 1250 µg/plate cytotoxicity in strain TA1537 was observed at 417 and 1250 µg/plate with and without S9 mix. Cytotoxicity was observed at 1250 µg/plate for the other strains with or without S9 mix. Precipitation of the test compound was observed at 417 and 1250 µg/plate with and without S9 mix. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[b] Batch no. TY0401. L5178Y cells were exposed for 3 hs or 24 h in the absence of S9 or 3 h in the presence of S9 mix. The highest concentrations in the 3 h exposures were 80 µg/mL for cultures without S9 mix or 180 and 160 µg/mL with S9 mix, based on marked cytotoxicity. No increases in mutant frequencies that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF) were observed. Positive and negative controls gave expected results in all experiments.

[c] Batch no. 3FZ0013G. In the experiment with S9 mix treatment, at 100–130 µg/mL the cell viability was reduced by approximately 50%. In the experiment without S9 mix treatment, pyrifluquinazon reduced cell viability by about 50% at 70 µg/mL after 6 h exposure, at 25 µg/mL at 1.5 cell cycle lengths exposure and at 25–50 µg/mL at 3 cell cycle lengths exposure. Precipitation of pyrifluquinazon was observed at concentrations higher than the cytotoxic concentration. Under all treatment conditions the cell viability at the evaluated highest concentration was reduced 50% or more compared with concurrent negative controls. The numbers of cells with structural chromosome aberrations was not changed by pyrifluquinazon treatment with or without S9 mix. However, the numbers of polyploid cells in pyrifluquinazon-treated cultures was significantly increased after 6 h with and without S9 mix. There was a clear concentration–response relationship especially without S9 mix. It is concluded that pyrifluquinazon did not induce structural chromosome aberrations in CHL/IU cells with and without S9 mix, but it has the potential to induce numerical chromosome aberrations in CHL/IU cells.

[d] Batch no. 1FZ0003P. Groups of ten male and ten female CD-1 mice received a single oral gavage administration of pyrifluquinazon at a dose of 0, 125, 250 or 500 mg/kg bw. Doses were based on a range-finding test using doses of 500, 750 or 1000 mg/kg bw administered to groups of two males and two females per dose. Severe clinical signs were observed at 750 and 1000 mg/kg bw (slow, shallow and irregular respiration, flat and hunched posture, over- and underactivity, abnormal gait, partially closed eyes, piloerection, prostrate posture, fasciculations, limited use of hind limbs and reduced body temperature) which resulted in the early termination of the animals. Similar clinical signs were observed in animals dosed at 500 mg/kg bw, however, all these animals survived to the scheduled termination.

In the micronucleus test, bone marrow smears were obtained from five mice per sex and per dose group at 24 and 48 h after dosing. An additional group of five male and five female mice received 12 mg/kg mitomycin C as a positive control and bone marrow smears were prepared 24 h after dose administration. The incidence of micronuclei in 2000 immature erythrocytes, the incidence of immature erythrocytes in at least 1000 total erythrocytes and the incidence of micronucleated mature erythrocytes was recorded. No mortality was observed. At 500 mg/kg bw fast, deep, slow and irregular respiration, unresponsiveness, flat and hunched posture, overactivity and underactivity, abnormal gait, partially closed eyes, piloerection and prostrate posture were observed. At 250 mg/kg bw male animals showed irregular respiration, flat posture, underactivity, abnormal gait, partially closed eyes and piloerection. Pyrifluquinazon induced no statistically significant changes in the incidence of micronucleated immature erythrocytes. There were statistically significant decreases in the proportion of immature erythrocytes in male mice treated with pyrifluquinazon at 250 or 500 mg/kg bw sampled at 48 h. These were considered to be indicative of bone marrow cell toxicity and therefore bone marrow exposure. The positive controls showed an appropriate increase in micronucleus formation.

[e] Batch no. 5023. In an unscheduled DNA synthesis (UDS) test in vivo groups of four male Wistar rats received a single oral gavage administration of pyrifluquinazon at doses of 0, 50 or 100 mg/kg bw. Doses were based on a range-finding test using doses of 100, 250, 500 or 1000 mg/kg bw administered to groups of two males and two females per dose. Severe clinical signs were observed at 250, 500 and 1000 mg/kg bw (abnormal posture, partially closed eyes, porphyrin-stained fur, piloerection, reduced spontaneous activity, apathy, tumbling, salivation, decreased frequency of breathing, hypothermia, loss of righting reflex and blinking, slight tonic-clonic seizures, moribundity) which resulted in the early termination of the animals. At 100 mg/kg bw piloerection, reduced spontaneous activity and apathy were observed but all animals survived to the scheduled termination. As there were no substantial differences in toxicity between sexes, only male rats were used in the UDS test.

In the main experiment, groups of four male rats per dose and per time interval received pyrifluquinazon by gavage in 1% w/v CMC at 10 mL/kg bw at doses of 0, 50 and 100 mg/kg bw (4 h or 16 h preparation interval). A group of four male rats received 80 mg/kg bw dimethylhydrazine (DMH, 4 h preparation interval) or 100 mg/kg bw 2-acetylaminofluorene (2-AAF, 16 h preparation interval) as positive controls. At scheduled intervals primary hepatocyte cultures were established and exposed for 4 h to methyl-3H-thymidine (3HTdR). The amount of incorporated radioactivity was determined by autoradiography. All animals survived to scheduled termination. At 50 and 100 mg/kg bw piloerection, reduced spontaneous activity and porphyrin-stained fur were observed. Pyrifluquinazon did not induce any biologically relevant or statistically significant UDS in the hepatocytes. The positive controls showed an appropriate increase in the number of nuclear and net grain counts. The viability of the hepatocytes was not substantially affected.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Study 1

In a range-finding, two-generation reproductive toxicity study, groups of eight male and eight female Sprague Dawley Jcl:SD rats were exposed to pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at dietary doses of 0, 100, 500, 1250 and 2500 ppm for the F0 parental generation, and 0, 100 and 500 ppm for the F1 parental generation. The dietary doses of 0, 100, 500, 1250 and 2500 throughout the experiment were equal to 0, 5.9, 30, 76 and 140 mg/kg bw per day for parental males, and 0, 5.9, 37, 98 and 128 mg/kg bw per day for parental females. The doses of 0, 100 and 500 ppm were equal to 0, 7.4 and 53 mg/kg bw per day for F1 males, and 0, 7.7 and 39 mg/kg bw per day for F1 females. Rats were mated after three weeks of exposure, and females allowed to complete gestation and lactation while being maintained on the treated diet. At four days post-partum litters were culled to eight pups. At weaning of the pups, all parental animals were euthanized. One male and one female from each litter were randomly selected from the 0, 100 and 500 ppm groups to become F1 parents of the F2 offspring and were given diets containing the test substance. Other pups from the F1 generation were euthanized at 26 days of age. F1 parental animals were reared for ten weeks and bred to obtain F2 pups. After completion of F2 pups examination on lactation day 4, all animals were euthanized. Clinical examinations were performed daily. Detailed observations were made weekly. Body weights and feed consumption of parental rats were recorded weekly. Individual pup weights were recorded on postnatal days (PNDs) 1, 4, 7, 14 and 21. Estrous cycle length, fertility parameters

and lengths of gestation were determined. All the litters were examined for number of pups, sex of pups, number of stillbirths, number of live births, viability index and gross anomalies. All pups were checked daily for mortality and clinical signs. Each pup received a detailed physical examination on PNDs 1, 4, 7, 14 and 21. For F2 pups, the anogenital distance was evaluated on day 4 of lactation. The culled pups and pups euthanized at weaning were macroscopically examined. After weaning of the F1 and F2 pups, the respective F0 and F1 parents were euthanized and necropsied. The weights of brain, pituitary, liver, kidneys, adrenals, spleen, thyroid, thymus, uterus with oviducts and cervix, seminal vesicles, prostate, epididymides, testes and ovaries from all parental F0 and F1 animals were recorded. In F1 and F2 weanlings, the weights of the brain, spleen, thymus, liver and uterus of one pup of each sex per litter were recorded. The ovaries, oviduct, uterus (horns and cervix), vagina, testes, seminal vesicles, coagulating glands, and prostate, pituitary, thyroids, liver, adrenals and epididymis (sections at caput, corpus, and cauda) of all F0 and F1 parental animals, except for those from the 2500 ppm group, were examined histopathologically. The testis was examined in detail for spermatogenesis. In addition, preserved livers from F1 weanlings were examined histopathologically.

No parental animals died. F0 parental females at 2500 ppm showed soiled fur in the nasorostral region. None of the F0 females in the 2500 ppm group were pregnant, thus only data on the premating period are available for this group.

In the 1250 and 2500 ppm groups, significantly decreased body weights, body weight gains and feed consumption were observed in F0 males and females, which were already observable during the first week of treatment. In high-dose males, body weight gains were lower than controls throughout treatment and final body weights were 30% lower than for controls. High-dose females actually showed a body weight loss (12 grams) during the three week premating period; this compared to a body weight gain (54 grams) in controls. At 1250 ppm males showed reduced body weight gain throughout the treatment period, with statistically significant differences during the first five weeks of treatment. At 1250 ppm females showed a body weight loss during the first week of treatment (2 g) as compared to a body weight gain in controls (21 g) and subsequently a reduced body weight gain throughout the treatment period, with statistically significant differences throughout the premating and gestation period. At 2500 ppm feed consumption was statistically significantly reduced throughout treatment in males and females (40 and 73% respectively during the first week). At 1250 ppm a slight reduction in feed consumption was observed in males during the premating period (up to 10%, reaching statistical significance during week 2) and a marked reduction was observed in females throughout the treatment period (up to 34%, 29% and 45% during the premating, gestation and lactation period respectively). In F0 females at 500 ppm, feed consumption was slightly, but statistically significantly reduced (9%) during the first week of treatment. Estrus cycle was generally not affected by treatment, although one high-dose female showed an abnormality of the estrous cycle. At 2500 ppm the mating index was reduced (only 50% compared to 100% in the controls and other dose groups) and none of the females was pregnant. At 1250 ppm all F0 females were pregnant, however, only half of them delivered pups, and the number of implantation sites was low (12 as compared to 17 in controls). Duration of gestation was slightly, but statistically significantly, increased in these females (23 days as compared to 22.3 days in controls). Balanopreputional separation was delayed in F1 males at 500 ppm. Macroscopic examination showed no treatment-related effects in F0 and F1 parental animals. At 2500 ppm, the absolute and relative weights of the brain, pituitary and kidneys, the absolute weight of the spleen, and the relative weights of the adrenals, testes, seminal vesicles and thyroids were significantly different when compared to the corresponding controls. At 1250 and 2500 ppm statistically significant differences were noted in the absolute weight of the epididymis and the relative weight of the liver in F0 males. At 500 ppm a statistically significant difference in the absolute brain weight (5%) was found in F1 males compared to controls. Histopathology showed hypertrophy of centrilobular hepatocytes in F0 parental animals at 1250 ppm as well as in one female at 500 ppm and in F1 weanlings at 1250 ppm. Hypertrophy of follicular cells in the thyroid was noted in three out of eight females at 1250 ppm.

At 1250 ppm the number of pups delivered (5.0) was significantly lower than that in the control group (14.1). Examination of the pups at 500 and 1250 ppm showed nipple retention in male pups during lactation days 8–14. Among these male pups, one at 1250 ppm showed hypospadia. At 1250 ppm, mean body weights of F1 male pups on and after lactation day 4 were statistically significantly lower than controls from lactation days 14–21 and in females on lactation day 21. The incidence of dilated renal pelvis was increased at 1250 ppm. Organ weight measurements in pups showed statistically significantly

increased relative liver weights at 500 and 1250 ppm, while absolute and relative weights of thymus and spleen in both sexes were decreased at 1250 ppm. Histopathology showed hepatocellular hypertrophy in pups of both sexes at 1250 ppm. In F2 male pups at 500 ppm statistically significant decreases in absolute (12%) and relative (14%) anogenital distance were found (Hojo, 2005a).

Study 2

In the main two-generation reproductive toxicity study, groups of 24 male and 24 female Sprague Dawley Jcl:SD rats were exposed to pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at dietary doses of 0, 30, 150 and 750 ppm. The corresponding dietary intakes of pyrifluquinazon are presented in Tables 18–20. Rats were mated after 11 weeks of exposure, and maintained on treated diet until the end of lactation. At four days post-partum, litters were culled to eight pups. At weaning of the pups, all F0 and F1 parental animals were euthanized. One male and one female from each litter were randomly selected to become F1 parents for the F2 offspring and were given diets containing the test substance. Other pups from the F1 and F2 generations were euthanized at 26 days of age. F1 parental animals were reared for ten weeks and bred to obtain F2 pups. Clinical examinations were performed daily. Detailed observations were made weekly. Body weights and feed consumption of parental rats were recorded weekly. Individual pup weights were recorded on PNDs 1, 4, 7, 14 and 21. Estrous cycle length, fertility parameters, and lengths of gestation were determined. For all F0 and F1 parental males except for the animals found dead during the study, sperm head and sperm were collected at necropsy from the testis and cauda epididymis (right side in principle) for counting and examination. All the litters were examined for number of pups, sex of pups, number of stillbirths, number of live births, viability index and gross anomalies. All pups were checked daily for mortality and clinical signs. Each pup received a detailed physical examination on PNDs 1, 4, 7, 14 and 21. For F2 pups, the anogenital distance was evaluated on day 4 of lactation. The culled pups and pups euthanized at weaning were macroscopically examined. After weaning of the F1 and F2 pups, the respective F0 and F1 parents were euthanized and necropsied. The weights of brain, pituitary, liver, kidneys, adrenals, spleen, thyroid, thymus, uterus with oviducts and cervix, seminal vesicles, prostate, epididymides, testes and ovaries from all parental F0 and F1 animals were recorded.

Histopathological examination of all F0 and F1 parental animals from the control and 750 ppm group was performed on ovaries, oviduct, uterus (horns and cervix), vagina, testis, epididymis (sections at caput, corpus, and cauda), seminal vesicles, coagulating glands, prostate, pituitary, adrenals, and the organs showing weight changes (the kidneys of both sexes in the F0 generation, the brain of both sexes in the F1 generation, and the kidneys of F1 females). The testis was examined in detail for abnormalities of spermatogenesis. In the ovary sections from F1 females, primordial follicles were counted. In F1 and F2 weanlings, the weights of the brain, spleen, thymus and uterus of one pup of each sex per litter were recorded. These organs and the thyroids, liver, kidneys and other reproductive organs, as well as organs or tissues with grossly visible abnormalities, were examined histologically.

At 750 ppm two parental males and three parental females in total for two successive generations died. Treatment related effects are presented in Tables 18–20. At 750 ppm statistically significant decreases were found in body weight gain (up to 17%) and feed consumption (up to 13%) of F0 and F1 parental females compared to controls. The number of pregnancies and gestation index were not affected by treatment. Estrous cycle length was slightly increased (7%) in F1 parental females at 150 and 750 ppm. Although the difference was statistically significant this finding was considered to be biologically insignificant because all F1 females in these groups had 4-day and/or 5-day estrous cycles, which are considered to be normal in rats. Gestation duration was statistically significantly increased, while litter sizes were decreased in F0 and F1 females at 750 ppm. The sexual development (time of preputial separation) of F1 parental males was statistically significantly delayed while the age and body weight at time of vaginal patency of F1 female pups was reduced at 750 ppm. Sperm examination revealed a reduction in normal sperm morphology in high-dose F0 and F1 males. Statistically significant increases in absolute and/or relative weights of liver, thyroids, adrenals, kidneys, seminal vesicles, pituitary and uterus, and decreases in brain weights were observed in males and/or females at 750 ppm. In addition increased absolute (up to 15%) and relative (up to 11%) thyroid weights were observed in F1 females at 150 ppm. At 750 ppm F1 and F2 pups showed no effect on body weight on PND 1, but decreased body weights on PND 21 (up to 15%), and PND 26 (up to 18%) compared to controls. In addition F2 pups in the 150 ppm group showed a reduced body weight (7%) on PND 21 compared to controls. In F1 and F2 pups at 750 ppm statistically significant reductions in absolute brain weight (up to 11%) were observed,

while relative brain weights were increased (up to 10%; statistically significant in F0 pups only). Anogenital distance was statistically significantly reduced in F1 and F2 male pups at 750 ppm (up to 16%) and in F2 male pups at 150 ppm (6%). In animals from the 750 ppm group histopathological examination showed centrilobular hepatocellular hypertrophy in both sexes in F0 and F1 parental animals, follicular cell hypertrophy of the thyroid in F0 and F1 parental females and pelvic dilatation in F1 parental females (Table 18). No test substance-related effects were observed on F0 and F1 parental rats in the 30 ppm group.

The NOAEL for parental toxicity was 30 ppm, equal to 2.4 mg/kg bw per day, based on increased thyroid weight in F1 females at 150 ppm (equal to 12 mg/kg bw per day).

The NOAEL for offspring toxicity was 30 ppm (equal to 2.3 mg/kg bw per day) based on reduced body weights in both sexes at 150 ppm (equal to 11 mg/kg bw per day).

The NOAEL for reproductive toxicity was 30 ppm (equal to 2.3 mg/kg bw per day) based on a reduced anogenital distance in F2 male pups at 150 ppm (equal to 11 mg/kg bw per day) (Hojo, 2006a).

Table 18. Summary of key results of the two-generation reproduction study in rats (1st generation)

	Males				Females			
Diet concentration (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
				F0 Parental animals				
Pyrifluquinazon intake (mg/kg bw per day)								
Premating (weeks 0–10)	0	2.0	10	52	0	2.3	12	59
Gestation and lactation	-	-	-	-	0	3.5	17	80
Body weight gain (g)								
Premating period	368	372	362	342	155	151	147	128**
Gestation	-	-	-	-	152	138	145	129**
Lactation	-	-	-	-	36	45	38	43
Feed consumption (g/rat per day)								
Week 10	25.7	26.7	26.4	26.3	17.7	17.9	18.0	17.8
Gestation period (days 14–20)	-	-	-	-	25.8	24.1	24.5	24.5
Lactation period (days 14–21)	-	-	-	-	74.5	75.1	73.3	65.0**
Mating and gestation performance								
Sperm morphology (% normal)	98.1	98.0	98.3	92.9**	-	-	-	-
Number of pregnancies	-	-	-	-	23/24	21/23	21/24	21/23
Gestation index (%)	-	-	-	-	100	95.2	95.2	95.2
Gestation duration (days)	-	-	-	-	22.0	22.2	22.1	22.7**
Mean litter size	-	-	-	-	15.0	13.8	14.3	10.7**
Organ weights								
Liver (g)	20.35	20.30	20.35	20.83	13.22	13.50	13.11	14.67*
Liver (% bw)	3.23	3.26	3.32	3.51**	4.23	4.35	4.29	4.91**
Pituitary (% bw)	0.0024	0.0022	0.0024	0.0025	0.0048	0.0051	0.0049	0.0052 **
Testes (mg)	1938	1984	2000	2077**	-	-	-	-
Testes (% bw)	0.310	0.320	0.330	0.353**	-	-	-	-
Thyroids (mg)	22.3	22.3	22.9	25.3	18.8	18.6	19.6	24.3**
Thyroids (% bw)	0.0036	0.0036	0.0038	0.0043**	0.0060	0.0060	0.0064	0.0081**

	Males				Females			
Diet concentration (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
				F1 Pup data				
Body weight (g) PND 1	6.7	6.9	6.7	6.8	6.3	6.5	6.4	6.3
Body weight (g) PND 21	63.9	63.9	62.0	54.3**	62.2	62.3	59.2	53.0**
At preputial separation/vaginal patency:								
Age (days)	41.1	41.3	42.5	46.3**	30.5	30.3	30.5	29.5
Body weight (g)	207	211	226**	253**	108	111	110	97**
Anogenital distances (mm) lactation day 4	5.52	5.50	5.70	4.78**	2.26	2.25	2.39	2.33
Organ weights at PND 26								
Body weight (g)	93	93	91	76**	88	87	85	72**
Brain (mg)	1556	1545	1556	1388**	1502	1510	1487	1341**
Brain (% bw)	1.67	1.66	1.72	1.84**	1.71	1.75	1.75	1.88**
Thymus (mg)	277	313*	272	209**	288	304	269	204**
Thymus (% bw)	0.298	0.335*	0.299	0.277	0.328	0.349	0.315	0.283**
Spleen (mg)	401	396	281	301**	367	351	347	282**

* $p < 0.05$ in comparison to controls ** $p < 0.01$ in comparison to controls

Source: Hojo, 2006a

Table 19. Summary of key results from the two-generation reproduction study in rats (2nd generation)

	Males				Females			
Diet conc. (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
				F1 Parental animals				
Pyrifluquinazon intake (mg/kg bw per day)								
Premating (weeks 0–10)	0	2.3	11	56	0	2.4	12	61
Gestation and lactation	-	-	-	-	0	3.4	17	82
Body weight gain (g)								
Premating period	443	444	446	430	217	219	222	197**
Gestation	-	-	-	-	151	158	152	136
Lactation	-	-	-	-	26	29	33	33
Food consumption (g/ rat/day)								
week 10	26.9	27.9	27.5	27.6	17.5	17.7	18.6	17.3
Gestation period (days 14-20)		-	-	-	26.4	26.2	26.7	27.0
Lactation period (days 14-21)		-	-	-	77.5	77.2	76.7	68.6**
Mating and gestation performance								
Estrous cycle length	-	-	-	-	4.1	4.2	4.4**	4.4**
Sperm morphology (% normal)	98.0	96.4	98.3	93.3*	-	-	-	
Number of pregnancies		-	-	-	18	23	21	18
Gestation index (%)	-	-	-	-	94.7	100	100	100
Gestation duration (days)		-	-	-	22.3	22.4	22.2	22.7*
Mean litter size	-	-	-	-	13.1	13.3	14.1	8.6**

	Males				Females			
Diet conc. (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Organ weights								
Adrenals (% bw)	0.0045	0.0041*	0.0045	0.0050*	0.0110	0.0101	0.0114	0.0120*
Brain (mg)	2176	2204	2190	2070**	1965	1967	1980	1808*
Brain (% bw)	0.356	0.356	0.361	0.347	0.609	0.590	0.601	0.569**
Liver (g)	20.65	20.39	20.08	21.10	13.45	14.01	14.48	16.24**
Liver (% bw)	3.37	3.26	3.28	3.52	4.16	4.19	4.39	5.08**
Testes (% bw)	0.318	0.311	0.331	0.341*	-	-	-	-
Thyroids (mg)	26.2	25.1	26.7	25.6	19.5	21.4	22.4*	25.0**
Thyroids (% bw)	0.0043	0.0040	0.0044	0.0043	0.0061	0.0064	0.0068*	0.0079**
Uterus (mg)	-	-	-	-	1034	966	1043	1205*
Uterus (% bw)	-	-	-	-	0.322	0.290	0.316	0.380*
				F2 Pup data				
Bodyweight (g)								
Postpartum day 1	7.1	7.4	6.8	7.4	6.8	7.0	6.4	7.0
Postpartum day 21	70.2	70.4	65.1	63.2*	67.7	67.9	62.8*	60.5**
Anogenital distance (mm) lactation day 4	6.03	5.97	5.65*	5.09**	2.54	2.52	2.39	2.48
Organ weights at PND 26								
Body weight (g)	99	101	95	90*	93	94	88	84**
Brain (mg)	1581	1592	1553	1456**	1509	1543	1509	1396**
Brain (% bw)	1.62	1.59	1.66	1.62	1.63	1.66	1.73*	1.68
Thymus (mg)	319	313	295	268*	308	319	286	266*

* $p < 0.05$ in comparison to controls ** $p < 0.01$ in comparison to controls

Source: Hojo, 2006a

Table 20. Summary of key histopathology data (incidence) from the two-generation reproduction study in rats

	Males				Females			
Diet concentration (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Thyroid, hypertrophy, follicular cell								
F0 parents	0/24	0/24	0/24	0/24	0/24	0/24	0/24	5/24*
F1 parents	0/24	0/24	0/24	0/24	0/24	0/24	2/24	21/24***
Liver, Hypertrophy, hepatocyte, centrilobular								
F0 parents	0/24	0/24	0/24	7/24**	0/24	0/24	0/24	12/24***
F1 parents	0/24	0/24	0/24	3/24	0/24	0/24	0/24	11/24***
Kidney, pelvic dilatation								
F0 Parents	0/24	0/1	-	0/24	0/24	-	1/24	1/24
F1 Parents	-	-	-	2/3	0/24	-	-	7/24*

Level of statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Source: Hojo, 2006a

(b) Developmental toxicity

Rat

Study 1

In a range finding developmental toxicity study, pyrifluquinazon (Batch no. 3FZ0012P; purity 96.4%; formulated in a 1% aqueous CMC suspension) was administrated by gavage to seven pregnant Sprague Dawley (Jcl:SD) rats from gestation day (GD) 6 to GD 19 at a dose of 0, 5, 10, 50 or 100 mg/kg bw per day. The dams were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded on GDs 0, 6, 9, 12, 15, 18 and 20. The fetuses were delivered by caesarean section on GD 20. The uteri and ovaries were examined and the numbers of live and dead fetuses, early and late resorptions, total implantations and corpora lutea recorded. Gravid uterine weights were recorded. Fetal body weights and placental weights were recorded and the fetuses sexed and examined for external, visceral and skeletal malformations, and any developmental variations.

Two high-dose females were found dead; one on GD 12 and one on GD 20. The cause of death could not be established. In the 100 mg/kg group, soiled fur (nasorostral region, chest, abdomen, extragenital region, or forelimbs) and eye discharge were observed. At this dose, three dams showed decreased or lost locomotor and prone position or side position from GD 10. Dams at 100 mg/kg bw per day barely gained weight over the study duration (8 g; −2 g when adjusted for gravid uterine weight) as compared to control (147 g; 52 g when adjusted for gravid uterine weight). Statistically significant reductions in body weight gains were also observed in dams at 50 mg/kg bw per day, from the start of treatment up to the end of the study. Final body weight of dams at 50 mg/kg bw per day were 8% lower than controls. Feed consumption was statistically significantly reduced in high dose dams (up to 59%) from GD 6–GD 20, and to a lesser degree (up to 18%, not statistically significant) in dams at 50 mg/kg bw per day. Numbers of corpora lutea and implants, and percent preimplantation losses in each treated group were comparable to, or higher than, controls. Gravid uterine weights in the 10 and 50 mg/kg groups were slightly lower than those of the control (not statistically significant). In the 100 mg/kg group, gravid uterine weight was markedly lower than that of the controls (10 g and 94 g, respectively) because four out of five dams bore no live fetuses and only showed resorptions and dead fetuses. In the other dose groups, numbers of live fetuses were comparable to control. In the 50 mg/kg bw per day group, fetal body weight and placental weight were markedly lower than those of the control group. External, visceral and skeletal examination showed no statistically significant differences in the incidences of litter having fetuses with malformations (Hojo, 2004).

Study 2

In the main developmental toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.03%; formulated in a 1% aqueous CMC suspension) was administrated by gavage to 25 pregnant Sprague Dawley (Jcl:SD) rats from GD 6 to GD 19 at a dose of 0, 5, 10 or 50 mg/kg bw per day. The dams were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded on GDs 0, 6, 9, 12, 15, 18 and 20. The fetuses were delivered by caesarean section on GD 20. At termination on GD 20 the dams were necropsied. The uteri and ovaries were examined, and the numbers of live and dead fetuses, early and late resorptions, total implantations and corpora lutea recorded. Gravid uterine weights were recorded. Fetal body weights and placental weights were recorded and the fetuses were sexed and examined for external, visceral and skeletal malformations and developmental variations.

No dams died during the study. No treatment-related clinical signs were observed. Main findings of this study are presented in Table 21. At 50 mg/kg bw per day, dams showed body weight loss (14 g) during the first three days of treatment while controls gained 4 g during this period. Body weights remained statistically significantly lower than controls throughout the duration of the study in this group. However, when adjusted for gravid uterine weights the differences were smaller and not statistically significant. Mean gravid uterine weights were statistically significantly decreased at 50 mg/kg bw per day (−75 g) as compared to controls (−94 g). During GDs 6–9 and GDs 9–12 in the high-dose dams feed consumption was statistically significantly reduced (by 33% and 24%, respectively) compared to controls. Necropsy examinations of uterine contents revealed no abnormalities. Numbers of corpora lutea and implants, and percent preimplantation losses in the treated group were comparable to controls. At 50 mg/kg bw per day the mean number of live fetuses (13.9) was slightly lower than in the controls (15.3), and mean percentage of resorptions and fetal deaths (12.7%) were slightly higher than in the control (5.8). However, the differences were not statistically significant. In the 10 and 50 mg/kg bw per

day groups absolute and relative values of anogenital distances were statistically significantly reduced in male live fetuses. The absolute (but not the relative) values of anogenital distance for female live fetuses at 50 mg/kg bw per day was also significantly reduced. In addition at 50 mg/kg bw per day fetal body weights and placental weights for both sexes were markedly lower than those in the control group. At 10 and 50 mg/kg bw per day significantly increased incidences of fetal skeletal variations (supernumerary rib and lumbosacral transitional vertebra) were noted. Reduced incidences of thymic remnant in the neck, observed at 10 and 50 mg/kg bw per day, were considered not treatment-related.

Table 21. Key findings of the teratogenicity study in rats

Dose (mg/kg bw per day)	0	5	10	50
Maternal body weight (g) GD 20	391	392	385	363*
Maternal weight gain, GD 6–20 (g)	112	112	108	82**
Maternal weight gain, days 6–9 (g)	4	3	3	−14**
Feed consumption, days 6–9 (g/rat per day)	18.0	18.4	17.8	12.1**
Feed consumption, days 9–12 (g/rat per day)	19.0	19.6	18.8	14.4*
% resorptions and fetal deaths	5.8	6.3	8.4	12.7
Mean litter size (live fetuses)	15.3	15.1	14.7	13.9
Mean fetal weight (g):				
Males	4.1	4.1	4.1	3.5**
Females	3.9	3.9	3.9	3.3**
Mean placenta weight (mg)	496	492	464	411**
Anogenital distances:				
Males	3.05	2.88	2.82**	2.18**
Females	1.29	1.23	1.25	1.21**
Fetal visceral findings				
Thymic remnant in the neck	5/172	2/175	0/ 161*	0/ 161*
Fetal skeletal findings				
Supernumerary rib	65/179	87/187	91/176**	93/173**
Lumbosacral transitional vertebra	0/179	2/187	3/176	6/173 *

* $p < 0.05$ in comparison to control ** $p < 0.01$ in comparison to control

Source: Hojo, 2006b

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduced feed intake and body weight gain at 50 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, based on reduction in anogenital distance in male fetuses and increased incidences in supernumerary ribs in both sexes at 10 mg/kg bw per day (Hojo, 2006b).

Rabbit

In a range-finding developmental toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%; formulated in a suspension of 1% CMC in purified water) was administered daily by gavage to groups of eight artificially inseminated female Japanese White rabbits (Kbl:JW) from GD 6 to GD 27 at a dose of 0, 5, 20, 50 or 100 mg/kg bw per day. The fetuses were delivered by caesarean section on GD 28. The does were checked daily for mortality and clinical signs. Body weights were recorded on GDs 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28. Feed consumption was recorded between days 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24, 24–27 and on day 28 of gestation. Uteri and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

At 100 mg/kg bw per day six females died during GD 15–23. The remaining two does aborted on GD 17. At this dose soiled fur, scleral congestion, nasal discharge, salivation, no faeces, red/brown discharge on the tray, emaciation and/or prone/side position were observed. At 50 mg/kg bw per day three does aborted during GD 18–24. At this dose soiled fur and/or no faeces, and occasionally loss of fur, loose stool or red/brown discharge on the tray were observed. Does at 100 mg/kg bw per day showed marked body weight loss from the start of treatment throughout the experiment. High-dose females virtually stopped feeding from the start of treatment onwards. No effects of treatment on body weight were observed in the other dose groups. At 50 mg/kg bw per day feed consumption was lower than for controls (35%) throughout treatment, reaching statistical significance during GD 18–21. At 20 mg/kg bw per day feed consumption was reduced by 11% compared to controls. Necropsy of high-dose animals showed that incidences of emaciation, soiled fur and hardened contents in the caecum were significantly higher than in the controls. Other gross pathological findings observed at this dose were subcutaneous haemorrhage in the abdominal region, hair bolus in the stomach, distended with diet in the stomach, mucosal haemorrhage in the caecum, distention in the caecum, yellowing of the liver, spot(s) on the liver, pale color of the kidney, and/or luminal distention of the uterus and vagina. Three maternal rabbits which aborted at 50 mg/kg bw per day, showed soiled fur, hair bolus in the stomach and/or yellowing of the liver.

Higher incidences of decreased defaecation were noted in the 20 and 100 mg/kg bw per day groups. Slightly lower body weight gains were noted during the first days of treatment (days 7–10) at 20 mg/kg bw per day (115 g, not statistically significant) and 100 mg/kg bw per day (52 g, statistically significant) compared with 145 g body weight gain in controls. Compared with controls, over the entire treatment period, net body weight gain was 119 and 145 g lower in does of the 20 and 100 mg/kg bw per day groups, respectively. These reductions were small but statistically significant. Feed consumption was reduced throughout the treatment period in the high-dose females, with the largest reductions during the first part of the study. Overall, the feed intake was reduced by 12% in this dose group. There was no effect on mean organ weights at any dose.

No treatment-related effects were observed on number of live fetuses, percent incidence of resorptions or fetal deaths, sex ratios, mean fetal weights or placental weights of live fetuses. No external abnormalities were found in any live fetuses in the control, 5, 20 or 50 mg/kg bw per day groups (Hojo, 2005b).

In the main developmental toxicity study, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%; formulated in a suspension of 1% CMC in purified water) was administered daily by gavage to groups of 25 artificially inseminated female Japanese White rabbits (Kbl:JW) from GD 6 to GD 27 at a dose of 0, 5, 10 or 20 mg/kg bw per day. The fetuses were delivered by caesarean section on GD 28. The does were checked daily for mortality and clinical signs. Body weights were recorded on GDs 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28. Feed consumption was recorded between days 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24, 24–27 and on day 28 of gestation. The uteri and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

Main findings from the developmental toxicity study are presented in Table 22. No mortality or treatment-related clinical signs were observed. No effects were observed on body weight gain, feed consumption or macroscopic examination of the dams. Mean gravid uterine weights were statistically significantly decreased (22%) at 20 mg/kg bw per day as compared to controls. This change was related to a significant decrease in number of live fetuses (6.5) compared to the controls (8.3). This was considered to be incidental as a reduction of similar magnitude, although not statistically significant, in the number of implantations per doe was also observed (7.7 at 20 mg/kg bw per day; 9.0 at 0 mg/kg bw per day). No statistically significant differences were found in percent incidence of resorptions and fetal deaths, sex ratio, fetal weights or placental weights. A significant increase in the fetal incidence of skeletal malformations in the 20 mg/kg group was observed (6/129 fetuses in 5/19 does) compared to controls (2/192 fetuses in 3/23 does). The study's author considered this finding not treatment-related as the incidence (4.7%) reportedly was within the background data range of the test facility (0–5.4%, historical background data not provided) and the types of observed skeletal malformations (bent clavicle in one fetus, fused sternebra in two fetuses, supernumerary lumber arch in one fetus, and misaligned caudal vertebra in two fetuses) were varied among individuals in this group.

Table 22. Key findings of the teratogenicity study in rabbits

Dose (mg/kg bw per day)	0	5	10	20
Gravid uterine weight (g)	461	248	455	361**
Number of implantations per doe	9.0	9.5	8.8	7.7
Resorption and fetal death (%)	7.6	16.7	4.9	17.5
Mean litter size (live fetuses)	8.3	7.9	8.3	6.5*
Mean fetal weight (g):				
Male	38.1	36.4	37.9	37.3
Female	37.3	36.4	34.9	35.8
Sex ratio (% males)	47	57	58	49
Fetuses with external malformations	0/192	1/190	0/175	0/129
Fetuses with skeletal malformation	2/192	2/190	0/175	6/129*
Fetuses with skeletal variations	63/192	68/190	66/175	43/129
Fetuses with visceral malformations	51/192	57/190	36/175	24/129

Level of statistical significance: * $p \leq 0.05$ ** $p \leq 0.01$

Source: Hojo, 2005c

The maternal and offspring NOAEL was 20 mg/kg bw per day, the highest dose tested. There was no evidence of teratogenicity (Hojo, 2005c).

2.6 Special studies

(a) Neurotoxicity studies

Study 1

In a preliminary acute neurotoxicity study, pyrifluquinazon (technical grade; batch and purity unknown) was administered by a single gavage dose in aqueous 0.5% CMC to groups of three male and three female Sprague Dawley (Crl:CD(SD)) rats at dose levels of 0, 15, 50 and 150 mg/kg bw. At 1, 2, 3, 6, 24, 48 and 72 h after treatment the rats were observed for clinical signs and placed in an open field arena for up to one minute to examine for alertness, alterations in posture and gait, and other unusual behaviours using a central nervous system (CNS) screen. Body weights and feed consumption were determined daily to day 4. On day 4 animals were euthanized, and all organs and tissues examined macroscopically.

Neither adverse clinical signs nor atypical posture, gait or behaviour were observed in any of the rats throughout the study period. No mortality or treatment-related effects on clinical signs, open field behaviour, body temperature, body weight, feed consumption or macroscopic findings were observed (Foss, 2006a).

In the main acute oral neurotoxicity study, groups of five male and five female (for 300 and 500 mg/kg bw) or ten male and ten female Sprague Dawley (Crl:CD(SD)) rats were given a single gavage dose of pyrifluquinazon (technical grade; Batch no. 3FZ0013G; purity 98.0%) at levels of 0, 30, 100, 300 and 500 mg/kg bw. Vehicle was 0.5% aqueous methoxymethylcellulose. The rats were checked daily for mortality and clinical signs. Detailed clinical observations were made before start of treatment, and on days 1 (6 h after dosing), 8, and 15. Body weights and feed consumption were recorded daily. The rats were subjected to a functional observational battery (FOB) and locomotor activity test before treatment and on days 1, 8 and 15. At termination on day 16, animals were necropsied and brains were weighed. Five rats per sex and per dose were examined neurohistologically.

One or two days after treatment, one male and one female rat at 300 mg/kg bw and two males and two females at 500 mg/kg bw were euthanized in moribund condition. Loss of the righting reflex was observed in all of these rats, also: limb tremors, fasciculations, and clonic convulsion in one male rat; ungroomed coat in one female rat; urine-stained abdominal fur in another female; discolored urine in two female rats. Apparent in other rats at 300 and 500 mg/kg bw were increased incidences

of dehydration, decreased motor activity, prostration, ataxia, and/or hyporeactivity, scant or no faeces, hunched posture, loss of righting reflex, coldness to touch, and lacrimation, bradypnea, and piloerection. Most of these effects were statistically significantly increased in both sexes. With the exception of scant faeces and dehydration, these clinical signs were not observed after day 3. In the FOB conducted six hours after treatment both the incidence and severity of sensorimotor reactivity, coordination, and autonomic processes were increased at 300 and 500 mg/kg. The rats showed unusual posture (either low carriage or prostrate), reduced rearing and arousal, slight to extreme alterations in the gait pattern (ataxia, splayed limbs, immobility), reduced palpebral closure, excessive lacrimation and bradypnea, reduced reaction to a visual and or tactile stimulus, poor performance in the visual placing response, righting response, grip tests, and landing footsplay. Body temperature was significantly reduced (2.5–4.9°C) in both sexes at 300 and 500 mg/kg bw. No alterations due to the test substance were observed when the surviving animals were examined 7 and 14 days after treatment. The FOB conducted six hours after treatment revealed one male rat at 100 mg/kg bw with unusual posture (low carriage) in the home cage and open field and a resultant gait that was described as moderately ataxic. The motor activity test at day 1 showed reductions of 46–92% in the cumulative values for the numbers of movements and time spent in movement for the 300 and 500 mg/kg bw compared with the controls. Male rats at 100 mg/kg bw had a significant reduction in one of the measures (time spent in movement) at one of the early blocks (intervals); this minimal difference was not considered evidence of neurotoxicity. The movement values in all treatment groups were comparable to controls at days 7 and 14. At 300 and 500 mg/kg significant body weight losses were observed from day 1 to day 4, followed by a rebound during days 4–12. Over the 16 days duration of the study the body weight gains were not statistically significantly different from controls. Feed consumption values were consistent with body weights. No treatment-related gross lesions or neurohistochemical changes were observed. Absolute brain weight was significantly reduced (7.9%), while relative brain weight was significantly increased (15%) in male rats at 500 mg/kg bw as compared to control.

The NOAEL was 100 mg/kg bw, based on moribundity, various clinical signs, body weight loss and changes in sensorimotor reactivity, coordination, autonomic processes and motor activity observed at 300 mg/kg bw per day in either one or both sexes (Foss, 2006b).

Study 2

In a 13-week dietary neurotoxicity study, groups of ten male and ten female Sprague Dawley (Crl:CD(SD)) rats were given pyrifluquinazon (technical grade; Batch no. 3FZ0013G; purity 98.0%) at 0, 30, 150 and 750 ppm (equal to 0, 1.8, 9.4 and 47 mg/kg bw per day for males, 0, 2.2, 11 and 53 mg/kg bw per day for females). The rats were checked daily for mortality and clinical signs. Detailed clinical observations were made before the start of treatment, and weekly during the treatment period. Body weights were recorded weekly, feed consumption was measured weekly during the first two weeks of treatment and every two weeks thereafter. Ophthalmological examinations were performed pretreatment and within four days of termination. The rats were subjected to a FOB and locomotor activity test before treatment and during weeks 2, 4, 8 and 13. At termination, animals were necropsied and brains weighed. Five rats per sex and per dose of the control and high-dose groups were examined neurohistologically.

No treatment-related mortality, clinical signs, ophthalmological changes or alterations in the FOB and motor activity test were observed. At termination females at 150 ppm had a slightly lower body weight (7%) as compared to controls. In particular, during the first week of treatment, body weight gain in these females (12.7 g) was lower (not statistically significant) than in the controls (17.9 g). At 750 ppm body weight was statistically significantly decreased at termination (12%) compared to the controls. A statistically significant reduction in body weight gain was already observed during the first week of treatment, and feed consumption values were statistically significantly decreased in these female rats early in the exposure period. Absolute brain weights in high-dose females were slightly (but statistically significantly) decreased by 6%. This was considered secondary in these females to the lower body weight when compared to controls, as the relative brain weight was increased (6%, not statistically significant). Neurohistological examination of high-dose rats revealed no effect of treatment.

The NOAEL for systemic toxicity was 150 ppm (equal to 11 mg/kg bw per day) based on reduced body weight gain at termination and reduced feed consumption early during treatment in females at 750 ppm (equal to 53 mg/kg bw per day). The NOAEL for neurotoxicity was 750 ppm (Foss, 2009).

(b) Pharmacological study

In a safety pharmacology study, a single gavage dose of pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0 %) as a suspension in 0.5% aqueous sodium CMC, was administered to groups of five F344/DuCrlCrlj female rats or groups of eight Crj:CD-1 (ICR) female mice at doses of 0, 5, 50 and 500 mg/kg bw. The rats were subjected to a FOB at 1, 2, 4, 6 and 24 h after dosing. At the same time points. motor activity, body temperature and pupil diameter were measured. Blood pressure and heart rate were measured at 1, 2, and 4 h after administration. Urine was collected from time of dosing to 6 h after administration for measurement of urinary Na^+, K^+, and Cl^- concentrations and osmotic pressure. The mice received the same treatment as the rats and one hour after administration hexobarbital (80 mg/10 mL per kg) was injected intraperitoneally. The time between loss of righting reflex and recovery from loss of righting reflex (sleep time) was measured. The animals that exhibited a sleeping time of more than 3× the mean sleeping time in the vehicle-treated group were regarded as exhibiting a sleep-reinforcing effect.

In rats treated at 500 mg/kg bw, all animals displayed prone or lateral position, abnormal respiration, lacrymation, low body temperature, ataxic gait (drag or staggering gait), decreased mobility (impossible gait), loss of approach, touch and sound responses, abnormal landing, loss of righting reflex, decreased muscle tone in the abdomen, and decreased grip strength in hind limbs. Four animals displayed decreased arousal, decreased muscle tone in the limbs, decreased grip strength in the forelimbs and three animals displayed lacrimation and urine incontinence. Two animals displayed pupillary mydriasis and one showed decreased pinna reflex. These signs had already been observed from 1–6 h after administration and became more serious 24 h after administration or later, when some animals displayed hunchback position, weak breathing, ptosis, salivation, loss of eyelid reflex, cyanosis and tiptoes gait. On each of days 3 and 4 one female died. The three surviving animals recovered 2–6 days after administration. At the high dose, motor activity was statistically significantly inhibited 1, 4, 6 and 24 h after administration. In addition there was observed a significant decrease in blood pressure at 2 h and 4 h after administration and a significant decrease of heart rates compared with the control group at 1, 2, and 4 h after dosing. High-dose rats lost body weight during the first two days and slowly gained body weight thereafter. Final body weight (112 g) was 15% lower than for controls (131 g). In rats treated at 50 mg/kg bw piloerection and decreased mobility were observed in two animals and decreased muscle tone in the abdomen and limbs in one animal, 4 and 6 h after administration. No effects were observed at 24 h or later. At 50 mg/kg bw there was a significant decrease in motor activity one hour after administration only. Body weight, blood pressure and heart rate were not affected at 50 mg/kg bw. No treatment-related effects were observed in rats at 5 mg/kg bw. No effects on renal function of rats were observed at any dose.

No effect on hexobarbital-induced sleeping time was observed in mice at 5 mg/kg bw. An extension of the hexobarbital-induced sleeping time was observed at 50 and 500 mg/kg bw (more than 3× the mean sleeping time of control mice was observed in 4/8 animals at 50 mg/kg bw and in all animals at 500 mg/kg bw).

For rats, the NOAEL was 50 mg/kg bw, based on mortality, body weight loss, marked clinical signs and decreased mobility observed at 500 mg/kg bw (Tsuchiyama, 2006).

(c) Immunotoxicity

In a four-week immunotoxicity study, pyrifluquinazon (technical grade; Batch no. 3FZ0013G; purity 98.0%) was administered for about four weeks to four groups of ten male and ten female Sprague Dawley (Crl:CD(SD)) rats at dietary concentrations of 0, 30, 150 and 750 ppm (equal to 0, 2.5, 12 and 62 mg/kg bw per day for males, 0, 2.7, 13 and 63 mg/kg bw per day for females). A fifth group of five male and five female rats served as a positive control and received an intraperitoneal dose of cyclophosphamide, at 25 mg/kg, once daily for four consecutive days prior to scheduled termination. Four days prior to necropsy, all animals in all five groups received a single intravenous dose of sheep red blood cells (sRBCs; 4 × 108 cells/mL) in 0.9% saline. The rats were checked daily for mortality and clinical signs. Body weights and feed consumption were measured weekly. At termination the rats were necropsied and weights of brain, spleen and thymus recorded. Spleens were collected for use in the antibody-forming cell assay (AFC assay). Splenocyte suspensions of each spleen from all animals were mixed with fresh sRBCs and guinea pig complement in a semisolid agarose matrix. Monolayers of this mixture were plated in Petri dishes and the cultures incubated at 37°C for at least 3 h. AFC was assessed by visual quantification of the resulting haemolytic plaques.

No mortality, clinical signs, effects on organ weights or macroscopic findings were observed. Decreases in body weight, body weight gain, and food consumption were noted for males given 150 ppm or 750 ppm and females given 750 ppm. Statistically significant decreases in body weight gain were observed at 750 ppm in both sexes during the second week and also in males during the third week. Final body weights were decreased by 7–8% compared to controls. There were no statistically significant changes in antibody-forming cells in response to sRBCs, as measured using the AFC assay, (that is AFC per spleen, AFC per million splenocytes or absolute and relative splenocyte viability) for any of the treated groups, compared to the control. As expected, animals given cyclophosphamide showed decreases in body weight gain, feed consumption, decreases in spleen and thymus weights and an ablated AFC response (indicative of immunosuppression), compared to controls. It was concluded that pyrifluquinazon did not cause immunotoxicity at doses up to 750 ppm (equal to 62 mg/kg bw per day) as assessed by the AFC assay (Arrington, 2009).

In order to establish a potential mechanism for the mononuclear cell infiltration in the nasal mucosa observed in the one-year repeated oral toxicity studies (Kuwahara, 2006c and Shibuya, 2008), an immunological study, using cell-mediated and antibody-mediated immunological examinations, was conducted using the blood and tissue samples obtained from Shibuya, 2008. Details on the study design can be obtained from the study evaluation described above (Shibuya, 2008). Blood samples obtained before treatment and at 13, 26, 39 and 52 weeks of treatment were analysed for lymphocyte subsets (pan T cell ($CD3^+$), pan B cell ($CD21^+$), helper T cell ($CD3^+CD4^+$), cytotoxic T cell ($CD3^+CD8^+$)) and measurements of immunoglobulin (IgE, IgM and IgG) were carried out. In addition, flow cytometric analysis of lymphocyte subsets (pan B cell ($CD21^+$), pan T cell ($CD3^+$), IgE positive B cell (IgE^+CD21^+), helper T cell ($CD3^+CD4^+$), cytotoxic T cell ($CD3^+CD8^+$)) was performed on the submandibular lymph node obtained at necropsy after 52 weeks of treatment.

No treatment-related changes were found in the flow cytometric analysis of lymphocyte subsets for peripheral blood or the submandibular lymph node. Nor were there effects of treatment in serum immunoglobulin (IgE, IgM and IgG) (Kosaka, 2008).

(d) Mechanistic studies – in vitro

Liver metabolism

A study was performed to investigate an observed prolongation of hexobarbital sleeping time in pyrifluquinazon-treated mice. Liver microsomes were prepared from untreated female F-344 rats. Inhibition of ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD) by pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) and by its metabolite IV-01 (Batch no. 4FZ6404P; purity 98.7%) at concentrations of 0.5, 2.5, 12.5, 62.5 and 312.5 µM was investigated. In a second part of the study male ddY mice received a single gavage dose of vehicle or pyrifluquinazon at 500 mg/kg bw and after one hour the liver was dissected to prepare microsomes. These mouse microsomes were used to evaluate metabolism of hexobarbital, measured by HPLC.

In rat liver microsomes no inhibition of PROD was observed following treatment with pyrifluquinazon or IV-01, whereas both substances inhibited EROD activity to about 20% of control values. In microsomes from mice the metabolism of hexobarbital was significantly inhibited by about 40% after a single dose of pyrifluquinazon. The study's author concluded that the prolonged hexobarbital sleeping time seen in mice could be the result of hepatic enzyme inhibition (Amanuma, 2006c).

Investigation of antiandrogenic effects

Androgen receptor binding affinity

A study was conducted to assess the binding affinity of pyrifluquinazon (Batch no. 2FZ0008P; purity 99.1%) and its metabolites IV-01 (Batch no. 4FZ6403P; purity 97.3%), IV-02 (Batch no. 4FZ6304P; purity 99.3%), IV-203 (Batch no. 4FZ0601S; purity 97.2%) and IV-208 (Batch no. 4FZ4501P; purity 99.7%) to the androgen receptor (AR) by measuring the displacement of ^{3}H-labelled synthetic androgen R1881. The competitive binding was tested, both to a commercially available recombinant rat AR-ligand binding domain (AR LBD) and to AR, in Sprague Dawley rat prostate homogenate, prepared 24 h after castration. Dihydrotestosterone (DHT) was used as positive control.

The assays showed that pyrifluquinazon and IV-01 have weak affinities (25 and 57% inhibition respectively of R1881 binding at 100 μM) to the AR LBD (25 and 57% inhibition respectively of R1881 binding at 100 μM) and rat prostate AR (20 and 25% inhibition respectively of R1881 binding at 100 μM). IV-02, IV-203 and IV-208 did not affect R1881 binding to the AR (Masaki, 2008a).

Androgen receptor reporter gene assay

A study was conducted to assess the antiandrogenic effect pyrifluquinazon and its metabolites IV-01, IV-02, IV-203 and IV-208 IV-01 by measuring the activation of luciferase, in the human breast cancer cell line MDA-MB-453-kb2 that was transformed with an AR-responsive luciferase reporter gene. This cell line consitutively expresses AR. Batch numbers and purities of pyrifluquinazon and its metabolites were not provided. Incubation of the cells with the test substances was conducted in the presence or absence of 0.2 nM DHT, to also assess receptor binding antagonism. Cell suspensions were incubated at 37°C in the presence or absence of test substances for approximately 20 h. Subsequently, cells were lysed, a luciferin-containing buffer was added, and luciferase activity measured.

Pyrifluquinazon and its metabolites did not induce inhibition of DHT-stimulated luciferase expression. On the contrary, a slight increase in DHT-independent luciferase expression (up to 150%) was observed with the test substances, indicating a weak induction through the AR or glucocorticoid receptors. Pyrifluquinazon and its metabolites did not show evidence of AR inhibition in this assay (Tamura, 2005).

A study was conducted to assess the antiandrogenic effect pyrifluquinazon (Batch no. 51201R; purity 98.7%) in rats. This was done by measuring the AR-mediated transcriptional activity and AR protein content in a reporter gene assay, employing forcedly expressed rat AR. Rat AR cDNA was cloned by the reverse transcription polymer chain reaction (RT-PCR) and cotransfected into the HEK293 (human kidney) cell line with a luciferase reporter (AR response element with luciferase gene). Cell cultures were incubated with pyrifluquinazon for 24 h in the presence and absence of DHT, and luciferase expression measured. AR protein was assayed by sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis and immunostaining followed by fluorescence autoimaging.

Pyrifluquinazon inhibited DHT-stimulated luciferase activity in a dose-dependent manner with a half-maximal inhibitory concentration (IC_{50}) of 7.2 μM. No effect was observed in the absence of DHT. The amount of rat AR protein in the HEK293-transfected cells was significantly decreased by pyrifluquinazon. The study's author concluded that pyrifluquinazon suppressed transcriptional activity mediated by rat AR, and this was accompanied by a decrease in the amount of AR protein, probably through enhanced degradation of rat AR protein (Masaki, 2010b).

A study was conducted to clarify the underlying mechanism of pyrifluquinazon's antiandrogenic effect and the nuclear localization of both rat and human AR. The nuclear localization of AR was assessed under the influence of pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) and metabolite IV-01 (Batch no. 4FZ6404P; purity 98.7%), in three cell lines. Rat AR was forcibly expressed in HEK293 cells. The human cell lines MDA-MB-453-kb2 (breast) and LNCaP (prostate) both constitutively express human AR. The cells were cultured for 8 h (2 h for LNCaP cells) in the presence of DHT to promote AR activation and translocation into the nucleus. Subsequently the cells were fixed in paraformaldehyde and incubated with an anti-rat AR polyclonal antibody, then tagged with a fluorescence-linked (Alexa 546) goat anti-rabbit second antibody, and visualized by fluorescence microscopy. AR and whole nuclei were visualized by staining with AR-specific antibody and 4′,6-diamidino-2-phenylindole (DAPI), respectively. The cell nucleus was counter-stained with DAPI. Emodin, a known inhibitor of AR nuclear translocation, was used as a positive control.

In the absence of DHT, rat and human ARs appeared broadly distributed in cytoplasm and nucleus. After DHT exposure, both human and rat ARs were confined to the nucleus, indicating translocation to the nucleus had occurred. After incubation with pyrifluquinazon and IV-01 at a concentration of 25 μM, rat AR remained partially in the cytoplasm. This effect was more significant with IV-01. Pyrifluquinazon and IV-001 did not affect nuclear translocation of the human AR in breast or prostate cell lines. Emodin increased cytoplasmic distribution of AR in the presence of DHT for both human and rat AR. The study's author concluded that pyrifluquinazon and IV-01 selectively inhibit nuclear translocation of rat, but not human AR (Masaki, 2010a).

A study was performed to clarify the involvement of the binding site in the interruption by pyrifluquinazon of the androgen-signaling pathway in rats. HEK293 human kidney cells were transfected with an antioxidant response element (ARE)-luciferase reporting element and a truncated rat AR cDNA lacking the ligand-binding domain. Twenty-four hours after transfection, cells were incubated for 10 h with pyrifluquinazon (10 or 25 μM; batch number and purity not reported) and DHT (1.0 nM). Hydroxyflutamide (1.0 μM) was used as a positive control.

Pyrifluquinazon inhibited DHT-stimulated luciferase expression with both the wild-type (source not reported) and truncated AR. Hydroxyflutamide inhibited luciferase expression of only the wild-type rat AR. The study's author concluded that pyrifluquinazon inhibited transactivation of rat AR even in the absence of the AR ligand-binding site (Masaki 2010d).

A study was performed to elucidate the antiandrogenic potential of pyrifluquinazon for humans by measuring AR protein levels. MDA-kb2 human breast cells, constitutively expressing human AR, were incubated for 24 h with pyrifluquinazon (0, 0.1, 1, 10 or 30 μM; Batch no. 51201R; purity 98.7%) in the presence and absence of DHT (concentration not reported). Cells were then lysed and centrifuged, and the supernatant denatured by boiling. The supernatant was subjected to SDS-PAGE electrophoresis (Western blot). After transfer to a polyvinylidene difluoride (PVDF) membrane, AR was identified by a (poorly described) primary anti-AR antibody, then tagged with a second anti-rabbit IgG antibody and visualized by a multi-imager.

Pyrifluquinazon had no clear effect on the levels of AR protein. The study's author concluded that the antiandrogenic potential of pyrifluquinazon could be insignificant for humans (Masaki, 2010c).

Steroid 5-α reductase inhibition

A study was performed to test the inhibitory effects of pyrifluquinazon (Batch no. 2FZ0008P; purity 99.1%) and its metabolites IV-01 (Batch no. 4FZ6403P; purity 97.3%), IV-02 (Batch no. 4FZ6304P; purity 99.3%), IV-203 (Batch no. 4FZ0601S; purity 97.2%) and IV-208 (Batch no. 4FZ4501P; purity 99.7%) on 5-α reductase activity in vitro in microsomes prepared from rat prostate obtained from ten-week-old male Sprague Dawley rats. Prostate tissue samples pooled from several animals were homogenized and S9 supernatant used for the assay. Conversion of ^{3}H-testosterone was evaluated by thin-layer chromatography using testosterone, DHT, finasteride and 4-androstene-3,17-dione as a reference standard.

Pyrifluquinazon and its metabolites IV-02, IV-203 and IV-208 at concentrations of 10 and 100 μM did not inhibit 5-α reductase activity. IV-01 inhibited 5-α reductase activity (IC_{50} = 5.7 μM), although less potent compared to finasteride (IC_{50} = 30 nM). In contrast to finasteride, the inhibition by IV-01 appeared to be non-competitive and varied with NADPH concentration. As plasma IV-01 concentrations of about 1.9 μg equiv./g, which is equivalent to 9.5 μM, were observed after a single oral dose of 100 mg/kg bw (Yoshizane, 2006a), it was concluded that concentrations of IV-01 in animal tissues could exceed the IC_{50} (Amanuma, 2008c).

Estrogen receptor binding assay

A study was performed to determine the binding affinity of pyrifluquinazon (Batch no. 4FZ0017P; purity 99.9%) and its metabolites IV-01 (batch no. 4FZ6403P, purity 98.7%), IV-02 (Batch no. 4FZ6304P; purity 99.3 %), IV-203 (Batch no. 4FZ0601S; purity 97.2%) and IV-208 (Batch no. 4FZ4501P; purity 99.7%) to purified recombinant human estrogen receptor (hER-α and hER-β), using a Fluormone ES2 competitive ligand-binding polarization technique. The recombinant hER was added to a fluorescence-labelled (Fluormone ES2) ER ligand to form a ligand-ER complex. If the test material competes with the ligand and displaces it from the ER ligand-binding site, the change in polarization characteristics of the preparation (high polarization for the large molecule when complexed with the ER receptor; low polarization for the small fluorescent ligand when displaced) can be measured. Initially the assays used ten-fold dilution factors to give a concentration–response curve. If the concentration curve showed an IC_{50}, a second assay was performed using two-fold dilutions close to the IC_{50} β-estradiol was used as a positive control.

For IV-208 an IC_{50} of 1.43×10^{-4}M for ERα was obtained: for ERβ it was 8.81×10^{-5}M. No displacement of the ligand was observed with pyrifluquinazon, IV-01, IV-02 or IV-203. These results indicate that IV-208 has binding affinity to hER-α and hER-β (Takeda, 2007).

(e) Mechanistic studies – in vivo

Investigation of effects on luteinizing hormone (LH)

Mouse

In a 13-week dietary study, performed to investigate whether interstitial (Leydig) cell tumours in the testes might be secondary to enhanced LH secretion, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered for 4, 8 or 13 weeks to groups of 20 male ICR (Crlj: CD1) mice at 0, 250, 500 or 1000 ppm (equal to 0, 33, 71 and 136 mg/kg bw per day). The animals were checked daily for mortality and clinical signs. Body weights and feed consumption were measured weekly. Blood samples were collected on the day before termination after 4, 8 or 13 weeks of treatment. Serum was assayed for LH and total testosterone. After 13 weeks of treatment DHT was also measured. At termination the mice were macroscopically examined and testes, epididymides and liver weighed and microscopically examined.

One mouse at 500 ppm died on day 50. This death was not considered related to treatment. No treatment-related clinical signs were observed. Small (up to 5%) but statistically significant lower body weight gains were observed from weeks 5–8 at 500 ppm, and at 1000 ppm throughout treatment. Feed consumption was not affected. Compared to controls, at 1000 ppm a decrease in absolute and relative epididimis weight (14 and 13%, respectively) was observed. Testis weight was not affected by treatment. At 250, 500 and 1000 ppm increases in absolute (12, 14 and 24%, respectively) and relative (8, 16 and 28%, respectively) liver weights were observed. An increase in serum LH was observed at 4, 8 and 13 weeks at 500 ppm (63–195%) and 1000 ppm (57–344%). At 250 ppm, an increase in LH (104%) was only observed during week 8. At 4, 8 and 13 weeks a non-dose-dependent increase in testosterone levels was observed at all doses (up to 146, 120 and 63% at 250, 500 and 500 ppm, respectively), occasionally reaching statistical significance at low and mid doses. At 13 weeks DHT was increased at 500 ppm (108%) and 1000 ppm (112%), reaching statistical significance at the high dose only. At necropsy, no treatment-related macroscopic findings were observed. After 13 weeks of treatment, testicular interstitial cell hypertrophy was observed in one animal at 500 ppm and in sixteen animals at 1000 ppm. No treatment-related histopathological changes were observed after 4 and 8 weeks of treatment.

Hormonal changes are considered to be a consequence of the stimulation of testosterone production in the interstitial cells by LH. The effects on the serum hormonal levels observed in this study are comparable with the results of the mechanistic study in rats (Amanuma, 2010). The study's author concluded that there is no difference between mice and rats with respect to the mechanism of testicular interstitial cell tumour induction by pyrifluquinazon (Nagai, 2011).

Rat

In a 13-week dietary study, performed to investigate whether rat Leydig cell tumours might be secondary to enhanced LH secretion, pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) was administered to groups of 15 male Fischer 344 rats at 0, 50, 350, 1300 or 2500 ppm (equal to 0, 6, 21, 77 and 145 mg/kg bw per day). The animals were checked daily for mortality and clinical signs. Body weights were measured weekly and feed consumption twice per week. Blood samples were collected under anaesthesia before the start of treatment, after 2, 4, 8 weeks and at termination. Serum was assayed for LH and total testosterone. At week 8 only, free testosterone and DHT were also measured. At termination after 13 weeks the rats were examined macroscopically and testes, epididymides, prostate, seminal vesicles with coagulating gland (SVCG) and pituitary were weighed, and microscopically examined.

Two rats died under anaesthetic (one after two weeks at 1300 ppm, and one after four weeks at 2500 ppm). These deaths were not considered treatment-related. At 2500 ppm, mainly in the first week of treatment, stained fur (periocular, perinasal and/or in the genital/anogenital region) was observed in all animals and lacrimation observed in two animals. Other sporadically observed clinical signs were not considered treatment-related. Statistically significant lower body weight gain was observed at 2500 ppm throughout the treatment period. Body weights were 12% lower compared to controls after one week and 24% lower compared to controls at termination. Feed consumption at the 2500 ppm dose level was decreased by about 20% throughout the treatment period. Body weight gain and feed consumption were not affected at lower doses. Compared to controls there was at 1300 and 2500 ppm a dose-related decrease in the weights of prostate (27 and 66%), SVCG (8 and 45%), and epididymides (13 and 49%), all being

statistically significant, except for the reduction in SVCG weight at 1300 ppm. There was no change in testis or pituitary weight. At 2500 ppm a rapid increase in serum LH (33% at week 2) and testosterone values (345% at week 2) was observed. At 1300 ppm total testosterone levels were increased (207%) at week 2. At week 8 all measures of serum testosterone (total, free, DHT) were increased at 1300 ppm (109, 134 and 61%, respectively) and at 2500 ppm (268, 371 and 189%, respectively). Macroscopy revealed increased incidences in accented lobular pattern at 1300 and 2500 ppm and dark-coloured liver and discoloration of the thyroid at 2500 ppm. Despite increased testosterone levels, an increase in testicular seminiferous tubular atrophy and of germ cell debris in the epididymides was found in most rats at 1300 ppm and in all rats at 2500 ppm. At the high dose vacuolation of tubular epithelial cell of the epidydimides was also observed in all rats.

The study's author concluded that an increased level of LH was seen in this study, consistent with an LH-promoted mode of action (MOA) for Leydig cell tumourigenesis (Amanuma, 2010).

Investigation of antiandrogenic effects

Study 1

The antiandrogenic activity of pyrifluquinazon was investigated in a Hershberger study. Groups of six young, castrated male Sprague Dawley rats received pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) by gavage in corn oil at 0, 50, 100 or 200 mg/kg bw per day for ten days. Rats were castrated at six weeks of age, and treatment started seven days thereafter. In the first part of the study, all animals received subcutaneous (s.c.) injections of testosterone propionate (TP) at 0.4 mg/kg bw per day simultaneously with pyrifluquinazon or a positive control substance. Finasteride at doses of 0.2, 1.0 and 5.0 mg/kg bw per day was used as a 5-α reductase inhibitor positive control. In the study's second part, TP was replaced by DHT at 1.25 mg/kg bw per day and finasteride at a dose of 5.0 mg/kg bw per day was used as a 5-α reductase inhibitor positive control, while flutamide at doses of 5 and 10 mg/kg bw per day as an androgen receptor antagonist positive control. At the end of the treatment period the rats were terminated and the ventral prostate, levator ani/bulbocarvernosus muscle (LABC), and SVCG were weighed. The results of the study are presented in Table 23.

Table 23. Effects of pyrifluquinazon in a Hershberger study in rats

Study Part 1		**Pyrifluquinazon**			**Finasteride**		
Dose (mg/kg bw per day)	**0**	**50**	**100**	**200**	**0.2**	**1.0**	**5.0**
Number of males	6	6	6	6	6	6	6
TP (mg/kg bw per day, s.c.)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Final body weight (g)	282	266	267	256*	278	274	286
Prostate (mg)	209	177	115**	53**	139**	142**	114**
SVCG (mg)	509	403	288**	100**	293**	262**	207**
LABC (mg)	578	475*	387**	534**	499*	523	519

Study Part 2		**Pyrifluquinazon**		**Finasteride**	**Flutamide**	
Dose (mg/kg bw per day)	**0**	**100**	**200**	**5.0**	**5.0**	**10**
Number of males	6	6	6	6	6	6
DHT (mg/kg bw/day, s.c)	1.25	1.25	1.25	1.25	1.25	1.25
Final body weight (g)	275	259	262	276	277	270
Prostate (mg)	111	85	56**	129	46**	32**
SVCG (mg)	184	114	87	222	81*	70*
LABC (mg)	473	361	262**	539	266**	237**

TP Testosterone propionate DHT Dihdrotestosterone SVCG Seminal vesicles with coagulating gland

LABC Levator ani/bulbocarvernosus muscle

Level of statistical significance: * $p \leq 0.05$ ** $p \leq 0.01$

In the first part of the study, the 5-α reductase inhibitor finasteride induced a decrease, as expected, in TP-stimulated prostate and SVCG weights, but had no effect on LABC weights, which was also as expected since the prostate lacks 5-α reductase. Treatment with pyrifluquinazon induced statistically significant weight decreases in all three organs. A minor decrease in body weight in rats receiving the top dose of pyrifluquinazon was considered unlikely to affect interpretation of the results, although accessory reproductive organ weights are sensitive to body weight change.

In the second part of the study, finasteride had no effect on DHT-stimulated accessory reproductive organ weights, as expected, since DHT bypasses the 5-α reductase enzyme inhibition of finasteride. Rats treated with flutamide showed a dose-dependent inhibition of DHT-stimulated organ weight in prostate, SVCG and LABC. Pyrifluquinazon also induced a decrease in DHT-stimulated prostate, SVCG and LABC weight.

The study's author concluded that the observed antiandrogenic effect was most likely due to androgen receptor blockade but some weak influence due to 5-α reductase enzyme inhibition may have also have occurred (Amanuma, 2006d).

Study 2

In a second Hershberger study the effect on the androgen receptor protein was investigated in male Sprague Dawley rats castrated at six weeks of age. Seven days after castration groups of four male rats were treated by gavage for ten days with corn oil (vehicle control), pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at 200 mg/kg bw per day, flutamide at 5 mg/kg bw per day, or finasteride at 5 mg/kg bw per day. All rats also received daily s.c. injections of TP at 0.4 mg/kg bw per day. One day after the ten-day treatment period, rats were terminated and the ventral part of the prostate weighed and frozen, the LABC muscle and SVCG (together with remaining part of the prostate) were fixed in formalin, separated and weighed the next day Samples of prostate were frozen and AR protein levels in prostate determined by Western Blot. The findings of the study are presented in Table 24.

Table 24. Effects of pyrifluquinazon in a Hershberger study in rats

		Pyrifluquinazon	Flutamide	Finasteride
Dose (mg/kg bw per day)	**0**	**200**	**5.0**	**5.0**
TP (mg/kg bw per day, s.c)	0.4	0.4	0.4	0.4
Final body weight (g)	292	283	292	298
Prostate (mg, ventral lobe only)	203	49***	45***	91***
SVCG (mg)	691	151***	109***	242***
LABC (mg)	647	268***	280***	618
AR content (% of control)	100	49***	61***	81

TP Testosterone propionate AR Androgen receptor SVCG Seminal vesicles with coagulating gland
LABC Levator ani/bulbocarvernosus muscle
Level of statistical significance: *** $p \leq 0.001$

Body weights were not affected by treatment with the various substances. Pyrifluquinazon induced statistically significant reductions in the weights of ventral prostate, SVCG, and LABC. Treatment with the antiandrogen flutamide induced a similar reduction in organ weights, while treatment with the 5-α reductase inhibitor finasteride induced decreases in prostate and SVCG weights but not LABC weight. Both pyrifluquinazon and flutamide induced a statistically significant decrease in the AR protein content of prostate samples. Finasteride induced a smaller, not statistically significant, reduction in prostate AR content. The study's author concluded that since an AR reporter gene assay showed no binding of pyrifluquinazon to the AR (Tamura, 2005), these results offer evidence that pyrifluquinazon may act by a different mechanism, affecting AR content (Amanuma, 2008a).

Groups of male Sprague Dawley rats (number of animals per group not reported), were administered a single gavage dose of pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at 0, 100 or 200 mg/kg bw. Additional groups were administered flutamide or finasteride (5 mg/kg bw) as reference controls. Rats were euthanized at 6, 12 or 24 h after dosing and the prostate dissected free and cut into two equal samples. One half was homogenized, centrifuged at 14 000 g, and the supernatant denatured

(mercaptoethanol and boiling). The denatured sample was subject to SDS-PAGE electrophoresis, and the protein bands transferred to a PVDF membrane by flat-plate electrophoresis. This was then incubated with rabbit anti-AR antibody and a mouse anti-tubulin antibody, then with HRP-linked anti-rabbit IgG or anti-mouse IgG. The prostatic AR levels were quantified by fluorescence measured by an auto-imager. The second half of each prostate was fixed in formalin for 24 h, and sections cut. The sections were incubated with rabbit anti-AR antibody and immunostained with a peroxidase substrate. Additional samples were homogenized, deproteinized with chloroform, and total RNA recovered for RT-PCR with primers for AR and β-actin. From prostate samples taken at 12 h, mRNA was subject to Northern blot analysis and hybridized with a ^{32}P-labelled probe against AR mRNA.

Table 25. Effect of a single dose on prostate AR

		Pyrifluquinazon		Flutamide	Finasteride
Dose (mg/kg bw/day)	**0**	**100**	**200**	**5**	**5**
Prostate weight (mg))					
6 hours	303	245	239	285	237
12 hours	236	264	261	224	251
24 hours	289	225	214	264	254
AR protein content (% control)					
6 hours	100	56*	40***	63*	83
12 hours	100	51**	47**	81	67*
24 hours	100	97	48**	104	129
AR mRNA content (% control)					
6 hours	100	100	97	159	146
12 hours	100	118	115	160*	124
24 hours	100	130	135	183*	104

AR Androgen receptor

Level of statistical significance: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Source: Masaki, 2008b

AR protein content in rats treated with pyrifluquinazon at 100 mg/kg bw decreased within 6 h of dosing, was still at the low level at 12 h, then recovered by 24 h after dosing, but not so at 200 mg/kg bw. Flutamide and finasteride also induced a reduction in AR protein content, however to a lesser extent. The reduction in AR protein in the prostate following pyrifluquinazon administration was also observed in immunostained sections. No clear effect on AR mRNA was detected after treatment with pyrifluquinazon and finasteride, while flutamide induced a small increase in AR mRNA levels. The study's author concluded that the data indicate that the antiandrogenic effect of pyrifluquinazon is a consequence of a decline in AR protein in accessory genital organs (Masaki, 2008b).

Investigation of estrogenic effects

A uterotrophic assay was performed to evaluate the possible estrogenic potential of pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%). Female Sprague Dawley (Crl:CD) rats were weaned at 20 days of age at which stage dosing commenced. To evaluate the estrogenic potential, groups of eight rats received pyrifluquinazon by gavage at doses of 0, 50, 100, 150 or 200 mg/kg bw per day for three consecutive days. An additional group received s.c. injections of 17-β-estradiol (17-β E2; 3 or 10 µg/kg bw per day) as a positive control. To evaluate antiestrogenic activity, groups of six rats receiving s.c. injections of ethinylestradiol (EE, 3 µg/kg bw per day) were administered pyrifluquinazon at 100 or 200 mg/kg bw per day, and the effects compared to s.c. injections of ICI 182,780 (50 or 200 µg/kg bw per day) as an antiestrogenic positive control. All animals were observed daily for clinical signs One day after the last dose the rats were weighed and euthanized and uterine weight measured, first with any luminal fluid, then as a blotted tissue weight.

In the test for estrogenic potential, one female at 200 mg/kg bw per day died during the study. Three additional females at this dose showed soiled fur in the genital region. Final body weight at this

dose was statistically significantly lower (15%) compared to controls. Other results of the study are presented in Table 26. Pyrifluquinazon did not cause a change in uterine weight. The positive control 17-β E2 induced an increase in uterine weight. In the test for antiestrogenic potential one female at 200 mg/kg bw per day showed exhaustion and was euthanized. At this dose two other females showed emaciation, one of which also displayed abnormal gait and reduced locomotor activity. Final body weight at the high dose was lower (14%) than controls without reaching statistical significance. Ethinylestradiol resulted in significantly increased uterine weight. The positive control, ICI 182,780, induced a dose-related inhibition of this increase. A moderate statistically significant inhibition (37%) of the ethinylestradiol-induced increase in absolute uterine weight was observed after treatment with pyrifluquinazon at 200 mg/kg bw per day, but not at 100 mg/kg bw per day. Effects on relative uterine weight showed similar results, but did not reach statistical significance. It was concluded that in this study pyrifluquinazon showed no direct estrogenic effect, but a weak antiestrogenic effect could not be excluded (Amanuma, 2008b).

Table 26. Uterotrophic assay in rats – key results

Estrogenicity assay	**Pyrifluquinazon**					**17-β E2**		
Dose (mg/kg bw per day)	0	50	100	150	200			
Dose (µg/kg bw per day, s.c.)						0	3	10
Number of females	8	8	8	8	7	6	6	6
Final body weight (g)	66	65	62	60	55***	69	68	65
Uterus, wet weight (mg)	31	35	35	31	29	36	99**	101**
Uterus, blotted (mg)	30	34	32	29	27	33	96***	98***

Antiestrogenicity assay		**EE**	**EE + pyrifluquinazon**		**EE + ICI 182,780**	
Dose of pyrifluquinazon (mg/kg bw/day)	0	0	100	200		
Dose ICI (µg/kg bw per day, s.c)					50	200
EE, µg/kg bw per day s.c	0	3	3	3	3	3
Number of females	6	6	6	5	6	6
Final body weight (g)	70	70	67	60	70	70
Uterus, wet weight (mg)	37	274###	245	171*	155**	63***
Uterus, blotted (mg)	34	157###	153	123	127*	58***

* $p < 0.05$ ** $p < 0.01$ *** $p \leq 0.001$ compared to respective control

$p \leq 0.001$ compared to non-EE control

Source: Amanuma, 2008b

Investigation of thyroid change

Study 1

A 14-day study in rats was performed to investigate the effect of pyrifluquinazon on serum levels of thyroid hormones and TSH and on hepatic uridine diphosphate glucuronosyltransferase (UDP-GT) activity. Groups of five male Fischer rats received a diet containing pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at dietary concentrations of 0, 100, 350 and 1300 ppm (equal to 0, 9.2, 32 and 117 mg/kg bw). The animals were checked daily for clinical signs. Body weights and feed consumption were measured weekly. Blood was collected pretreatment and on days 7 and 14 for measurement of serum levels of T_3, T_4 and TSH. At termination the animals were necropsied and their liver, thyroid and pituitary weighed and subjected to histopathological observation. In addition, hepatic UDP-GT activity was measured.

No effects of treatment on mortality, clinical signs, body weight or feed consumption were observed. Necropsy showed an enlarged thyroid in one high-dose male. Other effects of treatment are presented in Table 27. Increases in relative weight of liver and thyroid, and thyroid follicular cell hypertrophy and hypertrophic hepatocytes were observed at 1300 ppm. Induction of microsomal UDP-GT activity with

p-nitrophenol as substrate was observed in the liver microsomal fraction at 1300 ppm. Changes with androsterone as substrate were modest and inconsistent. Compared with controls, the serum levels of T_3 were statistically reduced after seven days of administration in all dose groups in a dose dependent manner. After 14 days of administration levels were comparable to the controls, other than for the 1300 ppm group, where they were increased. Serum levels of T_4 and TSH were slightly increased in the 1300 ppm group after 14 days, reaching statistical significance for T_4 only.

Table 27. Key dose-related findings of the 14-day study in rats

Diet concentration (ppm)	0	100	350	1300
Organ weights				
Liver (g)	7.1	7.3	7.3	7.9
Liver (g/100 g bw)	3.7	3.8	3.9	4.3**
Thyroid (mg)	15.4	15.4	16.3	17.7
Thyroid (mg/100 g bw)	8.0	8.1	8.7	9.7**
Microsomal UDP-GT activity in the liver				
p-Nitrophenol	39	48	47	83**
Androsterone	2.8	4.0**	3.4	3.8*
Thyroid-related hormone concentration in serum				
T_3 (ng/dL)				
day 0	170	135	114**	153
day 7	244	182*	123**	56**
day 14	118	74**	122	186**
T_4 (µg/dL)				
day 0	16.7	16.3	14.8	17.1
day 7	15.5	15.1	15.4	15.0
day 14	17.2	17.2	18.2	21.8**
TSH (ng/mL)				
day 0	8.2	8.8	8.1	8.5
day 7	11.0	10.8	13.2	13.9
day 14	10.9	12.0	11.9	16.7

Level of statistical significance: * $0.01 < p < 0.05$ ** $p < 0.01$ in comparison to control

Source: Amanuma, 2006b

The study's author suggests that increased thyroid weight and hypertrophy of thyroid follicular cells caused by pyrifluquinazon are secondary to the induction of hepatic UDP-GT that results in the stimulation of the thyroid. However, the Meeting concluded that the data were not entirely conclusive (Amanuma, 2006b).

Study 2

A second study was performed to further investigate the MOA underlying thyroid change seen in some studies. Groups of eight male F-344 rats received diet containing pyrifluquinazon (Batch no. 3FZ0013G; purity 98.0%) at concentrations of 0, 350, 1300 or 2500 ppm (equal to 0, 28, 103 or 179 mg/kg bw per day) for eight weeks. The animals were checked daily for clinical signs. Body weights were measured weekly and feed consumption was measured twice per week. Blood was sampled prior to treatment and at weeks 2, 4 and 8 for measurement of serum T_3, T_4 and TSH. At week eight only, free T_3 was also determined. At termination the animals were necropsied and liver, thyroid and pituitary weighed. A portion of liver was taken for biochemical assessment. Microsomes were prepared and UDP-GT activity determined using 4-nitrophenol as a substrate for T_4-glucuronyltransferse activity and using androsterone as a substrate for T_3-glucuronyltransferse activity. Liver, thyroid and pituitary were examined histologically.

No animals died during the study. Stained fur (genital or periocular) was observed on one animal at 1300 ppm and all animals at 2500 ppm while lacrimation was observed in some animals at 2500 ppm. These changes were observed during the first week of administration and disappeared in the second week. Discoloured eyeball, suggestive of anemia, was also observed in some animals at 2500 ppm during 3–5 weeks of treatment. Other effects due to pyrifluquinazon are presented in Table 28. A reduced body weight gain throughout the treatment period was observed at 2500 ppm. Final body weights at 2500 ppm were markedly lower (31%) than controls. Slight (up to 6%), but statistically significant lower body weights were also observed at 1300 ppm after one and two weeks of treatment. At 2500 ppm feed consumption was reduced by about one-third throughout the treatment period, while a slight reduction in feed consumption (8%) was observed during the first week at 1300 ppm. At necropsy the livers appeared enlarged, and the accessory sex glands (prostate and seminal vesicles with coagulating gland) appeared atrophic at 2500 ppm. Liver and thyroid weights were increased in the 1300 and 2500 ppm groups, whereas pituitary weight was reduced at 2500 ppm. At 2500 ppm an increase in TSH levels was seen at week 2, T_4 levels were consistently lower than the control, and T_3 and (at week 8, the only occasion measured) free T_3 levels increased. At 1300 ppm statistically significant increases in T_4 were seen at all time points. TSH was non-significantly increased at week 2. Microsomal UDP-GT activity showed a dose-related increase at all doses when nitrophenol was used as the substrate but no change when androsterone was the substrate, indicating that pyrifluquinazon has the potential to selectively induce the UDP-GT isozyme involved in conjugative metabolism of T_4 but not of T_3. Histology showed diffuse hepatocellular hypertrophy and periportal hepatocellular vacuolation in liver at 2500 ppm and centrilobular hepatocellular hypertrophy at 1300 ppm. Thyroid examination showed follicular cell hypertrophy and small-sized follicles at 1300 and 2500 ppm.

Table 28. Key findings of the thyroid study in rats

Dose (ppm in diet)	**0**	**350**	**1300**	**2500**
Body weight, week 8 (g)	279.9	281.6	275.7	192.4***
Pituitary weight (mg)	8.8	9.4	9.0	7.3*
Thyroid weight (mg)	17.5	19.4	22.8***	25.2***
Liver weight (g)	9.02	9.80	10.77***	10.30**
Liver weight (% bw)	3.22	3.47	3.89***	5.33***
Hormone levels, week 2				
T_4 (µg/dL)	6.2	5.8	4.4***	4.4***
T_3 (ng/dL)	138	166	177	262**
TSH (ng/mL)	7.6	8.3	10.6	11.3*
Hormone levels, week 4				
T_4 (µg/dL)	5.6	4.7*	3.6***	3.2***
T_3 (ng/dL)	134	137	150	210**
TSH (ng/mL)	10.2	11.0	13.2	9.5
Hormone levels, week 8				
T_4 (µg/dL)	5.8	5.1*	3.7***	2.7***
T_3 (ng/dL)	110	126	126	179**
TSH (ng/mL)	7.2	8.4	10.9	7.4
Free T_3 (pg/mL)	0.96	1.37	1.77	2.35**
Microsomal enzyme activities (nmol/min per mg protein)				
p-Nitrophenol UDP-GT	26.4	45.8**	57.5***	70.7***
Androsterone UDP-GT	0.24	0.27	0.24	0.22

Dose (ppm in diet)	0	350	1300	2500
Histopathology				
Liver: hepatocellular hypertrophy				
Diffuse	0	0	0	8
Centrilobular	0	0	8	0
Hepatocellular vacuolative change	0	0	0	8
Thyroid follicular cell hypertrophy	0	0	6	8

Level of statistical significance: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Source: Amanuma, 2009

The results for T_4 and TSH, taken together with liver and thyroid weight increases and induction of microsomal UDP-GT, are consistent with the hypothesis that hepatic enzyme induction depletes T_4. However, the increase in free T_3 remains unexplained. The study's author concluded that these results were sufficient to demonstrate that thyroid effects were secondary to hepatic enzyme induction. However, the Meeting noted that the data were not entirely conclusive (Amanuma, 2009).

(f) Studies with metabolites

Acute toxicity

Results of acute toxicity studies with metabolites of pyrifluquinazon are shown in Table 29.

Table 29. Results of acute toxicity studies with metabolites of pyrifluquinazon

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-1H (IV-01)	98.7	> 2000	Hurley, 2013a [a]
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-1H-imino (IV-02)	99.3	> 2000	Hurley, 2013b [b]
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-1H-4-oxo (IV-15)	98.6	> 2000	Hurley, 2013c [c]
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-1H-4-OH (IV-27)	88.1	> 2000	Hurley, 2013d [d]
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-1H-imino-4-OH (IV-28)	88.5	> 2000	Hurley, 2013e [e]
Rat	Sprague Dawley (Crl:CD)	F	Oral	AQW	99.96	>300–2000	Oda, 2006a [f]
Rat	Sprague Dawley (Crl:CD)	F	Oral	NNI-0101-quinazolinedione (IV-203)	99.7	> 2000	Toga, 2011 [g]
Rat	Sprague Dawley (Crl:CD)	F	Oral	IV-17	99.9	> 2000	Oda, 2006b [h]

LD_{50} Median lethal dose

[a] Five female rats were given a single oral gavage dose (Batch no. 4FZ6404P) of 2000 mg/kg bw of NNI-0101-1H (IV-01) suspended in 0.5% aqueous sodium CMC. One animal was euthanized in extremis 4 h after dosing. In the surviving animals dried red material around the nose and eyes and/or the forelimbs, dried yellow material on the urogenital and anogenital areas and decreased defecation were observed. Body weights were not affected and no treatment-related gross abnormalities at necropsy were found.

[b] Five female rats were given a single oral gavage dose (Batch no. 4FZ6304P) of 2000 mg/kg bw of NNI-0101-1H-imino (IV-02) suspended in 0.5% aqueous sodium CMC. No mortality was observed, but smaller faeces than normal in two animals on study day 1. Body weights were not affected and no treatment-related gross abnormalities were found at necropsy.

[c] Five female rats were given a single oral gavage dose (Batch no. 3FZ0303N) of 2000 mg/kg bw of NNI-0101-1H-4-oxo (IV-15) suspended in 0.5% aqueous sodium CMC. No mortality and no treatment-related clinical signs were observed. Body weights were not affected and no treatment-related gross abnormalities were found at necropsy.

[d] Five female rats were given a single oral gavage dose (Batch no. 3FZ0902N) of 2000 mg/kg bw of NNI-0101-1H-4-OH (IV-27) suspended in 0.5% aqueous sodium CMC. No mortality and no treatment-related clinical signs were observed. Body weights were not affected and no treatment-related gross abnormalities were found at necropsy.

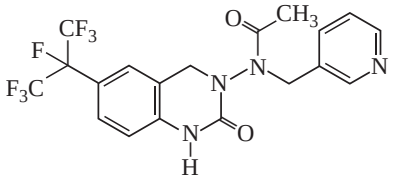
[e] Five female rats were given a single oral gavage dose (Batch no. 3FZ1302N) of 2000 mg/kg bw of NNI-0101-1H-imino-4-OH (IV-28) suspended in 0.5% aqueous sodium CMC. No mortality and no treatment-related clinical signs were observed. Body weights were not affected and no treatment-related gross abnormalities were found at necropsy.

[f] AQW (Batch no. 6FZ4703P) suspended in 0.5% aqueous sodium CMC, was administered by gavage to groups of three female rats at 300 mg/kg bw (first and second dose step), or 2000 mg/kg bw (third dose step). Abnormal gait (staggering) was observed from 4 h after administration at each dose level, and prone/lateral position and bradypnoea from the day following administration at 2000 mg/kg bw. No deaths occurred in the first or second dose step. After the third dose step 2 of the 3 animals died, seven and ten days after treatment. At necropsy the thymus and spleen of animals that had died were reduced in size.

[g] Two groups of three female rats were given a single oral gavage dose (Batch no. 7FZ0602S) of 2000 mg/kg bw of NNI-0101-quinazolinedione (IV-203) suspended in 0.5% aqueous sodium CMC. No mortality nor treatment-related clinical signs were observed. Body weights were not affected and no treatment-related gross abnormalities were found at necropsy.

[h] BR (IV-17; Batch no. 4FZ4102P) suspended in 0.5% aqueous sodium CMC, was administered by gavage to groups of three female rats, at 300 mg/kg bw (first and second dose step), or 2000 mg/kg bw (third and fourth dose steps). No mortality nor clinical signs were noted during the study. Body weights were not affected and no gross abnormalities at necropsy were found.

Structural formula:



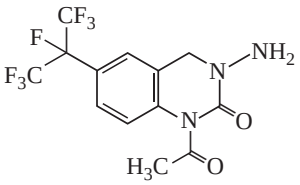
Genotoxicity of metabolites

Metabolites of pyrifluquinazon were tested for genotoxicity in a bacterial mutation test (Table 30).

Table 30. Overview of genotoxicity tests with metabolites of pyrifluquinazon

End-point	Test system	Metabolite	Concentrations/ doses tested	Purity (%)	Result	Reference
Reverse mutation	*S. typhimurium* (TA100, TA98, TA1535, and TA1537) *E. coli* (WP2*uvrA*)	AQA	39.1–1250 µg/plate in DMSO (− S9) 2.44–1250 µg/plate in DMSO (+ S9)	100.0	Negative	Oguma, 2006a [a]
		Pyrifluquinazon-quinazolinedione (IV-203)	39.1-5000 µg/plate in DMSO (± S9)	97.2	Negative	Oguma, 2011 [b]
		IV-208 (AQR; pyrifluquinazon-aminoquinazolinone-*N*-Ac)	1.22–1250 µg/plate in DMSO (− S9) 9.77–1250 µg/plate in DMSO (+ S9)	99.7	Negative	Oguma, 2006a [c]
		AQW	39.1–5000 µg/plate	99.96	Negative	Oguma, 2006c [d]
		QUA	39.1–5000 µg/plate in DMSO (− S9) 9.77–5000 µg/plate in DMSO (+ S9)	97.5	Negative	Oguma, 2006d [e]
		RFPAQ (pyrifluquinazon-imino (IV-102))	2.44–5000 µg/plate in DMSO (− S9) 9.77–5000 µg/plate in DMSO (+ S9)	99.6	Negative	Oguma, 2006e [f]
		RFPDQ (pyrifluquinazon-1H-imino (IV-02)	78.1–1250 µg/plate in acetone (± S9)	100.0	Negative	Oguma, 2006f [g]
		IV-17	9.77–1250 µg/plate in DMSO (± S9)	99.9	Negative	Oguma, 2006g [h]

[a] Batch no. 4FZ4601P. AQA chemical name:
1-acety 1-3-amino-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethy l]-3,4-dihydro-1H-quinazolin-2-one.
Structural formula:



In the presence of S9 mix, cytotoxicity was observed at 78.1 µg/plate and above for strain TA5137, at 313 µg/plate and above for strain TA1535 and at 625 µg/plate for TA100, TA98 and WP2*uvrA*. In the absence of S9 mix, cytotoxicity was observed in all strains at 625 µg/plate and above. Precipitation of the test compound was observed at 1250 µg/plate. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[b] Batch no. 4FZ0601S. In the presence and absence of S9 mix cytotoxicity was observed at ≥ 625 µg/plate for *S. typhimurium* strains only. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

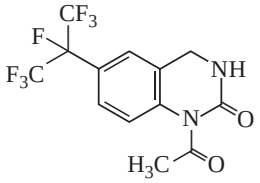
[c] Batch no. 4FZ4501P. In the presence of S9 mix, cytotoxicity was observed at 313 µg/plate for all *S. typhimurium* strains and at 1250 µg/plate for *E. coli* WP2*uvrA*. In the absence of S9 mix, cytotoxicity was observed at 78.1 µg/plate for strain TA1535, and at 625 µg/plate and above for strains TA100, TA98, TA1537 and WP2*uvrA*. No precipitation of the test compound was observed at concentrations used for mutagenicity testing. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[d] Batch no. 6FZ4703P. In the presence of S9 mix, cytotoxicity was observed at 625 µg/plate and above for all *S. typhimurium* strains and at 1250 µg/plate and above for *E. coli* WP2*uvrA*. In the absence of S9 mix, cytotoxicity was observed at 625 µg/plate and above for strains TA100, TA1535 and TA1537, at 1250 µg/plate and above for strain TA98 and at 2500 µg/plate and above for strain WP2*uvrA*. Precipitation of the test compound was observed at 1250 µg/plate and above in the absence and presence of S9 mix. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[e] Batch no. 4FZ4801P. QUA chemical name:

1-acetyl-6-[1,2,2,2-tetrafluoro-l -(trifluoromethyl)ethyl]-3,4-dihydro-lH-quinazolin-2-one.

Structural formula:



In the presence of S9 mix, cytotoxicity was observed at 156 µg/plate and above for *S. typhimurium* strains TA1535 and TA1537, at 313 µg/plate and above for strain TA98 and at 625 µg/plate and above for strain TA100 and *E. coli* strain WP2*uvrA*. In the absence of S9 mix, cytotoxicity was observed at 1250 µg/plate and above for strains TA1535 and TA1537 and at 5000 µg/plate for strains TA98 and TA100. There was no evidence of cytotoxicity in strain WP2*uvrA* in the absence of S9 mix. Precipitation of the test compound was observed at 1250 µg/plate and above in the absence of S9 mix and at 5000 µg/plate in the presence of S9 mix. There was a marginally greater than two-fold increase in revertant colonies following treatment of strain TA1537 in the presence of S9 in the first experiment, but as this was not dose-dependent and was not reproduced in a second experiment, it was judged not to be substance-related. It was concluded that QUA was not mutagenic under the conditions of this test. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[f] Batch no. 4FZ0401P. In the presence of S9 mix cytotoxicity was observed at 313 µg/plate and above in all *S. typhimurium* strains. In the absence of S9 mix, cytotoxicity was observed at 78.1 µg/plate for strain TA1535, at 156 µg/plate and above for strain TA1537, at 313 µg/plate for strain TA100 and at 1250 µg/plate for strain TA98. There was no evidence of cytotoxicity in *E. coli* WP2*uvrA* in either the absence or presence of S9 mix. Precipitation of the test compound was observed at 313 µg/plate and above in the absence of S9 mix, and at 2500 µg/plate and above in the presence of S9 mix. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[g] Batch no. 4FZ6304P. There was no evidence of cytotoxicity in any tester strain in either the absence or presence of S9 mix. Precipitation of the test compound was observed at 625 µg/plate and above in the absence of S9 mix and at 1250 µg/plate in the presence of S9 mix. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

[h] Batch no. 4FZ4102P. Cytotoxicity was observed at 156 µg/plate in strain TA1537 with S9 mix, at 313 µg/plate in strains TA1535 and TA1537 with and without S9 mix, and at 625 µg/plate and above in all strains with and without S9 mix. Precipitation of the test compound was observed at 5000 µg/plate with and without S9 mix. No significant increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used either with or without metabolic activation. The positive control substances employed for each bacterial strain significantly induced gene mutations in this study.

3. Observations in humans

No data are available

Comments

Biochemical aspects

Repeated dose studies of toxicokinetics were not performed. Following administration to rats of a single gavage dose of [^{14}C] pyrifluquinazon at 1 or 100 mg/kg bw, absorption was relatively rapid, with peak plasma concentrations after 1–3 h and 3–12 h, respectively. Pyrifluquinazon was relatively well absorbed from the GI tract: about 63% after a 1 mg/kg bw dose, based on bile, urine and carcass. The C_{max} values in blood and plasma for radioactivity and the AUC for radioactivity were calculated up to 168 h post dose, and were in general proportional to dose. Following a 1 mg/kg bw dose to bile duct-cannulated rats, 34.5% of the administered dose was excreted in the bile collected at 72 h post dose. Pyrifluquinazon was widely distributed, with highest levels of radioactivity in the liver, kidney, GI tract and the adrenals. In a study with pyrifluquinazon labelled on its quinazolinone phenyl, virtually no radioactivity remained in tissue after 168 h, whereas in a study with pyridine-labelled pyrifluquinazon, 18–31% of the radiolabel was recovered from the carcass at 168 h. As the pyridine moiety of pyrifluquinazon may be metabolized to nicotinic acid, nicotinamide and related molecules, it is hypothesized that the residual activity in the carcass at 168 h represents residues of the compound that are incorporated into organs and tissues. The major route of excretion was the faeces (75–80%), with smaller amounts in urine (15–20%). For both sexes, elimination was biphasic and almost complete by 48 h post dose for the 1 mg/kg bw dose (ca 80%), and by 144 h for the 100 mg/kg bw dose (ca 90%). In view of the rate of excretion and the distribution pattern, no potential for bioaccumulation is expected.

Pyrifluquinazon was extensively metabolized. No parent compound was found in urine and bile, and only low levels in faeces. The major routes of metabolism of pyrifluquinazon involve initial deacetylation of the nitrogen atom at the 1-position of the quinazoline ring, followed by hydroxylation of the 4-position in the quinazoline ring and conjugation by glucuronic acid, oxidation of the 1-position of pyridine ring, dehydrogenation of the amino group, hydroxylation of the 8-position in the quinazolinone ring, cleavage of the nitrogen–carbon bond, followed by acetylation of the nitrogen atom and cleavage of the quinazoline ring followed by conjugation with glucuronic acid.

In urine significant levels were found of only pyrifluquinazon-8-OH-quinazolinedione (IV-211) or its glucuronide (5–6% and 3–4% of the applied dose after 1 and 100 mg/kg bw, respectively), pyrifluquinazon anthranilic acid (IV-303) or its glucuronide (7% and 5–6% of the applied dose after 1 and 100 mg/kg bw, respectively) and pyrifluquinazon methylnicotinamide (IV-405, 18–21% after 1 mg/kg bw; 21% in males after 100 mg/kg bw), along with minor amounts (< 4%) of nicotinic acid (IV-403) and pyrifluquinazon-1*H*-imino oxide (IV-04). In bile the main metabolites were pyrifluquinazon-1H-4-OH (IV-27) or its glucuronide (9%), IV-211-glucuronide (8% of the applied dose of 1 mg/kg bw) and pyrifluquinazon aminoquinazolinone-*N*-Ac-4-OH (IV-212; 7% of the applied dose of 1 mg/kg bw) with lower levels of IV-303 (1.5% of the applied dose of 1 mg/kg bw). Taking into account the incomplete oral absorption of pyrifluquinazon, the metabolites IV-27, IV-211, IV-212, IV-303, IV-405 were formed in rats at more than 10% of the administered dose. The presence of metabolites in the GI of bile duct-cannulated rats suggested that microbial or chemical degradation occurs in the intestine. There were no significant differences in the toxicokinetic parameters or metabolism between the sexes.

Toxicological data

The acute oral LD_{50} in rats was 300–2000 mg/kg bw (Amanuma, 2013; Horiuchi, 2006a), the acute dermal LD_{50} was greater than 2000 mg/kg bw (Horiuchi, 2006b) and the acute inhalation LC_{50} was 1.2–2.4 mg/L (Janssen, 2005). Pyrifluquinazon was not irritating to the skin of rabbits (Horiuchi, 2006c), nor to the eyes of rabbits (Horiuchi, 2006d). Pyrifluquinazon was mildly sensitizing in a maximization test in guinea pigs (Amanuma, 2006a).

In repeated-dose oral toxicity studies with pyrifluquinazon in mice, rats and dogs, various effects were observed, most notably on the nasal cavity, reproductive organs, liver, adrenals, hormone levels (indicative of an antiandrogenic potential), clinical signs and behaviour.

In a 90-day study in mice using dietary pyrifluquinazon concentrations of 0, 60, 750 or 1500 ppm (equal to 0, 7.6, 102 and 206 mg/kg bw per day for males, 0, 9.1, 119 and 202 mg/kg bw per day for

females) the NOAEL was 60 ppm (equal to 7.6 mg/kg bw per day), based on clinical chemistry changes indicative of liver toxicity, and reductions in total serum protein, albumin, globulin and calcium, decreased total leukocyte and lymphocyte counts, and thyroid and epididymis weight, and increased liver weights and incidences of follicular cell hypertrophy in the thyroid and centrilobular hepatocellular hypertrophy in both sexes at 750 ppm (equal to 102 mg/kg bw per day) (Kuwahara, 2005).

In a 90-day study in rats using dietary pyrifluquinazon concentrations of 0, 50, 100, 500 or 2500 ppm (equal to 0, 2.9, 5.7, 29 and 155 mg/kg bw per day for males, 0, 3.2, 6.4, 33 and 159 mg/kg bw per day for females), the NOAEL was 500 ppm (equal to 29 mg/kg bw per day), based on effects on body weight, and a number of changes in haematology, clinical chemistry, organ weights and histopathological changes in liver, thyroid, kidney, pituitary, adrenal, bone marrow, pancreas, spleen, retina, ovary, uterus, vagina, testis and epididymis at 2500 ppm (equal to 155 mg/kg bw per day) (Nakashima, 2004).

In a 13-week oral toxicity study in dogs pyrifluquinazon was administered by gelatine capsule at doses of dose of 0, 2, 5 or 30 mg/kg bw per day. The NOAEL was 2 mg/kg bw per day, based on reduced body weight gain in both sexes, which was considered toxicologically relevant despite lack of statistical significance, at 5 mg/kg bw per day (Takeuchi, 2005).

In a one-year oral toxicity study in dogs administered pyrifluquinazon by gelatine capsule at a dose of dose of 0, 1.5, 5 or 15 mg/kg bw per day, the LOAEL was 1.5 mg/kg bw per day based on slight to moderate mononuclear cell infiltration of the lumina propria of the olfactory epithelium, which was considered to be an early indicator of alteration/necrosis of the olfactory epithelium observed at the mid and high doses (Kuwahara, 2006a).

In a second one-year oral toxicity study in dogs pyrifluquinazon was administered by gelatine capsule at doses of 0, 0.15, 0.5 or 5 mg/kg bw per day. The NOAEL was 0.5 mg/kg bw per day, based on mononuclear cell infiltration in the nasal cavity in both sexes at 5.0 mg/kg bw per day (Shibuya, 2008).

The overall NOAEL for the two one-year toxicity studies in dogs was 0.5 mg/kg bw per day, and the overall LOAEL was 1.5 mg/kg bw per day.

In an 18-month carcinogenicity study in mice using dietary pyrifluquinazon concentrations of 0, 60, 250 or 1000 ppm (equal to 0, 6.3, 27 and 122 mg/kg bw per day for males, 0, 5.8, 25, 120 mg/kg bw per day for females), the NOAEL was 60 ppm (equal to 6.3 mg/kg bw per day).This was based on an increased incidence of adrenal subcapsular cell hyperplasia in males at 250 ppm, equal to 27 mg/kg bw per day. At 1000 ppm (equal to 122 mg/kg bw per day) an increase in the incidences of interstitial cell hyperplasia and benign interstitial (Leydig) cell tumours in the testis was observed. There were no increases in the incidence of other neoplastic lesions. The NOAEL for carcinogenicity was 250 ppm (equal to 27 mg/kg bw per day) (Kuwahara, 2006a).

In a one-year study in rats using dietary pyrifluquinazon concentrations of 0, 100, 350 or 1300 ppm (equal to 0, 4.1, 14 and 56.5 mg/kg bw per day for males, 0, 5.0, 18, and 66 mg/kg bw per day for females), the NOAEL was 100 ppm, equal to 4.1 mg/kg bw per day, based on organ weight changes in both sexes and indications of kidney effects in females at 350 ppm (equal to 14 mg/kg bw per day) (Kuwahara, 2006b).

In a two-year carcinogenicity study in rats using dietary pyrifluquinazon concentrations of 0, 100, 350 or 1300 ppm (equal to 0, 3.5, 13 and 49 mg/kg bw per day for males, 0, 4.5, 16, and 60 mg/kg bw per day for females) the NOAEL was 100 ppm (equal to 3.5 mg/kg bw per day, based on histopathological changes in the eyes in both sexes, organ weight changes and macroscopic and histopathological changes in the male reproductive organs, including an increase in Leydig cell tumours set against a high background incidence. In females bile duct hyperplasia and uterine horn dilatation was noted at 350 ppm (equal to 13 mg/kg bw per day). Apart from the Leydig cell tumours, there were no increases in the incidence of other neoplastic lesions. Although the high background incidence in Leydig cell tumours in this study makes it difficult to assess with confidence any carcinogenic effect of pyrifluquinazon treatment in the testis, the Meeting noted that an increased incidence in this tumour type was also observed in the 18 month study in mice. Therefore the NOAEL for carcinogenicity was 3.5 mg/kg bw per day (Kuwahara, 2006c).

Studies were performed to investigate the MOA for the observed induction of interstitial (Leydig) cell hyperplasia and tumours in the testis of mice and rats (F-344 strain). Such tumours are common in certain rat strains, including F-344, and the relevance of a substance-related induction of such tumours for humans is questionable. However, interstitial cell tumours in testis of mice are rare and a substance-related induction of these tumours could not be discounted with respect to its relevance to humans. The hypothesis proposed by the sponsor is that antiandrogenic effects mediated through the AR give rise to sustained LH secretion due to disruption of negative feedback regulation, leading eventually to Leydig cell overstimulation. The meeting noted that, although the tumours in the mouse in particular are a matter of concern, most of the studies to substantiate the hypothesis were performed in rats or rat tissue. Pyrifluquinazon showed antiandrogenic activity in a two-generation reproductive toxicity study (Hojo, 2006a) and in two Hershberger assays in rats (Amanuma, 2006d; 2008a). In vitro studies showed that pyrifluquinazon has antiandrogenic potential, probably through disruption of the AR-mediated signaling pathway by decreasing the intracellular protein expression, rather than through competitive inhibition of androgen binding. In a 13-week dietary study in male mice using pyrifluquinazon concentrations of 0, 250, 500 or 1000 ppm (equal to 0, 33, 71 and 136 mg/kg bw per day) increases in LH, testosterone and DHT, decrease in absolute and relative epididymis weight and testicular interstitial cell hypertrophy were observed at 500 ppm and 1000 ppm (Nagai, 2011). In a 13-week dietary study in male rats, using pyrifluquinazon at concentrations of 0, 50, 350, 1300 or 2500 ppm (equal to 0, 6, 21, 77 and 145 mg/kg bw per day) several effects were observed at 1300 ppm and 2500 ppm There were increases in serum LH and testosterone values and a decrease in the weights of prostate, seminal vesicles with coagulating gland and epididymis, and an increase in testicular seminiferous tubular atrophy and of germ cell debris in the epididymis. At the high dose level, vacuolation of tubular epithelial cell of the epidydimides was also observed (Amanuma, 2010). The Meeting noted that Leydig cell tumours in rats occurred at lower dietary levels of pyrifluquinazon than the increase in LH in the 13-week MOA study in rats. The sponsor proposes that the hormonal changes in mice and rats are a consequence of the stimulation of testosterone production in the interstitial cells by LH and that the data support the hypothesis that pyrifluquinazon may increase levels of LH that would subsequently lead to interstitial (Leydig) cell tumours in mice and rats. The Meeting noted that although a number of studies had been performed in rats, only one had been performed in mice, which was the critical species and therefore it is not possible to establish the MOA in the mouse and hence the relevance to humans. With this in mind, it is not possible to dismiss the relevance to humans of the tumours observed in the testes of rats and mice.

The Meeting concluded that pyrifluquinazon is carcinogenic in male mice and male rats.

Pyrifluquinazon was tested for genotoxicity in an adequate range of vitro and in vivo assays. No evidence of genotoxicity was found (Inagaki, 2005, 2006; Gilby, 2015; Mason 2005; Dony, 2016). The Meeting concluded that pyrifluquinazon is unlikely to be genotoxic.

In view of the lack of genotoxicity, and the fact that tumours were observed only at doses unlikely to occur in humans, by a mechanism that would exhibit a threshold, the Meeting concluded that pyrifluquinazon is unlikely to pose a carcinogenic risk to humans via exposure from the diet.

In a two-generation reproductive toxicity study, rats were administered pyrifluquinazon in the diet at concentrations of 0, 30, 150 and 750 ppm (equal to premating doses of 0, 2.0, 10 and 52 mg/kg bw per day for F0 males, 0, 2.3, 12 and 59 mg/kg bw per day for F0 females; 0, 2.3, 11 and 56 mg/kg bw per day for F1 males, 0, 2.4, 12 and 61 mg/kg bw per day for F1 females, respectively). The NOAEL for parental toxicity was 30 ppm (equal to 2.4 mg/kg bw per day), based on increased thyroid weight in F1 females at 150 ppm (equal to 12 mg/kg bw per day). The NOAEL for offspring toxicity was 30 ppm (equal to 2.3 mg/kg bw per day), based on reduced body weights in both sexes at 150 ppm (equal to 10 mg/kg bw per day). The NOAEL for reproductive toxicity was 30 ppm (equal to 2.4 mg/kg bw per day), based on a reduced anogenital distance in F2 male pups at 150 ppm (equal to 12 mg/kg bw per day) (Hojo, 2006a).

In a developmental toxicity study of pyrifluquinazon in rats using gavage doses of 0, 5, 10 or 50 mg/kg bw per day from GD 6–19, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduced feed intake and body weight gain at 50 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, based on reduction in anogenital distance in male fetuses and increased incidences in supernumerary ribs in both sexes at 10 mg/kg bw per day (Hojo, 2006a).

In a developmental toxicity study in rabbits administered pyrifluquinazon by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day from GD 6–27, the NOAEL for maternal and offspring toxicity was 20 mg/kg bw per day, the highest dose tested (Hojo, 2005b).

The Meeting concluded that pyrifluquinazon is not teratogenic.

In an acute neurotoxicity study in which rats were administered pyrifluquinazon by gavage at a dose of 0, 30, 100, 300 or 500 mg/kg bw, and then observed for 14 days, the NOAEL was 100 mg/kg bw, based on moribundity, clinical signs, body weight loss and changes in sensorimotor reactivity, coordination, autonomic processes and motor activity observed at 300 mg/kg bw (Foss, 2006b).

An acute safety pharmacology study was conducted in which female rats received pyrifluquinazon by gavage at doses of 0, 5, 50 or 500 mg/kg bw and then observed for 24 hours. The NOAEL was 50 mg/kg bw, based on mortality, body weight loss, marked clinical signs and decreased mobility observed at 500 mg/kg bw (Tsuchiyama, 2006).

In a 13-week neurotoxicity study in rats using dietary pyrifluquinazon concentrations of 0, 30, 150 and 750 ppm (equal to 0, 1.8, 9.4 and 47 mg/kg bw per day for males, 0, 2.2, 11 and 53 mg/kg bw per day for females), the NOAEL for systemic toxicity was 150 ppm (equal to 11 mg/kg bw per day), based on reduced body weight gain at termination, reduced feed consumption early in treatment in females at 750 ppm (equal to 53 mg/kg bw per day). The NOAEL for neurotoxicity was 750 ppm, equal to 47 mg/kg bw per day, the highest dose tested (Foss, 2009).

There were no indications of neuropathological effects of pyrifluquinazon. The Meeting considered that the acute behavioural effects observed at high doses of pyrifluquinazon are due to severe systemic toxicity.

The Meeting concluded that pyrifluquinazon is not neurotoxic.

In a 28-day immunotoxicity study in rats using dietary pyrifluquinazon concentrations of 0, 30, 150 and 750 ppm (equal to 0, 2.5, 12 and 62 mg/kg bw per day for males, 0, 2.7, 13, 63 mg/kg bw per day for females), no signs of an immunotoxic effect were observed (Arrington, 2009).

The Meeting concluded that pyrifluquinazon is not immunotoxic

Mechanistic studies

In addition to studies investigating the antiandrogenic potential of pyrifluquinazon and the MOA causing the induction of interstitial cell tumours in mice and rats (as described above), studies were available in which the effects of this substance on liver enzymes and the possible secondary effect on the thyroid were investigated. The Meeting considered that these data provided no information that would impact on the evaluation of pyrifluquinazon.

Toxicological data on metabolites and/or degradates

Acute oral LD_{50} studies and mutagenicity studies were available for some metabolites of pyrifluquinazon. The acute oral LD_{50} values of metabolites IV-01, IV-02, IV-15, IV-17, IV-27, IV-28 and IV-203 were > 2000 mg/kg bw. For the metabolite AQW the LD_{50} was 300–2000 mg/kg bw. For the metabolites IV-02, IV-17, IV-102, IV-203, IV-208, AQA, AQW and QUA, negative results were obtained in reverse mutation test on bacteria (Ames tests).

The major residues in crops and livestock were pyrifluquinazon and its metabolites IV-01, IV-02, IV-03, IV-04, IV-15, IV-17, IV-203, IV-208 and IV-404 (nicotinamide, vitamin B compound). For the crop and livestock metabolites IV-03, IV-04, IV-404 and IV-208 no acute toxicity studies were available and for the the crop and livestock metabolites IV-01, IV-03, IV-04, IV-15 and IV-404 no reverse mutagenicity tests were available.

Apart from acute toxicity and reverse mutation tests, no specific toxicity studies on metabolites of pyrifluquinazon were available. In toxicokinetic studies in rats using pyrifluquinazon at single gavage doses of 1 or 100 mg/kg bw, metabolite levels were generally low. Significant levels (> 10% when taking the incomplete oral absorption into account) of the rat metabolites IV-211, IV-27, IV-303 and

their respective glucuronides, were found in urine and/or bile. The Meeting concluded that the toxicity of these rat metabolites would be covered by that of pyrifluquinazon. As crop and livestock metabolite IV-01 is an intermediate in the metabolic pathway leading to the formation of IV-211 or IV-27, and crop and livestock metabolite IV-203 is an intermediate in the metabolic pathway leading to the formation of IV-211 and IV-303, the toxicity of metabolites IV-01 and IV-203 would also be covered by that of pyrifluquinazon. The Meeting noted that, although the metabolites IV-02, IV-03, IV-04, IV-15, IV-17 and IV-208 retain the structural backbone of the parent and have undergone only minor structural changes when compared to pyrifluquinazon, it could not conclude on the toxicity of these compounds in view of the absence of repeated dose toxicity studies with these substances. For the metabolites IV-02, IV-17 and IV-208 the TTC approach (Cramer class III) could be applied for chronic toxicity, as reverse mutation tests on bacteria (Ames tests) were negative for these substances. For the metabolites IV-03, IV-04 and IV-15 the TTC for genotoxicity could be applied for chronic toxicity. IV-404 (nicotinamide) is a B vitamin and is not of toxicological concern.

Human data

No human data were available.

The Meeting concluded that the existing database on pyrifluquinazon was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an acute daily intake (ADI) of 0–0.005 mg/kg bw for pyrifluquinazon on the basis of an overall NOAEL of 0.5 mg/kg bw per day in two 1-year dog studies, based on a slight to moderate mononuclear cell infiltration of the lumina propria of the olfactory epithelium in both sexes at 1.5 mg/kg bw per day. A safety factor of 100 was used. The upper bound of the ADI gives a margin of about 24 000 relative to the LOAEL for the observed interstitial cell tumours in the testes of mice. The upper bound of the ADI gives a margin of about 2800 relative to the LOAEL for the observed interstitial cell tumours in the testes of rats.

The Meeting established an ARfD of 1 mg/kg bw for pyrifluquinazon on the basis of a NOAEL of 100 mg/kg bw in an acute neurotoxicity study of rats, based on moribundity, clinical signs, body weight loss and changes in sensorimotor reactivity, coordination, autonomic processes and motor activity observed at 300 mg/kg bw. A safety factor of 100 was used. This is supported by findings in the LD_{50} studies and a safety pharmacology study.

The ADI and ARfD also apply to the metabolites IV-01 and IV-203, expressed as pyrifluquinazon.

Levels relevant to risk assessment of pyrifluquinazon

Species	Study	Effect	NOAEL	LOAEL
Mouse	13-week study of toxicity [a]	Toxicity	60 ppm, equal to 7.6 mg/kg bw per day	750 ppm, equal to 102 mg/kg bw per day
	18-month study of carcinogenicity [a]	Toxicity	60 ppm equal to 6.3 mg/kg bw/day	250 ppm, equal to 27 mg/kg bw per day
		Carcinogenicity	250 ppm, equal to 27 mg/kg bw per day	1000 ppm, equal to 122 mg/kg bw per day
Rat	13-week study of toxicity [a]	Toxicity	500 ppm, equal to 29 mg/kg bw per day	2500 ppm, equal to 155 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	100 ppm, equal to 3.5 mg/kg bw per day	350 ppm, equal to 13 mg/kg bw per day
		Carcinogenicity	100 ppm, equal to 3.5 mg/kg bw per day	350 ppm, equal to 13 mg/kg bw per day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	30 ppm equal to 2.3 mg/kg bw per day [b]	150 ppm equal to 11 mg/kg bw per day [b]
		Parental toxicity	30 ppm equal to 2.4 mg/kg bw per day [b]	150 ppm equal to 12 mg/kg bw per day
		Offspring toxicity	30 ppm equal to 2.3 mg/kg bw per day	150 ppm equal to 10 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	10 mg/kg bw per day	50 mg/kg bw per day
		Embryo and fetal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
	Acute neurotoxicity study [b]	Neurotoxicity	100 mg/kg bw	300 mg/kg bw
	Acute safety pharmacology study [b]	Neurotoxicity	50 mg/kg bw	500 mg/kg bw
	Thirteen-week neurotoxicity study [a]	Toxicity	150 ppm, equal to 11 mg/kg bw per day	750 ppm, equal to 53 mg/kg bw per day
		Neurotoxicity	750 ppm, equal to 47 mg/kg bw per day [c]	-
Rabbit	Developmental toxicity study [b]	Maternal toxicity	20 mg/kg bw per day [c]	-
		Embryo and fetal toxicity	20 mg/kg bw per day [c]	-
Dog	One-year study of toxicity [d, e]	Toxicity	0.5 mg/kg bw per day	1.5 mg/kg bw per day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

[d] Capsule administration

[e] Two or more studies combined

Acceptable daily intake (ADI), applies to pyrifluquinazon, IV-01 and IV-203, expressed as pyrifluquinazon

0–0.005 mg/kg bw

Acute reference dose (ARfD), applies to pyrifluquinazon, IV-01 and IV-203, expressed as pyrifluquinazon

1 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to pyrifluquinazon

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Relatively rapid (T_{max} 1–3 hours and 3–12 hours after 1 or 100 mg/kg bw dose, respectively) and incomplete (63% at 1 mg/kg bw, based on levels in urine, bile and carcass) (rat)
Dermal absorption	19%, 13% and 4% at 0.001, 0.01 and 0.1 mg/cm^2, respectively (in vivo, rat)
Distribution	Widely distributed; highest concentrations found in liver, kidneys, GI tract and adrenals
Potential for accumulation	None
Rate and extent of excretion	Relatively rapid; 80% in 48 hours after 1 mg/kg bw
Metabolism in animals	Extensively metabolized; major metabolites are IV-27 or its glucuronide, IV-211 or its glucuronide, IV-212, IV-303 or its glucuronide, IV-405
Toxicologically significant compounds in animals and plants	Pyrifluquinazon, IV-01, IV-02, IV-03, IV-04, IV-15, IV-17, IV-203, IV-208
Acute toxicity	
Rat, LD_{50}, oral	> 300–2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 1.2–2.4 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea pig, dermal sensitization	Mildly sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Olfactory epithelium
Lowest relevant oral NOAEL	0.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	0.042 mg/L (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Eyes, liver, male and female reproductive organs
Lowest relevant NOAEL	3.5 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice and rats [a]
Genotoxicity	No evidence of genotoxicity [a]
Reproductive toxicity	
Target/critical effect	Reduced anogenital distance in male pups
Lowest relevant parental NOAEL	2.4 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	2.3 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	2.3 mg/kg bw per day (rat)

Developmental toxicity	
Target/critical effect	Reduced anogenital distance in male fetuses; increased incidences of supernumerary ribs
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	5 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	100 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	53 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	
Lowest relevant NOAEL	62 mg/kg bw per day, highest dose tested (rats)
Studies on toxicologically relevant metabolites and impurities	
IV-01, IV-02, IV-15, IV-27, IV-28 and IV-203 (metabolites) and IV-17	LD_{50} > 2000 mg/kg bw
AQW	LD_{50} > 300–2000 mg/kg bw
IV-02, IV-17, IV-102, IV-404, IV-203, IV-208, AQA, AQW and QUA	No evidence of genotoxicity in Ames test
Human data	No data

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.005 mg/kg bw [a]	One-year toxicity studies in dogs [b]	100
ARfD	1 mg/kg bw [a]	Acute neurotoxicity study in rats	100

[a] Applies to pyrifluquinazon and IV-01 and IV-203 expressed as pyrifluquinazon.

[b] Two or more studies combined

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TOLCLOFOS-METHYL

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Explanation

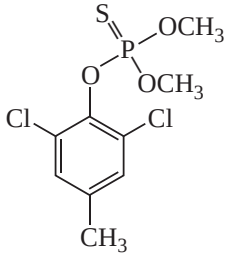
Tolclofos-methyl is the ISO-approved common name for *O*-2,6-dichloro-*p*-tolyl *O*,*O*-dimethyl phosphothioate (IUPAC) with the Chemical Abstract Service number 57018-04-9.

Tolclofos-methyl is used for the control of soil-borne fungal diseases of potatoes but may also be used for the treatment of lettuce and other crops. Unlike other organophosphorous pesticides that are used as insecticides, tolclofos-methyl is a fungicide and its pesticidal mode of action (MOA) is via inhibition of phospholipid biosynthesis.

Tolclofos-methyl was last evaluated by JMPR in 1994. At that time, an acceptable daily intake (ADI) of 0–0.07 mg/kg body weight (bw) was established on the basis of reduced brain cholinesterase activity in a two-year study of toxicity and carcinogenicity in mice. New studies submitted since the last Joint FAO/WHO Meeting on Pesticide Residues (JMPR) evaluation include in vitro comparative metabolism (human and rat microsome), one-generation reproduction toxicity, neurotoxicity, immunotoxicity, phototoxicity, and in vitro endocrine toxicity studies.

Tolclofos-methyl was re-evaluated by the present Meeting within the periodic review programme of Codex Committee on Pesticide Residues (CCPR). The majority of studies considered by the current Meeting were evaluated during the 1994 JMPR meeting and, where appropriate, the text from the previous monograph has been adopted here verbatim. Some of the critical studies do not comply with good laboratory practice (GLP), as the data were generated before the implementation of GLP regulations. Overall, however, the database was considered adequate for the risk assessment.

Figure 1. Structure of tolclofos-methyl



Evaluation of acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

Mouse

In a study designed to assess the metabolism of tolclofos-methyl, seven-week old male and female ICR mice were administered by gastric intubation a single 5 mg/kg bw dose of [^{14}C-4-methyl]-tolclofos-methyl (radiochemical purity, 99%) dissolved in corn oil. Elimination of radioactivity was measured 1, 2, 4, and 7 days after exposure. Thin layer chromatography (TLC) analysis was performed to identify metabolites and will be discussed in Section 1.2 of this monograph.

One day after treatment 82.4 and 74.2% of the radiolabel administered to males and females, respectively was recovered in urine, faeces and expired air. The major route of elimination was urine accounting for 75.9% of excretion in males and 69.3% of excretion in females. This was followed in importance by faecal elimination at 4.4–5.8% of the administered radiolabel. Less than 1% of the administered radiolabel was recovered in the expired air. Seven days after exposure the total radioactivity recovered was 90.9% of the administered dose for males and 87.3% for females. Similarly to the pattern observed one day post exposure, after seven days urine accounted for the majority of the recovered radioactivity (83.3% and 81.5% in males and females, respectively) followed by faecal elimination (6.7% and 5.2% in males and females, respectively) with expired air accounting for < 1% (Mihara, Ohkawa & Miyamoto, 1980).

Rat

A single 5 mg/kg bw dose of [^{14}C-4-methyl]-tolclofos-methyl (radiochemical purity, 99%) dissolved in corn oil was administered by gavage to five-week old male and female Sprague Dawley rats. Elimination of radioactivity in excreta was measured 1, 2, 4, and 7 days after exposure. Whole body radiography was conducted in male rats 1 or 6 hours post dosing. At termination (seven days post exposure), 27 tissues were examined to quantify the amount of radioactivity retained. In addition, TLC analysis was performed to identify metabolites and this will be discussed in Section 1.2.

Radiography revealed that most of the radioactivity 1–6 hours post dose is present in the gastrointestinal (GI) tract followed by the kidneys and liver. Tolclofos-methyl absorption and elimination was rapid with radioactivity recovery reaching 83.3% in males and 83.2% in females one day after exposure. The majority of the radioactivity was recovered in the urine (66.7% in males and 62.1% in females) followed by faeces (16.4% in males and 20.5% in females). Expired air and tissue residues each accounted for < 1% of the administered radioactivity (Mihara, Ohkawa & Miyamoto, 1980).

Five mg/kg bw of [^{14}C]-tolclofos-methyl, labelled uniformly in the benzene ring (radiochemical purity ≥ 99%), was administered by gavage to three Sprague Dawley SPF male and female rats per observation time point. Animals were sacrificed 30 minutes, 1, 2, 4, 8, 24, or 72 hours after administration. Several tissues including heart, lung, liver, kidney, brain, plasma, and blood were collected for determination of ^{14}C-tissue concentrations. To evaluate the elimination profile for tolclofos-methyl, bile cannulated-rats received ^{14}C-tolclofos-methyl and bile was collected 1, 2, 4, 6, 8, 12, 24, or 48 hours post dose. Urinary excretion was measured 6, 12, 24, or 48 hours after treatment while faecal radioactivity elimination was measured 12, 24, or 48 hours post dose. The metabolic profile of tolclofos-methyl in the blood, liver, kidney, bile, and faeces was investigated and will be discussed in Section 1.2.

For most tissues in male rats, peak concentration was reached within 2 hours after administration of the compound with the exception of the testis and epididymis (peak concentration at 4 hours) and fat (peak concentration at 8 hours). The highest concentration of radioactivity was localized to the kidney which was approximately four-fold higher than the ^{14}C concentration in plasma and liver. For the remainder of the tissues, radioactivity concentration was 35% or less than in plasma. In females, peak radioactivity in the kidneys occurred within 30 minutes of exposure while ^{14}C concentration reached its highest levels in skin and uterus 4 hours after exposure and 8 hours after exposure in the fat. Similarly to the distribution pattern reported in males, the concentration of radioactivity was highest in the kidney (approximately three- to five-fold higher than plasma and liver). For the remainder of the tissues, concentration of radioactivity was 29% or less than that of plasma. Seventy-two hours after administration, < 2% of the administered dose remained in the tissues and carcass of males and < 3% in females.

Forty-eight hours cumulative excretion of [^{14}C]-tolclofos-methyl in males reached 5.8% in bile, 46.7% in urine, and 42.3% in faeces. In females, the percentage of administered dose excreted was 11.7%, 59.4%, and 23.7% in bile, urine, and faeces, respectively (Esumi, 1989).

The absorption, distribution, and elimination profile of [^{14}C-phenyl]-tolclofos-methyl (radiochemical purity > 98.9%,) dissolved in corn oil, was evaluated in groups of five male and five female Sprague Dawley CD rats exposed to a single low dose (5 mg/kg bw), single high dose (200 mg/kg bw) or multiple days (14 consecutive days) low dose (5 mg/kg bw per day) followed by a single oral dose of [^{14}C-phenyl]-tolclofos-methyl . The control groups consisted of three male and three female Sprague Dawley CD rats treated with vehicle only. The elimination of radiolabel in the urine and faeces was measured 0.5, 1, 2, 3, 4, 6, and 7 days after treatment. At termination (seven days after exposure), radioactivity levels were quantified in blood, bone (femur), brain, fat, heart, kidney, liver, lungs, muscle, ovary, skin, spleen, testes, uterus, and carcass. In addition, metabolite profiles of 0–48 hours urine and faecal samples were evaluated by TLC to determine the nature and quantity of the metabolites formed.

Urinary and faecal excretion was rapid with greater than 95% of the administered dose recovered within 48 h. Seven days after exposure the total radiolabel recovery was 103.8% ± 4.5%. The primary route of elimination was the urine, with a radiolabel recovery range of 85–91% regardless of sex, dose, or treatment paradigm. Faecal output accounted for 9–20% of the administered radiolabel; elimination

via expired air accounted for < 0.1% of the administered radiolabel. Residues in all tissues collected seven days post exposure accounted for < 1.0% of the administered dose. Similarities in the elimination profile across all test groups indicate that absorption and excretion are not saturated at doses as high as 200 mg/kg bw. The identification of tolclofos-methyl and its metabolites in tissues and excreta is described in Section 1.2 of this monograph (Krautter et al., 1988a, b).

1.2 Biotransformation

Mouse

The excreta (urine and faeces) of seven-week-old male and female ICR mice administered 5 mg/kg bw [^{14}C]-tolclofos-methyl (radiochemical purity, 99%) by gavage were evaluated by one dimensional TLC to identify and quantify the metabolites excreted during 24 h following test compound administration. Thirteen metabolites were identified with no significant differences in the metabolic profile between males and females. Major amongst these were the following:

- 2,6-dichloro-4-methyl phenol (9% of administered label),
- *O*,*O*-dimethyl *O*-(2,6-dichloro-4-carboxyphenyl)phosphate (11% of administered label),
- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-carboxyphenyl)phosphate (12% of administered label),
- 3,5-dichloro-4-hydroxybenzoic acid (12% of administered label), and
- 3,5-*O*-dichloro-4-hydroxybenzyl glycine (13% of administered label).

The major biotransformation reactions are oxidative desulfuration to oxon and related derivatives, oxidation of the 4-methyl group to alcohols and acids, cleavage of P-O-aryl and P-O-methyl linkages and conjugation of the resultant acid with glycine. (Mihara, Ohkawa & Miyamoto, 1980).

Rat

The biotransformation of a single dose of 5 mg/kg bw ^{14}C-tolclofos-methyl (radiochemical purity, 99%) following administration by gavage was evaluated in five-week-old Sprague Dawley rats. Fourteen metabolites were identified in the urine and faeces. Thirteen of these metabolites were also identified in mice as described in the previous paragraph. The only metabolite unique to rats was DM-TM-COOH (S10). The metabolic profile identified for mice and rats is shown here in Fig. 2 (Mihara, Ohkawa & Miyamoto, 1980).

The metabolism of [^{14}C]-tolclofos-methyl labelled uniformly in the benzene ring (radiochemical purity ≥ 99%) and administered by gavage to groups of bile cannulated-rats (Sprague Dawley SPF, three male and three female) at a dose of 5 mg/kg bw (98–104 µCi/kg bw) was evaluated. Two hours post dose the blood, liver, and kidneys were analysed by TLC to quantify and identify any parent compound and metabolites present. Bile and faecal samples were collected for 24 h post dose. The two hour time point for collection of tissue samples was selected because radioactivity concentration peaked in plasma and tissue at this time point. Metabolites were isolated from the faeces, urine, bile and major tissues by chromatography and identified by co-chromatography with authentic standards and/or by spectroanalysis. Two hours after oral administration, the major metabolites in blood, liver and kidney were:

- *O*,*O*-dimethyl-*O*-(2,6-dichloro-4-carboxyphenyl) phosphorothioate (see Fig. 2),
- 3,5-dichloro-4-hydroxybenzaldehyde,
- *O*-methyl *O*-hydrogen *O*-(2,6-dichloro-4-methylphenyl) phosphorothioate, and
- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-hydroxymethylphenyl) phosphorothioate.

Only a small amount of the parent compound was detected in the liver. The major biotransformation reactions were oxidative desulfuration to oxon and related derivatives, oxidation of the 4-methyl group to alcohols and acids, cleavage of the P-O-aryl and P-O-methyl linkages and conjugation of the resultant acids and phenols with glucoronic acids (Mihara, Ohkawa & Miyamoto, 1980; Krautter et al. 1988a, b; Esumi, 1989).

The metabolite profile of [^{14}C-phenyl]-tolclofos-methyl (radiochemical purity > 98.9%) after administration to Sprague Dawley male and female rats at a single dose of 5 mg/kg bw, 200 mg/kg bw, or 14 consecutive doses at 5 mg/kg bw per day, was analysed by TLC and co-chromatography.

More than ten metabolites were detected in the excreta. No marked differences were seen in relation to sex. The major metabolites detected in the excreta were:

- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-methylphenyl) phosphate (Fig. 2; 10–26% of urinary ^{14}C),
- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-hydroxymethylphenyl) phosphorothioate (12–25%),
- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-carboxyphenyl) phosphorothioate (11–35%), and
- *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-methylphenyl) phosphorothioate (12–44%).

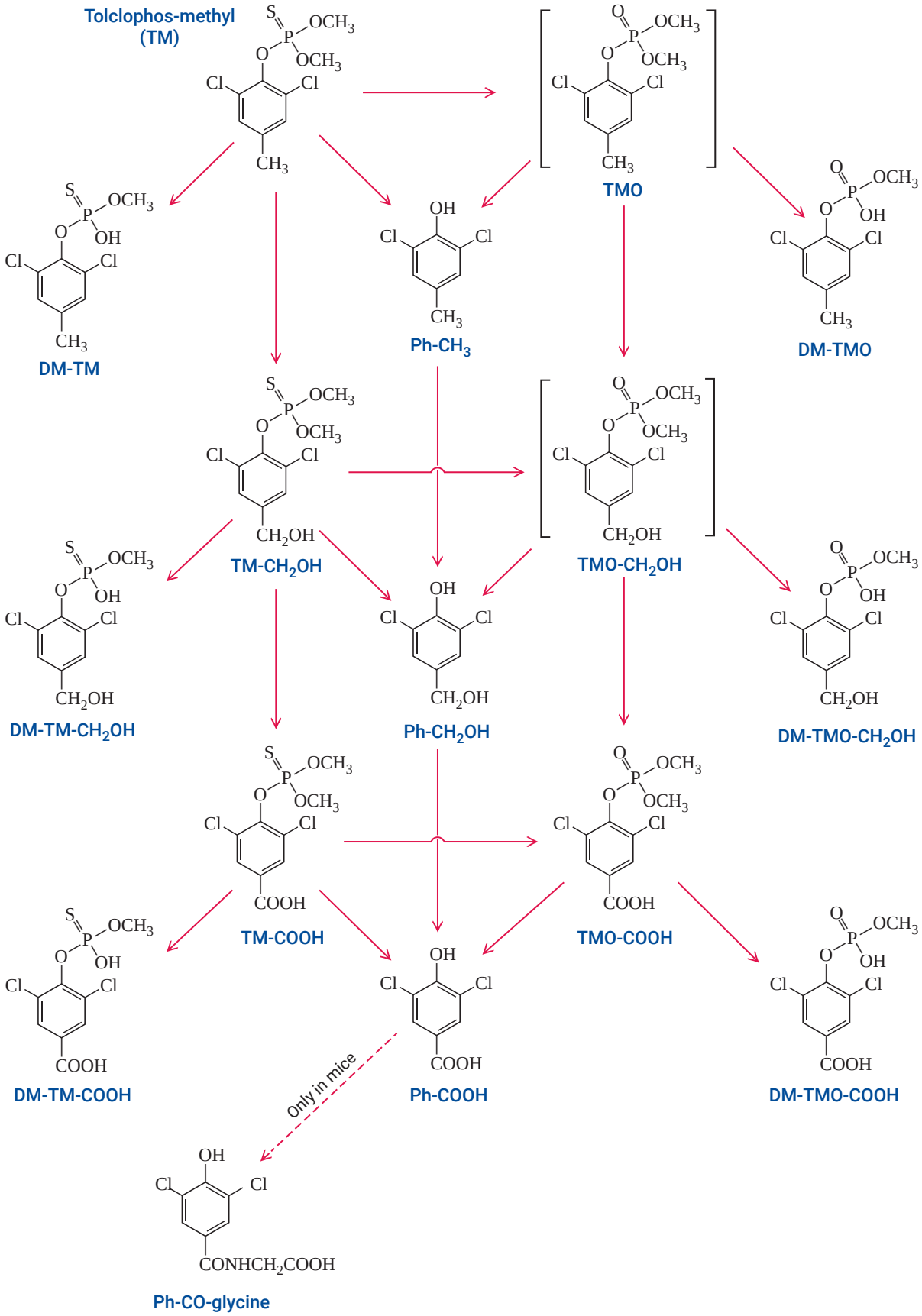
In rats with bile cannulas, most of the radiolabel excreted into the bile within 24 h after administration was associated with polar metabolites. The major metabolites in the bile were *O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-hydroxymethyl phenyl) phosphorothioate and 2,6-dichloro-4-methyl phenol glucuronides. Radiocarbon excreted into the faeces within 24 h after administration was associated only with the parent compound (Esumi, 1989).

Table 1. Identity of [phenyl ^{14}C]-tolclofos-methyl metabolites in the rat

Designation	Chemical name
TM (parent compound)	*O*,*O*-dimethyl *O*-(2,6-dichloro-4-methylphenyl) phosphorothioate
DM-TM	*O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-methylphenyl) phosphorothioate
TM–CH_2OH	*O*,*O*-dimethyl *O*-[2,6-dichloro-4-(hydroxymethyl)phenyl] phosphorothioate
DM-TM–CH_2OH	*O*-methyl *O*-hydrogen-*O*-[2,6-dichloro-4-(hydroxymethyl) phenyl] phosphorothioate
DM-TM-COOH	*O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-carboxyphenyl) phosphorothioate
ph-CH_3	2,6-dichloro-4-methyl phenol
ph-COOH	3,5-dichloro-4-hydroxybenzoic acid
TMO	*O*,*O*-dimethyl *O*-(2,6-dichloro-4-methylphenyl) phosphate
ph-CH_2OH	3,5-dichloro-4-hydroxybenzyl alcohol
TMO-COOH	*O*,*O*-dimethyl *O*-(2,6-dichloro-4-carboxyphenyl) phosphate
DM-TMO	*O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-methylphenyl) phosphate
DM-TMO-CH_2OH	*O*-methyl *O*-hydrogen-*O*-[2,6-dichloro-4-(hydroxymethyl)phenyl] phosphate
DM-TMO-COOH	*O*-methyl *O*-hydrogen-*O*-(2,6-dichloro-4-carboxyphenyl) phosphate

The proposed metabolic pathway for tolclofos-methyl is shown in Figure 2 on the next page.

Figure 2. In vivo metabolic pathway for tolclofos-methyl



Comparative in vitro metabolism

A comparative in vitro metabolism study using rat and human liver microsomes was conducted in which they were exposed to 10 µM [phenyl ^{14}C]-tolclofos-methyl (radiochemical purity 99.9%) for 15 minutes to investigate the potential differences in metabolism between rats and humans. After incubation, the metabolites were quantified by high performance liquid chromatography (HPLC) radiochromatography and identified by HPLC and TLC co-chromatography with authentic standards and by liquid chromatography–mass spectrometry (LC-MS) analysis. A preliminary study was conducted to establish the optimal conditions for the definitive study. In the preliminary study, duplicate rat liver microsome preparations were exposed to 1, 10, or 100 µM [phenyl ^{14}C]-tolclofos-methyl for 0, 15, 30, 60, or 180 minutes. A positive control [4-^{14}C]-testosterone was used to test for the metabolic capacity of the liver microsome preparations. Tolclofos-methyl depletion reached linearity at concentrations of ≤ 10 µM within 15 minutes of incubation. To maximize the potential to detect metabolite levels in radioanalysis, a 15 minute reaction time was selected for the main study.

In the main study five metabolites were identified in human liver microsomes and seven in rat microsomes. The predominant metabolites in both species were TM-CH_2OH (35% and 11% of total radiochromatogram in human and rat liver microsomal preparations, respectively) and TMO- CH_2OH accounting for 17% and 43% of total radiochromatogram in human and rat liver microsomal preparations, respectively. Three other metabolites (ph-CH_2OH, 2,6-dichloro-*p*-cresol, and TM-COOH were also identified in both rat and human liver microsomes. These three metabolites accounted for 8–16% of the total radiochromatogram from human liver microsomes and 10–11% from the rat microsome preparations. Two metabolites unique to the rat were also detected — an unidentified metabolite (M2) and TMO-COOH. Together these accounted for 3–5% of the total radiochromatogram. The formation rate for the two main metabolites was 230 and 71 pmol/min per mg of protein in humans and rats, respectively for TM-CH_2OH and 113 and 288 pmol/min per mg of protein in humans and rats, respectively for TMO-CH_2OH. The metabolic pathway in both rat and human liver microsomes proceeds as follows:

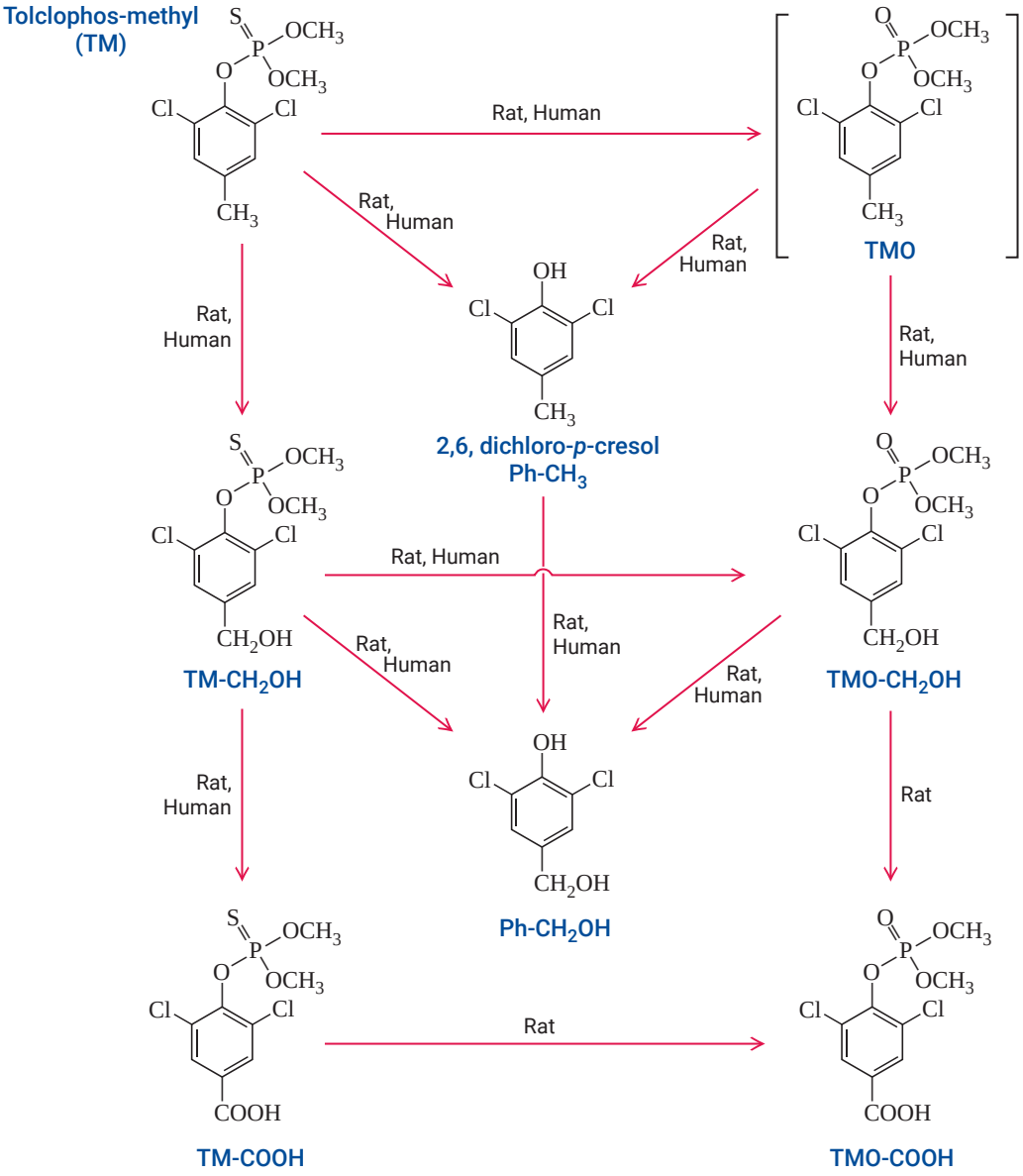
[1] oxidative desulfuration of the P=S group to P=O,

[2] oxidation of the 4-methyl group, and

[3] cleavage of the P-O-aryl linkage.

The proposed metabolic pathway(s) of [phenyl ^{14}C]-tolclofos-methyl in human and rat liver microsomes is presented on the next page in Fig. 3 (Nagahori, 2015).

Figure 3. Proposed in vitro metabolic pathway(s) for [phenyl^{14}C]-tolclofos-methyl in human and rat liver microsomes



2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with tolclofos-methyl are summarized in Table 2.

Table 2. Summary of acute toxicity studies with tolclofos-methyl

Species	Strain	Sex	Route	Purity (%)	LD_{50} or LC_{50}	Reference
Mouse	DD	M+F	Oral	97	3500 mg/kg bw (M) 3600 mg/kg bw (F)	Segawa (1978)[a]
Mouse	DD	M+F	Dermal	97	5000 mg/kg bw (M+F)	Segawa (1978)[a]
Rat	SD	M+F	Oral	97	5000 mg/kg bw (M+F)	Segawa (1978)[a]
	SD	F	Oral	98.6	> 5000 mg/kg bw	Parcell, Crook & Gibson, (1994)[b]
	SD	M+F	Oral	97.7	> 5000 mg/kg bw (M+F)	Kynoch (1985)
Rat	SD	M+F	Dermal	97	> 5000 mg/kg bw (M+F)	Segawa, (1978)[a]
Rat	SD	M+F	Dermal	97.5	> 2000 mg/kg bw (M+F)	Moon, (2010)
Rat	Wistar	M+F	Inhalation	97.4	> 3.32 mg/L[d]	Hardy et al., (1986)[a]
Rat	SD	M+F	Inhalation	97.4	> 2.07 mg/L	Lowe (2012)[e]
Rabbit	NZW	M+F	Dermal	97.7	> 2000 mg/kg bw (M+F)	Kynoch & Parcell (1985)[c]
Dog	NR	M+F	Oral	98.7	> 1000 mg/kg bw (M+F)	Pence et al., (1978)[a]

LC_{50} median lethal concentration LD_{50} median lethal dose

DD dopamine deficient SD Sprague Dawley NZW New Zealand White NR Not reported;

F female M male

[a] This study was previously considered by the 1994 JMPR Meeting

[b] Plasma and brain cholinesterase data were collected at termination. No changes were noted at the highest dose tested (5000 mg/kg bw). Piloerection and hunched posture noted at all doses tested (2, 200, and 5000 mg/kg bw) and partially closed eyes for animals treated at 5000 mg/kg bw.

[c] 1/5 female rabbits exposed to 2000 mg/kg bw died on observation day 5 after exhibiting noisy respiration and nasal exudate on day 4. Another female rabbit in the 2000 mg/kg bw treatment group showed decreased food consumption on days 9–13 and piloerection on day 11.

[d] Four-hour whole body exposure

[e] Four-hour nose only exposure

(b) Dermal irritation

Six male albino Japanese rabbits received 500 mg of tolclofos-methyl (purity, 97%, incorporated into Vaseline) on clipped intact or abraded dorsal skin for four hours under an occlusive dressing. No signs of irritation were seen at any of the application sites seven days after treatment (Matsubara, Hara & Kadota, 1978).

Six female New Zealand White rabbits received 500 mg tolclofos-methyl (purity 97.7%) moistened in distilled water and applied to clipped intact skin under an elastic adhesive dressing (Elastoplast) for four hours. No signs of irritation were observed throughout the four-day observation period. (Liggett & Parcell, 1985a).

Three male New Zealand White rabbits received 500 mg tolclofos-methyl (purity 97.5%) dissolved in corn oil placed onto a lint patch, applied to clipped intact skin for four hours and occluded by surgical tape. Very slight erythema and oedema were noted in all three rabbits within an hour post exposure. These signs of irritation resolved three days after the exposure. It is noteworthy that this is the only dermal irritation study in the tolclofos-methyl database to report signs of dermal irritation (Ota, 2010a).

(c) Ocular irritation

In an eye irritation study 50 mg tolclofos-methyl (purity 97%) was applied to the conjunctival sac of one eye of eight male New Zealand White rabbits for either five minutes or 24 h, with the untreated eye serving as a control. After the exposure period the eyes were washed with physiological saline for two minutes. The eyes were examined after 1, 24, 48, or 72 hours, or seven days of exposure. There were no signs of irritation observed at any time period (Matsubara, Hara & Kadota, 1978).

Eye irritation was also evaluated after 75 mg tolclofos-methyl (purity 97.7%) was applied to conjunctival sac of one eye of six female New Zealand White rabbits with the contralateral untreated eye serving as a control. The study report did not indicate if the eyes were rinsed after application of the test material. The eyes were examined after one hour, then after 2, 3, 4 and 7 days after exposure. Conjunctivae effects marked by slight redness and chemosis, as well as slight to moderate increase in eye discharge, were noted in all animals one hour after exposure. These effects resolved by the second day after exposure (Liggett & Parcell, 1985b).

Tolclofos-methyl (purity 97.5%) was instilled into one eye of three New Zealand White rabbits at 69 mg. The eye was not rinsed after application of the test material. Observations for signs of eye irritation were conducted 1, 24, 48, 72, and 96 h after application and scored according to the method of Draize. Conjunctival redness and chemosis (grade 1: slight redness and swelling) was reported for all three animals at the one-hour observation. After 24 h one of the rabbits exhibited a slightly worsening condition with a partial eversion of the eyelid (grade 2) and discharge. Indications of eye irritation in this animal were still apparent at the 48 and 72 h observations but had resolved by the 96 h observation (Ota, 2010b).

(d) Dermal sensitization

In a study designed to assess the dermal sensitization potential of tolclofos-methyl (purity 98%), 20 female Hartley guinea pigs were given an intradermal injection of 5% tolclofos-methyl in corn oil and Freund´s complete adjuvant/distilled as the first step in the induction process. One week after the injection the second induction was performed by applying a patch containing 0.4 mL of tolclofos (25% in acetone) to the skin of the sensitized animals for 48 h under an occluded dressing. Control groups each consisting of ten animals received the same treatment, but without test substance for the vehicle control, and with α-hexylcinnamaldehyde (HCA) as the positive control. Two weeks after the second induction a patch saturated with 0.2 mL of 10% tolclofos-methyl or 10% HCA was applied for 24 h under an occlusive dressing as a challenge. The patch was removed 24 or 48 h later and the animals were examined for signs of skin sensitization by the method of Magnusson and Kligman. Slight to moderate erythema was observed in seven out of 20 animals and slight to moderate swelling was reported for five out of 20 animals in the tolclofos-methyl-sensitized group when challenged with the test material (Nakamura, 2001).

A 1985 dermal sensitization study was not included in this monograph as the stability of the compound had not been determined (Seaber, 1985).

2.2 Short-term studies of toxicity

(a) Oral administration

Rat

Groups of ten male and ten female Sprague-Dawley rats were fed for 32–34 days, diets containing tolclofos-methyl (purity, 97.9%) at 0, 200, 1000, 5000 or 20 000 ppm (equal to 0, 16, 79, 414, or 1635 mg/kg bw per day for males, 0, 18, 88, 452, or 1830 mg/kg bw per day for females). Animals were observed twice daily for mortality and clinical signs, while food consumption and body weight were recorded weekly. Ophthalmoscopic examination, standard haematological tests and clinical chemical analyses were performed at week 4, and certain tests were repeated at termination. Plasma and erythrocyte cholinesterase activities were determined in the control and high-dose groups at week 4 and in all animals at termination, when brain cholinesterase activity was also measured. At terminal sacrifice, relevant organs were weighed, and most organs were examined microscopically.

There were no treatment-related deaths. Reductions in food consumption (–16% in males and –6% in females) and body weight gain (–45% in males and –37% in females) were observed at 20 000 ppm. Body weight gain was slightly reduced in animals of each sex at 5000 ppm during week 1. Haematological parameters were unaffected by treatment. Cholesterol levels were increased (by 116–138%) in animals of each sex at 20 000 ppm, and total protein and albumin levels were slightly higher in males from this group. Other differences between treated and untreated animals were detected, but the values were within the normal range. Plasma cholinesterase activity was reduced by 14% in males and by 50% in females at 20 000 ppm on week 4 of the study. Erythrocyte cholinesterase activity was reduced by 9–19% in animals of each sex at 5000 and 20 000 ppm, however, no clear dose–response relationship was seen. Brain cholinesterase activity was lower in males (by 12–31%) than that in the controls in all treated groups, and in females (by 20%) at 5000 and 20 000 ppm. Although the reduction was statistically significant in all male groups, no clear dose–response relationship was found, and the activity in controls was slightly higher than usual. There were no ophthalmoscopic alterations or gross changes at necropsy. The relative weights of livers were increased by 12% in females at 5000 ppm, and in males and females at 20 000 ppm (by 27% in males and 39% in females). Hypertrophy of hepatocytes was observed in animals of each sex at 20 000 ppm. Relative weights of the kidneys were slightly increased in animals of each sex at 5000 and 20 000 ppm, but no treatment-related histopathological changes were seen. The no-observed-adverse-effect level (NOAEL) was 1000 ppm, equal to 79 mg/kg bw per day, on the basis of increased relative kidney weights and decreased brain cholinesterase activity at 5000 ppm, equal to 414 mg/kg bw per day (Colley et al., 1982). [*This text was adopted verbatim from the 1994 JMPR monograph.*]

Groups of 12 male and 12 female Sprague Dawley rats were fed diets for 13 weeks containing tolclofos-methyl (96.6% pure) at 0, 100, 1000 or 10 000 ppm (equal to 6.5, 66 or 653 mg/kg bw per day for males, 7.1, 71 or 696 mg/kg bw per day for females). Animals were observed daily for mortality and clinical signs; body weight as well as food and water consumption were determined weekly. Ophthalmological analysis, urinalysis, standard haematological tests, clinical chemical analyses and measurement of plasma, erythrocyte and brain cholinesterase activities were undertaken at termination, when relevant organs were weighed, and most organs examined microscopically.

No deaths and no significant abnormal clinical or ophthalmological signs were noted during the study. Depression of body weight gain of males (–25%) and females (–27%) at 10 000 ppm was seen throughout the treatment period and was associated with similar decreases in food and water consumption. A slight decrease in food consumption was noted in females at 1000 ppm during week 1. Decreased plasma (16–53%), brain (8–9%) and erythrocyte (19–20%) cholinesterase activities were noted in animals of each sex at 10 000 ppm. A minimal decrease in erythrocyte cholinesterase activity (10%) was also seen in females at 1000 ppm. Liver weight was increased in animals of each sex at 10 000 ppm, and this was found at histopathological examination to be associated with hypertrophy of hepatocytes. Changes in some clinical chemical parameters were observed at 10 000 ppm, which included increased levels of cholesterol (48% in males, 102% in females) and phospholipids (37% in males, 64% in females) and slightly increased α-2 and β globulin levels in animals of each sex. Other changes, although statistically significant, were minor and of questionable biological significance. At 10 000 ppm, increased relative kidney weights were seen in animals of each sex, increased blood urea nitrogen levels were seen in males only and decreased urinary pH was seen in males and females. At 1000 ppm, relative kidney weights were slightly increased in females and relative liver weights in males. The Meeting considered that the increased weight ratios observed at 1000 ppm were not biologically relevant and that the NOAEL was 1000 ppm (equal to 66 mg/kg bw per day), based on decreased cholinesterase activity and changes in clinical chemistry parameters at 10 000 ppm (equal to 653 mg/kg bw per day) (Kimura, 1990). [*This text was adopted verbatim from the 1994 JMPR monograph.*]

Dog

Groups of six male and six female beagle dogs were fed diets for 26 weeks containing tolclofos-methyl (purity, 98.7%) at 0, 200, 600 or 2000 ppm (equal to 7.4, 23 or 69 mg/kg bw per day in males, 4.1, 21 or 65 mg/kg bw per day in females). Animals were observed daily for mortality and weekly for clinical signs, body weight and food consumption. Ophthalmoscopic examinations were performed before treatment and at termination of the study. Standard haematological tests and clinical chemical analyses

were performed at regular intervals until termination. Erythrocyte and plasma cholinesterase activities were determined at weeks 2, 4, 8, 13 and 26. Brain cholinesterase was determined at termination, when relevant organs were weighed, and most were examined histologically.

There were no deaths and no overt signs of toxicity. Body weight gain was reduced by 54% in males and by 46% in females at 2000 ppm, although food consumption was unaffected by treatment. There were no treatment-related ocular changes. Although the haematocrit was within the normal range, decreased values in comparison with controls (–11% in males and –10% in females) were observed at 2000 ppm. The mean corpuscular volume and mean corpuscular haemoglobin concentration were not affected by treatment. Alkaline phosphatase activity was increased throughout the study by 139–295% in animals of each sex at 2000 ppm. Total bilirubin was not increased in treated animals. At 2000 ppm, plasma cholinesterase activity was decreased by 19–26% in females throughout the study, but no significant decreases were seen in erythrocyte cholinesterase activity in animals of either sex, nor in plasma cholinesterase activity in males. Brain cholinesterase activity was unaffected by treatment. Urinalysis gave unremarkable results, and there were no gross changes at necropsy. Liver weights were increased (by 59% in males and 43% in females) at 2000 ppm, but there were no concomitant histological changes, and there were no treatment-related histopathological changes. The NOAEL was 600 ppm, (equal to 21 mg/kg bw per day), on the basis of reduced body weight gain at 2000 ppm, (equal to 65 mg/kg bw per day; Pence, 1979). [*This text was adopted verbatim from the 1994 JMPR monograph.*]

Groups of six male and six female beagle dogs were fed diets for 52 weeks containing tolclofos-methyl (purity, 96.7%) at 0, 80, 400 or 2000 ppm (equal to 0, 2.2, 11 or 59 mg/kg bw per day for males, 0, 2.6, 11.2, or 62 mg/kg bw per day for females). Animals were observed daily for mortality and weekly for clinical signs. Ophthalmoscopic examinations were performed before treatment and at weeks 26 and 52. Standard haematological tests, clinical chemical analyses and determinations of plasma and erythrocyte cholinesterase activity were performed one week before treatment and at weeks 13, 26 and 52. Brain cholinesterase activity was determined at termination, when relevant organs were weighed, and most were examined histologically.

No clinical signs of toxicity were observed. Decreased food consumption (–9% in males and –16% in females) and body weight gain (–32% in males and –54% in females), slightly lower erythrocyte counts (–19% in males and –9% in females), haematocrit value (–17% in males and –8% in females) and haemoglobin concentration (–17% in males and –8% in females), slightly decreased albumin and total protein levels (significant at some time points) and elevated alkaline phosphatase levels (> 200%) were found at 2000 ppm. Plasma cholinesterase activity was slightly, but not significantly, lower than that in controls in females at the highest dose.Erythrocyte and brain cholinesterase activities were not affected by the treatment. No treatment-related ophthalmoscopic alterations were observed. Increases in mean liver weights, both absolute (61% in males and 32% in females) and relative (74% in males and 52% in females), were seen in dogs at 2000 ppm. Microscopic examination of the livers revealed increased incidences of hepatocytic hypertrophy and intracytoplasmic homogeneous material, and an increased amount of hepatocytic pigment in these animals. The amount of this pigment was also increased at 400 ppm, and it was present in control animals. The nature of the pigment has not been elucidated, but it does not contain iron or bilirubin. These dogs also had decreased absolute (–46%) and relative (–40%) prostate weights, and the absolute and relative weights of the pancreas were increased by 16–27% and 28–49%, respectively. The NOAEL was 400 ppm, (equal to 11 mg/kg bw per day), on the basis of reduced body weight gain at 2000 ppm (equal to 59 mg/kg bw per day) (Cox et al., 1988; Moore, 1993).

(b) Dermal application

Tolclofos-methyl (purity, 97.7%) dissolved in acetone was applied to the skin of five male and five female New Zealand White rabbits at doses of 0, 30, 300 or 1000 mg/kg bw per day for six hours per day, on five days per week for 21 days.

There were no deaths and no treatment-related clinical signs; neither body weight nor food consumption were affected by treatment. Dermal irritation (slight erythema) was seen in some animals in all treated groups beginning on day 6 of treatment. Eosinophil counts were increased in males at 1000 mg/kg bw per day, and the level of inorganic phosphorus was decreased (by 14–20%) in animals of each sex at this dose. Brain cholinesterase activity was about 15% lower than that in controls in

males and about 15% higher in females at 300 and 1000 mg/kg bw per day levels. These changes were considered not to be of biological significance. Erythrocyte cholinesterase activity was lower than in controls in males at the same two doses, but there was no dose–effect relationship. Plasma cholinesterase activity was lower (by 22–29%) than in controls in animals of each sex at 300 and 1000 mg/kg bw per day. The relative weights of the kidneys were increased (by 20%) in females at 1000 mg/kg bw per day. Macroscopic findings consisted of accumulation of the compound on treated skin; microscopic examination revealed hyperkeratosis, acanthosis and subepidermal pleocellular infiltration of the skin (Gargus, 1986). [*This text was adopted verbatim from the 1994 JMPR monograph.*]

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

Groups of 15 male and 15 female ddY mice were fed diets for nine months containing tolclofos-methyl (purity, 97%) at 0, 10, 30, 100 or 3000 ppm (equal to 1.2, 3.8, 12 or 510 mg/kg bw per day for males, 1.4, 4.1, 14 or 560 mg/kg bw per day for females). Three satellite groups of five males and five females each were similarly treated and killed at 2, 4 and 13 weeks to determine cholinesterase activity. Animals were observed daily for mortality and signs of toxicity. At the end of exposure, an ophthalmological examination and clinical chemical analyses (including plasma and erythrocyte cholinesterase) were undertaken, and brain cholinesterase activity was measured. Most organs were weighed and examined microscopically. There were no overt signs of toxicity and no deaths. Body weights were reduced by about 20% and body weight gain by about 35% in animals of each sex at 3000 ppm, but at this dose food consumption was increased in males and females and water intake was increased in males. No treatment-related ophthalmological changes were observed, and haematological parameters were unaffected by treatment. The cholesterol level was increased by 35% in females at 3000 ppm. A dose-related decrease in plasma cholinesterase activity was found in animals of each sex, with activities that were 63–94% of the control values in mice at 30 ppm, 30–56% at 100 ppm and 4–35% (61% in one case) at 3000 ppm. At the high dose, the activity reached a plateau after four weeks. Erythrocyte cholinesterase activity was decreased to 39–46% of the control level in males at 3000 ppm at weeks 13 and 40, and to 57–75% of the control level in females at this dose at all time points. Brain cholinesterase activity was unaffected by treatment in females, but was reduced by about 24% in males at 3000 ppm at weeks 13 and 40. Organ weights in the 3000 ppm group were reduced, but neither gross nor histopathological changes attributable to treatment were observed. The NOAEL was 100 ppm (equal to 12 mg/kg per day), on the basis of decreased erythrocyte and brain cholinesterase activity and an effect on body weight at 3000 ppm (equal to 513 mg/kg bw per day) (Suzuki et al., 1978). [*This text was adopted verbatim from the 1994 JMPR monograph.*]

Groups of 70 male and 70 female B6C3F1 mice were fed diets containing tolclofos-methyl (purity, 94.3%) at 0, 10, 50, 250 or 1000 ppm (equal to 1.3, 6.5, 32 or 134 mg/kg bw per day in males, 1.3, 6.8, 34 or 137 mg/kg bw per day in females) for up to 104 weeks. Animals were observed twice daily for clinical symptoms and mortality. Body weight was measured weekly during the first 13 weeks and then once every four weeks. Food intake was measured weekly. Standard urinalysis was performed on ten animals from each group at 6, 12 and 18 months and in all survivors at termination of the study. Standard haematological tests were performed on 10 animals from each group at 12 months and in all survivors at termination. Standard clinical chemical analyses and measurements of serum, erythrocyte and brain cholinesterase activities were done on ten animals from each group at 6 and 12 months and in all animals at termination. Animals in the control and 1000 ppm groups were subjected to ophthalmologic examination at 12 and 24 months. Ten animals from each group were necropsied at six and at 12 months, at termination or when found dead or moribund. Relevant organs were weighed and examined microscopically.

There were no treatment-related effects on mortality and no overt signs of toxicity, including ophthalmologic changes. Body weight gain was slightly reduced in females at 1000 ppm up to week 52; food consumption was also slightly decreased in this group after 52 and 104 weeks. Food conversion efficiency, water intake, urinary parameters and the haematological profile were unaffected by treatment. Serum cholinesterase activity was decreased in animals of each sex at 250 ppm (by 25–52%) and at 1000 ppm (by 43–81%) at both times, and erythrocyte cholinesterase activity was decreased by 11–28% at 250 ppm and by 13–47% at 1000 ppm, with the greatest extent of decrease seen at termination .

A dose-related decrease was found in brain cholinesterase activity in females (by 7–24% at 250 ppm and 9–33% at 1000 ppm) and males (by 10–13% at 250 ppm and 17–26% at 1000 ppm). Serum and brain (but not erythrocyte) cholinesterase activities were lower than in controls (by 12 and 18%, respectively) in females on 50 ppm at week 28; but controls at week 28 had higher levels than at week 52 and at termination. Glucose levels were increased in males and were slightly increased in females at 1000 ppm at week 104. Absolute and/or relative kidney weights were increased in animals of both sexes at 1000 ppm, in males on 250 ppm at week 52, and in females on 250 ppm at week 104. Thymus weights were decreased in females on 1000 ppm at weeks 52 and 104, and pituitary weights were increased in females at this dose at week 104. No treatment-related changes were seen at necropsy or on histopathological examination at any time. Although an increase in the incidence of follicular cell carcinomas was noted at 1000 ppm (equal to 42 mg/kg bw per day), in the absence of any indication of thyroid toxicity at higher doses in the remainder of the tolclofos-methyl database this observation was, based on the weight of the evidence, considered to be a spurious finding. Tolclofos-methyl was not found to be carcinogenic. The NOAEL was 50 ppm, equal to 6.5 mg/kg bw per day, on the basis of reduced erythrocyte and brain cholinesterase activity and increased absolute and relative kidney weights at 250 ppm (equal to 32 mg/kg bw per day) (Satoh et al., 1983).

Table 3. Cholinesterase activity in male mice

Tolclofos-methyl in diet (ppm)	**0**	**10**	**50**	**250**	**1000**
	Cholinesterase activity (µmol/mL per minute) ± Standard error				
Plasma					
Week 28	6.23	6.06	5.62	3.69 ± 0.19*** (41%) [a]	1.83 ± 0.07*** (71%) [a]
Week 52	6.33	6.07	6.05	4.27 ± 0.18*** (33%) [a]	2.92 ± 0.08*** (54%) [a]
Week 104	8.89	8.89	8.42	6.66 ± 0.3*** (25%) [a]	5.06 ± 0.37*** (43%) [a]
Erythrocytes					
Week 28	5.49	5.33	5.60	4.03 ± 0.18*** (27%) [a]	3.88 ± 0.17*** (29%) [a]
Week 52	5.22	5.13	5.39	4.28 ± 0.19** (18%)[a]	3.67 ± 0.12*** (30%)[a]
Week 104	5.21	5.13	5.14	4.54 ± 0.18* (13%) [a]	4.53 ± 0.17* (13%) [a]
Brain					
Week 28	20.16	18.39	18.42	18.21	16.70
Week 52	18.85	18.43	19.00	16.20 ± 0.76* (14%) [a]	14.27 *± 0.70** (24%) [a]
Week 104	17.77	17.83	16.59	15.41 ± 0.82* (13%) [a]	13.16 ± 0.43*** (26%) [a]

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

[a] Numbers in parenthesis represent percent decrease relative to concurrent negative control group

Table 4. Cholinesterase activity in female mice

Tolclofos-methyl in diet (ppm)	0	10	50	250	1000
	Cholinesterase activity (µmol/mL per minute) ± Standard error				
Plasma					
Week 28	9.46	9.20	8.28 ± 0.17*** (12%) [a]	4.51 ± 0.1*** (52%) [a]	1.79 ± 0.17*** (81%) [a]
Week 52	8.51	8.33	7.56	4.83 ± 0.11*** (43%) [a]	2.68 ± 0.07*** (69%) [a]
Week 104	9.01	9.28	8.58	6.04 ± 0.14*** (33%) [a]	3.68 ± 0.12*** (59%) [a]
Erythrocytes					
Week 28	5.87	5.79	5.62	4.20 ± 0.18*** (28%) [a]	3.10 ± 0.09*** (47%) [a]
Week 52	5.13	4.97	5.10	3.96 ± 0.2* (23%) [a]	3.19 ± 0.16*** (38%) [a]
Week 104	5.25	5.08	5.11	4.67 ± 0.15* (11%) [a]	4.04 ± 0.16*** (23%) [a]
Brain					
Week 28	21.40	19.63	17.52± 0.9* (18%)[a]	16.32 ± 0.85** (24%) [a]	14.42 ± 0.84*** (33%) [a]
Week 52	18.77	17.83	19.86	16.45 ± 0.72* (12%) [a]	15.89 ± 0.56** (15%) [a]
Week 104	17.92	17.89	18.71	16.61 ± 0.5 (7%) [a]	16.24 ± 0.5* (9%) [a]

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

[a] Numbers in parenthesis represent percent decrease relative to concurrent negative control group

Rat

Study 1

Groups of 15 male and 15 female Sprague Dawley rats were fed diets containing tolclofos-methyl (purity, 97%) at 0, 300, 1000, 3000 or 10 000 ppm (equal to 16, 51, 164 or 540 mg/kg bw per day in males, 18, 65, 184 or 623 mg/kg bw per day in females) for 28 weeks. Animals were observed daily for clinical signs; body weight and food consumption were measured weekly. Ophthalmologic examinations were carried out after three months and at termination. Standard haematological tests and clinical chemical analyses were performed at termination. Urinalysis was carried out in 12 animals per group after three months and at termination. Plasma and erythrocyte cholinesterase activities were determined after 2, 4 and 13 weeks, and at termination; brain cholinesterase was determined only at termination, when relevant organs were weighed, and several were examined histologically.

There were no overt signs of toxicity and no treatment-related deaths. Although the body weights of animals of each sex at 10 000 ppm were lower than those of controls throughout the study, their initial body weights were also statistically significantly lower. Body weight gain was significantly reduced (by 18%) in females at 10 000 ppm. Food consumption was unaffected by treatment. Treatment did not affect ophthalmologic, urinary, haematological or clinical chemical parameters. Plasma cholinesterase activity was lower (by 23–41%) in females at 10 000 ppm than in controls at weeks 2, 4, 13 and 28. Erythrocyte cholinesterase activity was highly variable and was reduced by 15–19% in comparison with controls in males at 10 000 ppm at weeks 2, 4 and 28. Brain cholinesterase activity was unaffected by treatment. Kidney and liver weights were slightly increased in animals of each sex at 10 000 ppm, and in females at 1000 and 3000 ppm, but the biological significance of these findings was questionable. No gross abnormalities were seen at necropsy. Bile duct proliferation was found in the livers of 3/15 females

at 10 000 ppm and oval cell proliferation was seen in 2/15 females at 3000 ppm and 3/15 at 10 000 ppm. No histopathological changes were seen in the kidneys. The NOAEL was 1000 ppm (equal to 65 mg/kg bw per day) on the basis of histopathological changes in the livers of females at 3000 ppm (equal to 164 mg/kg bw per day) (Hiromori et al., 1978).
[*This text was adopted verbatim from the 1994 JMPR monograph.*]

Study 2
Groups of 65 male and 65 female Fischer 344 rats were fed diets containing tolclofos-methyl (purity, 94.9–98.7%) at 0, 100, 300 or 1000 ppm (equal to 0, 4.2, 12, or 42 mg/kg bw for males, 0, 4.8, 15, or 49 mg/kg bw for females) for 122 weeks (males) or 129 weeks (females). At week 52, 10 males and 10 females from each group were killed. Animals were observed twice a day for mortality and signs of morbidity. Body weight and food consumption were recorded weekly (weeks 0–26), every two weeks (weeks 27–52) then every four weeks until termination. Ophthalmoscopic examinations were performed every 26 weeks and at termination. Standard haematological tests, clinical chemical analyses and measurements of erythrocyte and plasma cholinesterase activity were conducted in ten animals of each sex in each group one week before treatment, at weeks 4, 13, 26, 52, 78 and 104, and at termination. Brain cholinesterase activity was determined in ten animals of each sex per group at week 52 and at termination. All animals killed or found dead were necropsied, and relevant organs weighed; most organs were then examined histologically.

There were no treatment-related effects on mortality and no overt signs of toxicity. A slight decrease in body weight gain was observed in males at 1000 ppm. Food consumption and food conversion efficiency were unaffected by treatment, as was the haematological profile. Statistically significant ($p = 0.05$) decreases in alkaline phosphatase ranging from 16 to 46% were noted in males at ≥ 300 ppm beginning on week 13 of the study. In females, statistically significant decreases in alkaline phosphatase were reported at 1000 ppm during the week 13 and week 104 evaluations (21% and 46%, respectively). Alkaline phosphatase was also decreased by 21–27% at other time points in females exposed to1000 ppm tolclofos-methyl, however, these decreases were not statistically significant. Plasma, erythrocytes, and brain cholinesterase activity was sporadically decreased and did not show a dose–response relationship thereby precluding a meaningful interpretation of the data. Urinalysis revealed no remarkable findings, organ weights were unaffected by treatment, and there were no treatment-related gross or histopathological changes at necropsy. Tolclofos-methyl was not carcinogenic in this study. No NOAEL could be identified, given the variability of the data on cholinesterase activity (Pence et al., 1982).
[*This text was adopted verbatim from the 1994 JMPR monograph.*]

Study 3
Groups of 30 male and 30 female Fischer 344 rats were fed diets containing tolclofos-methyl (purity, 98.3–97.3%) at 0, 100, 300 or 1000 ppm (equivalent to 0, 5, 15, or 50 mg/kg bw per day) for 104 weeks, and brain, erythrocyte and plasma cholinesterase activities were assessed. Ten males and ten females from each group were sacrificed at week 52. Animals were observed daily for mortality, and weekly for body weight, food consumption and gross signs of toxicity until week 26, then every two weeks during weeks 26–52 and finally every four weeks during weeks 52–104. Blood samples were taken for determination of plasma and erythrocyte cholinesterase activities at initiation of the study, at weeks 5, 14, 27, 53, 79 and at termination. Brain cholinesterase activity was determined at week 52 and at termination.

There were no treatment-related effects on survival and the incidences of clinical signs and palpable tissue masses were comparable among the groups. Mean plasma cholinesterase activities were statistically significantly decreased (by 9–16%) in males when compared with controls at 300 and 1000 ppm at week 27. Mean erythrocyte and brain cholinesterase activities were comparable among the groups throughout the study. Plasma cholinesterase activity was reduced (by < 20%) at certain times in treated animals, but no dose–response relationship was found. Gross pathological findings were unrelated to treatment. Doses < 1000 ppm did not depress erythrocyte or brain cholinesterase activity or induce gross pathological effects. The NOAEL was 1000 ppm, equivalent to 50 mg/kg bw per day, the highest concentration tested (Pence et al., 1985a).

2.4 Genotoxicity

(a) In vitro studies

All in vitro genotoxicity studies considered in this monograph had been previously evaluated by the 1994 JMPR meeting. The results of the 1994 evaluation are presented in Table 5 below.

Table 5. Overview of the genotoxicity tests with tolclofos-methyl

Endpoint	Test Object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537	10–2000 µg/plate (±S9)	97	Negative	Suzuki & Miyamoto, 1978
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537; *E. coli* WP2uvrA	10–5000 µg/plate (±S9)	98.7	Negative	Moriya, Ohta & Shirasu, 1981
Reverse mutation	*B. subtilis* H17 rec^+, M45 rec^-	1–1000 µg/disc	97	Negative	Suzuki & Miyamoto, 1978
Reverse mutation	*B. subtilis* H17 rec^+, M45 rec^-	20–5000 µg/disc	98.7	Negative	Moriya, Ohta & Shirasu, 1981
Host-mediated assay	*S. typhirium* in ICR mice	870, 1750 mg/kg bw	97	Negative	Suzuki & Miyamoto, 1978
Chromosomal aberration	CHO-K1 cells	0, 10, 20, 40 µg/mL (–S9) 0, 37.5, 75, 150 µg/mL (+S9)	96.6	Negative[a]	Kogiso et al., 1990
Unscheduled DNA synthesis	Male Sprague Dawley rat hepatocytes	0.3, 1, 3, 10, 20, 40 µg/mL	96.6	Negative[b]	Hara et al., 1990
Unscheduled DNA synthesis	Human carcinoma cells (HeLa)	0.3, 3, 30, 300 µg/mL	NR	Negative	Monaco & Nunziata, 1981
Gene mutation	Chinese hamster V79 lung cells	1.5, 15, 150, 1500 µg/mL (±S9)	NR	Negative[c]	Monaco & Nunziata, 1981
In vivo					
Chromosomal aberration	Male ICR mouse bone marrow cells	0, 500, 1 000, 2000, 4000 mg/kg bw	99.8	Negative[d]	Suzuki &, Hara 1981
Dominant lethal mutation	Male Sprague Dawley rats	62.5, 208.3, 625 mg/kg	NR	Negative[e]	Brusick, 1981

NR Not recorded

[a] Positive controls mitomycin C (without metabolic activation), and cyclophosphamide (with metabolic activation) yielded expected positive result

[b] Positive control (2-acetylaminofluorene) yielded expected positive results

[c] Positive controls (methyl methanesulfonate and *N*-nitrosodimethylamine) yielded expected positive results

[d] Positive control (2-acetylaminofluorene) yielded expected positive results

[e] Positive controls (methyl methanesulfonate and urethane) yield expected positive results

(b) In vivo studies

Two of the three available in vivo genotoxicity tests with tolclofos-methyl were previously reviewed by the 1994 JMPR meeting and their results are summarized in Table 5 above. The only new in vivo genotoxicity study is summarized below.

In a range-finding study designed to identify the maximum tolerated dose for a micronucleus test in mice, groups of three ICR mice of each sex received a single dose of 0, 500, 1000, or 2000 mg/kg bw tolclofos-methyl (97.5%) via gavage. Observations for clinical signs of toxicity were conducted at 1, 3, 5, 24, and 48 h after dosing. All animals survived to the end of the study. All three males treated at the 2000 mg/kg bw dose exhibited clinical signs including decreased motor activity (3/3), ataxia and crawling posture (1/3), and piloerection (3/3). No clinical signs of toxicity were reported for females.

In the micronucleus assay, males only (five/dose per time point) were administered 0, 500, 1000, or 2000 mg/kg bw tolclofos-methyl (purity 97.5%) via gavage and their bone marrow collected at 24 hs post dosing. In the control and 2000 mg/kg bw groups bone marrow from five additional males per group was collected 48 h after treatment. A positive control group comprised of five males received an intraperitoneal injection of mitomycin C at a dose of 0.5 mg/kg bw. The frequency of micronucleated polychromatic erythrocytes and the ratio of polychromatic erythrocytes were determined: the smears were examined at 1000-fold magnification by light microscopy according to the method described by Schmidt.

Consistent with the clinical observations in the range-finding study, animals in the highest dose group exhibited decreased motor activity and piloerection (4/5 animals). However, there was no evidence of increased micronucleated polychromatic erythrocytes at any dose level or observation period. At the 24 h observation time point the frequency of micronucleated polychromatic erythrocytes at the 2000 mg/kg bw dose was 0.23% compared to 0.33% in the control group. Similarly, at 48 h after treatment the frequency of micronucleated erythrocytes at 2000 mg/kg bw was 0.17% compared to 0.23% in the concurrent control group. In contrast, the positive control group frequency of micronucleated polychromatic erythrocytes was statistically significantly increased from the concurrent control (2.73% compared to 0.33% in the control group) (Matsumoto, 2013).

Tolclofos-methyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Study 1

A three-generation study of reproductive toxicity was performed in Sprague Dawley rats fed diets containing tolclofos-methyl (purity, 97.9–98.7%) at 0, 100, 300 or 1000 ppm (equal to 0, 6.9, 20.5, and 70.6 mg/kg bw per day for F0 males, 0, 8.9, 26.2, or 90.5 mg/kg bw per day for F0 females), with two litters per generation. The F0 generation consisted of 30 rats of each sex per group. The F1a, F2a and F3a generations were sacrificed at weaning and underwent gross necropsy. Five rats of each sex in each group were selected at weaning from the F1b, F2b and F3b litters for histopathological examination, and 25 rats of each sex from each group were selected from the F1b and F2b litters to breed the following generation. A 15-week growth period was allowed before mating of the 'a' litters, and a minimal 10-day rest period was allowed between weaning of the 'a' litters and mating to produce the 'b' litters. After birth of the 'b' litter, all parental animals were necropsied.

There were no treatment-related deaths or overt signs of toxicity. Pregnancy rates were low at times in all groups, but the pattern was not related to dose. The lowest pregnancy rate (52%) was observed in the F1b generation at the middle dose. Pregnancy rates were below 70% in three of the six generations in the controls, in two generations at the low dose, in four generations at the middle dose and in one generation at the high dose. The body weights of F2a and F2b pups at 300 and 1000 ppm were lower than those of controls at the start of the growth period. However, these reductions were not dose-related, and the growth rates and body weights in other groups were comparable throughout the study. There were no treatment-related gross changes at necropsy in any group. The weights of the ovaries were increased in non-pregnant adult F2b females at 1000 ppm, but this was not associated with

histomorphological alterations. There was an increased incidence (not statistically significant) of fine cytoplasmic vacuolation of the adrenal cortex at 1000 ppm in adult F2b females that became pregnant at both matings. The aetiology and toxicological significance of this finding, given its occurrence in the controls, was unknown. There was no effect on reproductive parameters or on offspring survival and development in any litter, and there were no treatment-related gross or histopathological findings in the litters. The NOAEL for parental, reproductive, and offspring toxicity was 1000 ppm (equal to 70.6 mg/kg bw per day), the highest concentration tested (Pence et al., 1985b).

Study 2

Tolclofos-methyl (purity 97.1%) was administered to groups of ten males and ten females Sprague Dawley rats at concentrations of 0, 2500, 5000, or 10 000 ppm (equal to 0, 173, 338, or 680 mg/kg bw per day for males, 0, 178, 353, or 668 mg/kg bw per day for females) for two weeks prior to mating and continuously until termination. Parental (F0) females were sacrificed after weaning of their pups while F0 males were sacrificed after 28 days of exposure. Offspring (F1) received a diet containing tolclofos-methyl at the same concentrations as the F0 animals from the time of weaning until termination at 8 weeks of age. F0 animals were observed daily for clinical signs of toxicity or mortality and weighed at the start of treatment and weekly thereafter until termination. Body weight gains were calculated for dams on the first day of treatment, and day 0 of gestation (GD 0) and lactation. Food consumption was measured during the premating period for F0 animals. Dams were observed for signs of abnormal nursing behaviour and subjected to gross pathological examination of all organs at termination. Reproductive tract organs as well as the liver, kidneys and brain of the F0 animals were collected and weighed. However, no histopathological examination was conducted for F0 animals. Reproductive parameters, such as number of pups delivered, stillbirth, sex of pups delivered, were measured. F1 pups were examined for external abnormalities and anogenital distance measured at birth. On post-natal day (PND) 4, the litters were standardized to eight pups (four per sex); the other pups were sacrificed at this time, subjected to a gross pathological examination and their gender determined. Pups were examined for preputial separation and vaginal opening beginning on PND 35 and 28, respectively. Body weights were measured at birth and on PNDs 4, 7, 14, and 21 and weekly thereafter until termination. At termination, the brains, thymus, spleens and uteri of F1 pups were collected and weighed. In addition, the reproductive organs and tissues of F1 animals in the control and high-dose groups were examined histopathologically, including an assessment of oocyte and ovarian follicle counts and classification into primordial, growing, or antral follicles.

Statistically significant decreases (11–20%) in maternal body weights were reported at 10 000 ppm during lactation, consistent with a marked decrease in body weight gain (≥ 84%). A concomitant decrease in food consumption ranging from 18–35% was also reported for this group. No differences in estrous cyclicity or reproductive parameters were noted at any concentration. At necropsy, statistically significant increases in absolute liver weights in both sexes (17–25%) and decreases in ovarian and uterine weights (34% and 48%, respectively) were noted at 10 000 ppm. These absolute organ weight changes were also reflected in the relative organ:body weight ratios. Relative liver weights were increased (21–51%) in both sexes, while relative ovarian and uterine weights decreased by 21% and 39%, respectively. Also noted at termination was a decrease in brain cholinesterase activity at ≥ 5000 ppm in males (6–11%) and females (11–18%).

Most indications of offspring toxicity occurred at the 5000 ppm concentration and above. The exception being a decrease in spleen weights which was seen at all concentrations during the PND 21 examination. Decreases in body weight (12–46%) were seen in both sexes at concentrations ≥ 5000 ppm beginning during the lactation period and persisting until the time of sacrifice. Body weight gains were also reduced (9–50%) at these concentrations along with a decrease in food consumption (10–46% in males and 14–33% in females). There were no differences in anogenital distance, time to vaginal opening or ovarian follicle counts at any concentration. However, preputial separation was delayed by three days at 10 000 ppm. Notably, this delay was seen in conjunction with a 30% lower body weight. At the highest concentration tested, decreases in absolute thymus (47%), uterine (35%), and ovarian (21%) weights were observed in females. In males, thymus (54%), kidney (15%), seminal vesicle (27%) and epididymal (21%) absolute weights were decreased. Absolute spleen weight decrements (18–67%) were reported at all concentrations in both sexes. Consistent decreases in absolute brain weight were noted

during both the PND 21 and PND 56 examinations at concentrations ≥ 5000 ppm in males (5–16%) and females (6–21%). Decreases were noted in relative spleen weights in males at all doses (18–41%) and females at ≥ 5000 ppm (18–35%), brain weights at ≥ 5000 ppm in both sexes (12–56%), and thymus weights at 10 000 ppm in males (17%). Spleen weight decreases were noted at all dose levels in the offspring, but in the absence of any corroborative data in this or any other study in the tolclofos-methyl database to suggest that the spleen or the immune system are target organs/systems, the spleen weight decreases were not considered adverse. The parental NOAEL was 5000 ppm (equal to 338 mg/kg bw per day) on the basis of decreased body weight, ovarian, uterine, and liver weights at 10 000 ppm (equal to 680 mg/kg bw per day). The reproductive NOAEL was 10 000 ppm (equal to 680 mg/kg bw per day), the highest concentration tested. The offspring NOAEL was 2500 ppm (equal to 173 mg/kg bw per day), on the basis of decreased body weight, body weight gain, and food consumption at 5000 ppm (equal to 338 mg/kg bw per day) (Kawabe, 2005) .

(b) Developmental toxicity

Rat

Study 1

Groups of 21–26 pregnant Fischer 344 rats were administered 0, 5, 15 or 50 mg/kg bw per day tolclofos-methyl (purity, 94.9%; dose adjusted to 100%) in 0.5% w/v methylcellulose by gavage on GDs 6–15. There were no overt signs of toxicity, and body-weight gain and food consumption were unaffected by treatment. There were no treatment-related gross lesions in the dams at necropsy. Pregnancy rates were slightly higher in all treated groups (77–87%) than in controls (70%). There were no treatment-related changes in the number of fetal deaths, fetal viability or size or the incidences of visceral or skeletal anomalies or variations. The NOAEL was 50 mg/kg bw per day, the highest dose tested. It should be noted, however, that since the highest dose tested failed to elicit a response there is uncertainty as to whether the animals were adequately challenged (Pence et al., 1979).

[*This text was adopted verbatim from the 1994 JMPR monograph.*]

Study 2

Groups of 23 pregnant Sprague Dawley rats were administered 0, 100, 300 or 1000 mg/kg bw per day tolclofos-methyl (purity, 96.7%) in 0.5% w/v methylcellulose by gavage on GDs 6–15. On GD 20 all animals were sacrificed, and caesarean sections performed. Rats were observed daily for mortality and clinical signs and weighed. At termination dams were examined for gross visceral abnormalities, and their uteri weighed and examined for implantations and resorptions. Live fetuses were weighed, sexed and examined for external, visceral and skeletal abnormalities. All dams survived to the day of scheduled sacrifice with no clinical signs of toxicity.

Mean body weight gain in animals at 100 and 1000 mg/kg bw per day was significantly less than in controls on days 6–11 (–11% and –28%, respectively). Both mean body weight gain (days 6–16) and mean net body weight gain (days 0–20) in rats at 1000 mg/kg bw per day were lower (by 10 and 14%, respectively) than the corresponding control value. In addition, mean food consumption in animals at 1000 mg/kg bw per day on days 6–16 and 16–20 was slightly below the control value. There were no treatment-related differences in implantation efficiencies, and mean fetal viability, sex ratio and fetal body weight were similar in all groups. The number of fetuses with unossified fifth and/or sixth sternebrae was significantly greater in the 1000 mg/kg bw per day group than in the control group. However, the incidence of total fetal skeletal variations was similar in all groups. Other variations in development were not related to dose. Two to three malformed fetuses were found in each group, but neither the type nor the frequency of malformations indicated a teratogenic or embryotoxic response. Tolclofos-methyl was found to be neither teratogenic nor embryotoxic in this study at doses up to and including 1000 mg/kg bw per day. The NOAEL for maternal toxicity was 300 mg/kg bw per day based on decreased body weight gain at 1000 mg/kg bw per day. The embryo/fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested (Morseth, 1987).

[*This text was adopted verbatim from the 1994 JMPR monograph.*]

Rabbit

Groups of 13–17 pregnant New Zealand White rabbits were administered 0, 300, 1000 or 3000 mg/kg bw per day tolclofos-methyl (98.7% pure) in 5% w/v carboxymethylcellulose by gavage on GDs 6–18. The animals were observed daily for mortality and clinical signs and weighed. At termination, dams were observed for gross visceral abnormalities, and their uteri weighed and examined for implantations and resorptions. Live fetuses were weighed, sexed and examined for external, visceral and skeletal abnormalities.

One rabbit at 3000 mg/kg bw per day died on GD 14 of an undetermined cause. There were no overt signs of toxicity in any group. Spontaneous abortion occurred in one dam at 1000 mg/kg bw per day and in two at 3000 mg/kg bw per day on or after GD 21. Mean body weights of animals in the treated groups were significantly lower than the control values throughout the study, including day 0. Body weight gain was reduced by 76% in animals at 3000 mg/kg bw per day; at termination, weight gain (days 0–29) was 19% lower than in controls. At 1000 mg/kg bw per day, body weight gain was reduced by 56% during treatment, but body weight gain was similar to that of controls at termination. Food consumption was decreased during treatment by up to 38% in animals at 1000 and 3000 mg/kg bw per day. There were no treatment-related changes in maternal organ weights. One animal at 3000 mg/kg bw per day resorbed her entire litter. There were no other treatment-related changes in implantation efficiency, mean fetal viability, size, sex ratio, fetal body weight or external, visceral or skeletal development. Tolclofos-methyl was not teratogenic in this study at doses up to and including 3000 mg/kg bw per day, which was toxic to dams. The maternal toxicity NOAEL was 300 mg/kg bw per day on the basis of decreased body weight gain and food consumption at 1000 mg/kg bw per day. The embryo/fetal NOAEL was 3000 mg/kg bw per day, the highest dose tested (Kashima, 1991).

2.6 Special studies

(a) Neurotoxicity

Rat

Study 1

In a time-to-peak-effect study designed to elucidate the effects of tolclofos-methyl exposure on cholinesterase activity, tolclofos-methyl (purity 97.5%) was administered by gavage at a single dose of 0 or 2000 mg/kg bw in corn oil (vehicle) to groups of 20 Sprague Dawley rats per sex. For analysis of plasma, red blood cell (RBC) and whole brain cholinesterase activity, five rats/sex per dose were euthanized by carbon dioxide at approximately 1, 2, 4, and 8 h following dose administration. Blood samples were collected from the inferior vena cava, the whole brains were harvested and weighed, and plasma, RBC, and brain homogenates analyzed for cholinesterase activity.

All animals survived to the scheduled termination; no test substance-related clinical findings were noted. Cholinesterase activity (RBC, plasma, whole brain) was unaffected for males and females in the 2000 mg/kg group at approximately 1, 2, 4 and 8 h (no different from the control group across all time points observed). The absence of a consistent reduction in cholinesterase activity through the eight hours following dose administration precluded the determination of a time-of-peak-effect cholinesterase activity. The NOAEL was 2000 mg/kg bw, the highest dose tested (Beck, 2010 a, b).

Study 2

In an acute neurotoxicity study, groups of 12 Sprague Dawley rats of each sex were given a single oral dose of tolclofos-methyl (purity 97.5%) in corn oil by gavage at doses of 0, 200, 700, or 2000 mg/kg bw and observed for 14 days. Neurobehavioural assessment (functional observational battery and motor activity testing) were performed prior to the initiation of dose administration (study day –8**),** at the time of peak effect on study day 0 (approximately 4 hs after dose administration), then again on study days 7 and 14. Based on the results of the time-to-peak-effect study, cholinesterase activity was not measured. At study termination, all rats were euthanized and perfused in situ for neuropathological examination. Of the perfused animals, six animals per sex from the control group and the 2000 mg/kg group were selected randomly and subjected to histopathological evaluation of brain and peripheral nervous system tissues.

No test substance-related clinical findings were observed during the study. All animals survived to scheduled termination (study day 15). Mean body weights and body weight gains were

unaffected by treatment. In the 700 and 2000 mg/kg bw groups, mean overall motor activity (total and ambulatory counts) was decreased in males by 28–32% and females by 29–15% , respectively at the time of peak effect on study day 0, but activity scores were similar to the control group on study days 7 and 14. Total motor activity was unaffected by test substance administration at a dose level of 200 mg/kg bw (see Table 6). Notably, total motor activity changes were not dose-dependent. Decreases in mean locomotor activity (ambulatory counts only), were noted on study day 0 at doses ≥ 700 mg/kg bw in males (33–45%) and females (26–40%) (Table 7). These decreases, however, were only dose-dependent in females. There were no test substance-related macroscopic or microscopic changes. Brain weights and measurements were unaffected by administration of the test compound at any dose level. The NOAEL was 200 mg/kg bw on the basis of decreased motor activity at 700 mg/kg bw (Beck, 2010c).

Table 6. Total motor activity per test day (total activity counts per session ± standard deviation)

Tolclofos-methyl dose (mg/kg bw)	0	200	700	2000
			Males	
Pre-test	2614 ± 610	2809 ± 900	2571 ± 610	2838 ± 416
Day 0	2207 ± 520 [24%][a]	1975 ± 699	1491 ± 500** (–32%)[b]	1587 ± 484** (–28%)[b]
Day 7	2823 ± 910	2516 ± 717	2314 ± 682	2920 ± 597
Day 14	2594 ± 563	1977 ± 790	2232 ± 947	2523 ± 564
			Females	
Pre-test	2753 ± 835	2854 ± 387	2939 ± 676	2925 ± 780
Day 0	2947 ± 535 [18%][a]	2783 ± 726	2103 ± 628 (–29%)[b]	1796 ± 793 (–15%)[b]
Day 7	3419 ± 1036	3402 ± 1140	3431 ± 879	3912 ± 1130
Day 14	3452 ± 1467	3272 ± 1081	3249 ± 1302	3486 ± 1085

** $p < 0.01$

[a] Numbers in square bracket represent coefficient of variation

[b] Numbers in parenthesis represent percent change relative to concurrent negative control group

Table 7. Locomotor activity per test day (ambulatory activity counts per session ± standard deviation)

Tolclofos-methyl dose (mg/kg bw)	0	200	700	2000
			Males	
Pre-test	496 ± 140	550 ± 224	451 ± 162	503 ± 165
Day 0	440 ± 140 [32%][a]	384 ± 190	240 ± 83** (−45%)[b]	295 ± 93** (−33%)[b]
Day 7	639 ± 240	566 ± 269	489 ± 220	676 ± 212
Day 14	528 ± 157	439 ± 248	446 ± 237	607 ± 201
			Females	
Pre-test	526 ± 252	582 ± 221	627 ± 302	626 ± 177
Day 0	698 ± 249 [36%][a]	700 ± 346	518 ± 255 (–26%)[b]	421 ± 247** (–40%)[b]
Day 7	946 ± 427	924 ± 430	937 ± 298	1090 ± 393
Day 14	917 ± 497	872 ± 375	898 ± 453	933 ± 302

** $p < 0.01$

[a] Numbers in square bracket represent coefficient of variation

[b] Numbers in parenthesis represent percent change relative to concurrent negative control group

Study 3
In a subchronic neurotoxicity study, tolclofos-methyl (purity 96.8%) was administered continuously in the diet for 90 days to groups of 12 male and 12 female12 Alpk:AP:fSD (Wistar-derived) rats at dose levels of 0, 300, 1800, or 10 000 ppm (equal to 0, 20.6, 122.3, 735.7 mg/kg bw per day for males,0, 23.1, 135.8, and 762.7 mg/kg bw per day for females). Neurobehavioural assessment (functional observational battery and motor activity testing) was performed in all main study animals during week 1, and in weeks 2, 5, 9 and 14. Cholinesterase activity was measured five rats/sex per dose, in brain and RBCs at weeks 2, 5 and 9 (satellite phase) and week 14 (main study). Plasma cholinesterase was not evaluated. At study termination (14 weeks), five animals/sex per dose were subjected to neuropathological examination. Brains from all of the perfused animals were weighed and histopathological evaluation of the brain and peripheral nervous system tissues conducted.

Effects were only seen at the 10 000 ppm dose level. They included decreases in body weight (≤ 16% and ≤ 9% in males and females, respectively), body weight gain (18–113%) and food utilization (–26% in males). A statistically significant 32% decrease in the motor activity of males exposed to 10 000 ppm tolclofos-methyl was reported during the week 14 observation. RBC and brain cholinesterase activity were also decreased at this dose level. RBC cholinesterase activity was statistically significantly decreased in males (17–23%) and females (14–17%). Brain cholinesterase activity was also statistically significantly reduced in males (14%) and females (≤ 14%). It is noteworthy that while reduced RBC cholinesterase activity was consistently seen beginning at the week 5 assessment until termination, brain cholinesterase activity decreases were sporadic (only during week 5 in males and weeks 2 and 14 in females). There were no treatment-related neurobehavioural (functional observation battery), brain weight, neuropathological or microscopic findings in the tissues examined. The systemic NOAEL was 1800 ppm (equal to 122.3 mg/kg bw per day) on the basis of decreased body weight, body weight gain, and food utilization at 10 000 ppm (equal to 735.7 mg/kg bw per day). The neurotoxic NOAEL was 1800 ppm (equal to 122.3 mg/kg bw per day) on the basis of decreased motor activity at 10 000 ppm (equal to 735.7 mg/kg bw per day) (Moxon, 2007).

Hens

Groups of ten Leghorn hens were administered 0 or 8000 mg/kg bw tolclofos-methyl (purity 97%) or 500 mg/kg bw tri-*ortho*-cresyl phosphate orally in corn oil. After a 21-day observation period, a second dose of vehicle or tolclofos-methyl was administered. Animals were sacrificed 21 days after the second dose. There were no deaths in the groups given the vehicle or tolclofos-methyl. Plasma cholinesterase activity in the group treated with tolclofos-methyl was decreased by nearly 50% eight days after the first dose, but had recovered to the pretreatment level 21 days after dosing. Hens treated with tolclofos-methyl had no signs of leg weakness or paralysis and no histopathological changes in their nervous tissues. Birds treated with tri-*ortho*-cresyl phosphate had the typical clinical and histopathological signs of delayed polyneuropathy (Okuno et al., 1982).
[*This text was adopted verbatim from the 1994 JMPR monograph*.]

(b) Immunotoxicity

In a preliminary immunotoxicity study, groups of eight female CD-1 mice received diets containing tolclofos-methyl (purity 97.5%) at a concentration of 0, 100, 2000, or 4500 ppm (equal to 0, 19.6, 413, or 749 mg/kg bw per day) for four weeks. A positive control group consisting of eight female CD-1 mice was administered cyclophosphamide at a dose of 20 mg/kg bw per day via gavage for five days (days 22–26, inclusive of the study). Animals were observed twice a day for signs of toxicity and detailed physical examinations were conducted weekly. Body weights were assessed twice during the acclimation period, on the first day of treatment and twice weekly thereafter. Four days before necropsy all animals received intravenous injections of sheep red blood cells (sRBCs) until termination. At the end of the study, the animals were sacrificed and splenocytes obtained from each animal to conduct a plaque-forming assay (PFC) to assess the animals' ability to mount a T-lymphocyte-dependent antibody response (TDAR). In addition, all animals were subjected to a full necropsy including an examination of lymph nodes, Payer's patches, and thymus tissues. Blood and brain samples were also collected at termination for cholinesterase activity assessments.

Though not statistically significant, body weight decreases of 10% were noted at the 4500 ppm concentration level, beginning on study day 18. Overall body weight gain was reduced at all concentrations by 30–57% attaining statistical significance ($p < 0.01$) at ≥ 2000 ppm. Food consumption was decreased by 18% (not statistically significant) at 4500 ppm. Plasma cholinesterase activity was statistically significantly ($p < 0.01$) reduced by 31–89% at all concentrations in a dose-dependent manner. In contrast, a modest but statistically significant 7% decrease in brain cholinesterase activity was reported at 4500 ppm. With regards to the immune system, no statistically or biologically significant changes in viable cells/spleen, PFC/10^6 viable cells, or PFC/spleen were reported at any concentration. The positive control group exhibited the expected response. The systemic NOAEL was 100 ppm (equal to 19.6 mg/kg be per day) on the basis of body weight gain decreases at 2000 ppm (equal to 413 mg/kg bw per day). The immunotoxicity NOAEL was 4500 ppm (equal to 749 mg/kg bw per day), the highest concentration tested (Brown, 2010).

In an immunotoxicity study, tolclofos-methyl (purity 97.5%) was administered to groups of ten female Crl:CD-1(ICR) mice via diet at dose levels of 0, 500, 1500, or 4500 ppm (equal to 0, 91,273, or 811 mg/kg bw per day) for 28 days. The positive control consisted of eight female mice which were administered 20 mg/kg bw per day cyclophosphamide via gavage on days 22–26. On day 25, all animals were immunized with a 0.5 mL intravenous injection of sRBCs at a concentration of 4×10^8 cells/mL On day 29, all animals were sacrificed. The necropsy included the examinations of all major organs, tissues and body cavities. Spleens from all animals were harvested for evaluation of spleen anti-sRBC antibody response with a PFC assay. Other parameters evaluated were: clinical observation, mortality, body weight, body weight gain, food consumption, spleen and thymus weights.

There were no unscheduled deaths or treatment-related clinical signs. The mean final body weight (day 29) for animals receiving 4500 ppm was lower (–11.2%) than for controls. Overall mean body weight gains (days 1–29) for animals receiving 4500 ppm were lower (–40%) than in the controls. Food consumption for females receiving 4500 ppm was lower (–11%) than in controls. There was no treatment-related effect on water consumption, organ weights or macropathology. For immunotoxicity, there were no statistically significant changes in the numbers of cells/spleen, PFC/10^6 viable cells or PFC/spleen in any of the tolclofos-methyl-treated groups when compared to the control. The positive control with cyclophosphamide resulted in a significant reduction of the PFC response. The numbers of PFC/10^6 viable cells and PFC/spleen (both $p < 0.001$) were significantly reduced when compared to the control. High interindividual variability was noted in all the treatment groups as well as in the control group. Evaluation of individual animal data from this study did not show any trend or distribution that would demonstrate significant suppression of anti-SRBC plaque-forming-cell response. The positive control group demonstrated a statistical decrease in PFC response as compared to the vehicle controls. Natural killer (NK) cell activity was not evaluated in this study.

The toxicology database for tolclofos-methyl does not show any evidence of treatment-related effects on the immune system. The overall weight of evidence suggests that this chemical does not directly target the immune system. The immunotoxicity NOAEL was 4500 ppm (equal to 811 mg/kg bw per day), the highest concentration tested. The systemic NOAEL was 1500 ppm (equal to 273 mg/kg bw per day) on the basis of decreased body weight, body weight gain, and food consumption at 4500 ppm (equal to 811 mg/kg bw per day) (Brennan, 2010).

(c) Endocrine disruption

Study 1

In an in vitro study evaluating the impact of nine pesticides on human estrogen receptors α and β (hERα and hERβ) mRNA and MCF-7BUS cells (a human breast cancer cell line), were exposed to tolclofos-methyl (purity 98.8%) at concentrations of 0, 5 or 25 μM for 48 h. The concentration and duration of exposure were selected based on previous studies in which the same investigators concluded that the 5 μM and 25 μM concentrations represented the lowest- and maximum-observed-effect concentrations, respectively (LOEC and MOEC) in either the E-screen or ER transactivation assay, and that the maximum response for E2 (17β-estradiol) on ER mRNA steady state occurred after 48 h of exposure. For a positive control in this study, E2 was used at concentrations of 0.06 nM (EC_{50}[a] in ER transactivation assay) or 10 nM. Exposure to tolclofos-methyl alone did not lead to any statistically significant changes in the

[a] $EC_{10/40/50/100}$ is the molar concentration that produces 10/40/50/100% of the maximum response for a given test substance.

levels of hERα or hERβ mRNA at either concentration. However, when cells were coexposed with 0.06 nM E2, a statistically significant decrease (ca 24%) in hERα mRNA levels was noted at both the 5 and 25 nM tolclofos-methyl concentrations. In contrast, a statistically significant increase (93%) in hERβ mRNA was reported at the 5 nM tolclofos-methyl concentration but not at 25 nM. The E2 positive control elicited the expected effects for estrogen exposure (Gruenfeld & Bonefeld-Jorgensen, 2004).

Stably transfected MVLN cells (a recombinant human breast cancer cell line) and MCF-7BUS cells (transiently transfected human breast cancer cell line) were exposed to tolclofos-methyl (purity unknown) at a concentration range of 0.5–25 μM to evaluate the potential for estrogen receptor transactivation in vitro. The maximum concentration tested was selected based on evidence of cytotoxicity at concentrations > 25 μM. As a positive control E2 was used, while ICI (an estrogen antagonist) was used as a negative control. Transactivation was measured using the chemically-activated luciferase expression (CALUX) assay. A comparison of the E2 dose response for both cell lines indicated that for MVLN cells the EC_{50} is 33 pM while the EC_{100} is 150 pM (EC_{40} = ca 25 pM). For MCF-7BUS the EC_{50} is 43 pM and the EC_{100} 10 000 pM (10 nM). MVLN cells exposed to tolclofos-methyl at a concentration of 25 μM reached 80% of the luciferase activity induced at the EC_{40} for E2 and 45% of the CALUX activity at the the EC_{100} for E2. Notably, a dose–response relationship for ER transactivation was observed in the 5–25 μM concentration range. For the MCF-7BUS cells, 10 μM tolclofos-methyl reached 26% of the CALUX activity induced at the the EC_{100} for E2. Interestingly, when either of the cell lines were coexposed to tolclofos-methyl and E2 there were no increases in luciferase activity beyond that elicited by E2 alone. The tolclofos-methyl EC_{50} was determined to be 4.3 μM and 3.1 μM for the MVLN and MCF-7BUS cell lines, respectively. Based on the EC_{50} values, the difference in estrogenic activity between tolclofos-methyl and E2 is > 72 000-fold (Bonefeld-Jorgensen, Gruenfeld & Gjermandson, 2005).

Study 2

In a study conducted to evaluate the potential estrogenic activity of tolclofos-methyl (purity 99%), $BG1Luz4E_2$ cells (human ovarian carcinoma cell line stably transfected with an estrogen-inducible receptor gene) were exposed to tolclofos-methyl (concentrations not provided in publication). Estrogenic activity was measured using an estrogen-mediated chemical-activated luciferase gene expression (E-CALUX) expression assay. This publication provided little detail on the conduct of the study or the results. According to the authors, the EC_{10} for tolclofos-methyl was 7.7×10^{-5} M compared to an EC_{10} for E2 of 3.2×10^{-12}. This constitutes a > 24 million-fold difference between the activity of tolclofos-methyl and E2 (Kojima et al., 2005).

Study3

COS-7 cells (monkey kidney cell line) transiently transfected with an expression plasmid for human or mouse pregnane X receptor (hPXR or mPXR, respectively) as well as a reporter-responsive firefly luciferase plasmid, were exposed tolclofos-methyl (purity > 97%) at concentrations ranging between 1×10^{-7} and 1×10^{-5} M to investigate its potential to act as a PXR agonist. Cells were also exposed to one of two positive controls: rifampicin and pregnenolone-16α-carbonitrile (PCN) at concentrations of 10^{-8} to 10^{-5} M. Activity was assessed as the concentration required to elicit a 20% of the maximal response of the positive control and expressed as a REC_{20} (20% relative effective concentration) or an RLA (relative luciferase activity). The rifampicin REC_{20} for hPXR was estimated at 4.3×10^{-7} M (hPXR was not activated by PCN at the highest concentration tested). For mice, the PCN REC_{20} for mPXR was estimated at 5.7×10^{-8} M (mPXR was not activated by rifampicin at the highest concentration tested). The tolclofos-methyl REC_{20} was 9.8×10^{-6} M for hPXR and elicited no response in mice. This response in the hPXR suggests a ca 23-fold difference in the potential for tolclofos-methyl to activate the hPXR relative to rifampicin (Kojima et al., 2011).

(d) Phototoxicity

In a study conducted to evaluate tolclofos-methyl's potential to elicit phototoxicity, Balb/3T3 clone A31 mouse cells were exposed to tolclofos-methyl (purity 97.5%) followed by a 50 minute irradiation with a solar simulator (Xenon arc lamp) at room temperature for the +UV assay, or maintained at room temperature in a dark place (−UV assay). Cells from the −UV or +UV assay were then incubated in DMEM culture medium for an additional 20 hours and 50 minutes, before measuring for cell viability using the Neutral red assay (i.e., measuring of optical density of the Neutral red extract at 540 nm; the OD_{540} value).

In the dose-range phase of the study the tolclofos-methyl concentrations tested were 0.586, 1.17, 2.34, 4.69, 9.38, 18.8, 37.5, and 75 µg/mL. At the highest concentration tested (75 µg/mL), precipitation was observed at the beginning of testing, the beginning of irradiation, and at the end of treatment. As a result, for the definitive study the tolclofos-methyl concentrations tested were 0.293, 0.586, 1.17, 2.34, 4.69, 9.38, 18.8 and 37.5 µg/mL. A positive control (chlorpromazine hydrochloride) was also tested at concentrations ranging from 0.313 to 40 µg/mL, while 100 µL of dimethyl sulfoxide (DMSO; negative control) was tested. Since a half-maximal inhibitory concentration (IC_{50}) could not be identified at tolclofos-methyl concentrations that did not precipitate out of solution, a photo irritation factor (PIF) could not be calculated. Hence, the mean photo effect (MPE) value was used to evaluate the phototoxicity potential of tolclofos-methyl. An MPE value < 0.1 is considered to indicate a substance is nonphototoxic. The IC_{50} for the positive control (chlorpromazine hydrochloride) was 28.79 µg/mL in the absence of irradiation but 1.46 µg/mL after irradiation with a PIF of 19.9, indicating that the positive control did induce phototoxicity. In contrast, for tolclofos-methyl the MPE was –0.008 indicating that tolclofos-methyl is not phototoxic (Nakagawa, 2013).

3. Observations in humans

The results of medical surveillance of workers employed in tolclofos-methyl production have not revealed any toxic effects. The medical records of 20 workers in Japan were reviewed. All workers had been engaged continuously in packaging operations since manufacture of technical-grade tolclofos-methyl began in 1988, for an average of 4 h/day. No occupation-related problems were observed or reported. Plasma and erythrocyte cholinesterase activities were not measured (Murayama, 1991). A similar survey on the health status of workers engaged in the production of technical grade tolclofos-methyl between 2004 and 2014 revealed no health issues attributable to tolclofos-methyl exposure (Nishioka, 2015).

There are no reports of poisoning incidents and no epidemiological studies available for tolclofos-methyl.

Comments

Biochemical aspects

After oral administration [^{14}C-4-methyl]-tolclofos-methyl was rapidly absorbed from the GI tract of mice and rats and was extensively distributed throughout the body.

In mice exposed to a single dose of tolclofos-methyl at 5 mg/kg bw, 74–83% of the administered radiolabel was recovered in urine, faeces, and expired air within the first day of exposure. The major route of excretion was the urine, accounting for 69–76% of the administered dose, while faecal excretion accounted for < 6% (Mihara, Ohkawa & Miyamoto, 1980).

In a study in rats exposed to a single 5 mg/kg bw dose of [^{14}C-4-methyl]-tolclofos-methyl, absorption and elimination was rapid with radioactivity recovery reaching 83% one day after exposure. The majority of the radioactivity was recovered in the urine (> 62%) followed by faeces (> 16%). For most tissues, peak concentration was reached within two hours of administration. The oral absorption was at least 63% within 48 h. The highest concentration of radioactivity was localized to the kidney (3–5 times higher than plasma and liver). For the remainder of the tissues, radioactivity concentration was < 29% of plasma. By 72 h post exposure, < 3% of the administered dose remained in the tissues and carcass (Mihara, Ohkawa & Miyamoto, 1980; Krautter et al., 1988a, b; Esumi, 1989).

Tolclofos-methyl undergoes extensive metabolism in mammals, proceeding through a pathway involving stepwise oxidative desulfuration to an oxon and related derivatives, oxidation of the 4-methyl group to alcohols and acids, cleavage of P–O–aryl and P–O–methyl linkages, and conjugation of the resultant acid with glycine. In rats, the major metabolites were Ph-CH_3 (12%) and Ph-COOH (29%). In mice, the major metabolites were Ph-COOH (12%), Ph-CO-glycine (13% ; unique to mice) and DM-TMO-COOH (12%). An in vitro metabolism study comparing the metabolic profile of rat microsomes to human microsome preparations, indicated that the rodent and human metabolic pathways are virtually identical with only two unique minor metabolites (< 5% of radioactivity) identified in rat, but not in human microsome preparations (Nagahori, 2015).

Toxicological data

The acute median lethal dose (LD_{50}) of tolclofos-methyl was > 5000 mg/kg bw in rats (Segawa, 1978; Parcell, Crook & Gibson, 1994; Kynoch, 1985), > 3500 mg/kg bw in mice (Segawa, 1978) and > 1000 mg/kg bw in dogs (Pence et al., 1978). The dermal LD_{50} was > 5000 mg/kg bw in rats and mice (Segawa, 1978) and > 2000 mg/kg bw in rabbits (Kynoch & Parcell, 1985). The median lethal concentration (LC_{50}) for inhalation in rats was > 2.07 mg/L (Lowe, 2012). No signs of ocular irritation were noted (Matsubara, Hara & Kadota, 1978). Slight dermal irritation in rabbits was observed at 500 mg/kg bw (Ota, 2010a). There was evidence of dermal sensitization as assessed by the Magnusson and Kligman methodology (Nakamura, 2001).

In general, tolclofos-methyl exhibited relatively low toxicity. Body weight decrements, decreases in cholinesterase activity and liver weight changes were the most commonly observed effects.

In a 32 to 34-day toxicity study in rats exposed to dietary concentrations of 0, 200, 1000, 5000, or 20 000 ppm (equal to 0, 16, 79, 414, and 1635 mg/kg bw per day for males, 0, 18, 88, 452, and 1830 mg/kg bw per day for females), the NOAEL was 1000 ppm, (equal to 79 mg/kg bw per day), on the basis of increased relative kidney weights and reduced brain cholinesterase activity at 5000 ppm, (equal to 414 mg/kg bw per day) (Colley et al., 1982).

In rats exposed for 13 weeks to tolclofos-methyl at dietary concentrations of 0, 100, 1000 or 10 000 ppm (equal to 0, 6.5, 66 or 653 mg/kg bw per day for males, 0, 7.1, 71 or 696 mg/kg bw per day for females), the NOAEL was 1000 ppm (equal to 66 mg/kg bw per day), based on marginal reduction in erythrocyte cholinesterase activity and changes in clinical chemistry parameters at 10 000 ppm (equal to 653 mg/kg bw per day) (Kimura, 1990).

In a 26-week study, dogs were exposed to tolclofos-methyl at dietary concentrations of 0, 200, 600 or 2000 ppm (equal to 0, 7.4, 23 or 69 mg/kg bw per day in males, 0, 4.1, 21 or 65 mg/kg bw per day in females). The NOAEL was 600 ppm (equal to 21 mg/kg bw per day), on the basis of reduced body weight gain at 2000 ppm (equal to 65 mg/kg bw per day) (Pence et al., 1979).

In a 52-week study, dogs were exposed to tolclofos-methyl at dietary concentrations of 0, 80, 400 or 2000 ppm (equal to 0, 2.2, 11 or 59 mg/kg bw per day for males, 0, 2.6, 11.2, or 62 mg/kg bw per day for females). The NOAEL was 400 ppm (equal to 11 mg/kg bw per day), on the basis of reduced body weight gain at 2000 ppm, equal to 59 mg/kg bw per day (Cox et al., 1988; Moore, 1993).

The overall NOAEL for dogs after short-term exposure was 600 ppm (equal to 21 mg/kg bw per day), on the basis of reduced body weight gain at 2000 ppm (equal to 59 mg/kg bw per day).

In a nine-month study, mice were exposed to tolclofos-methyl at dietary concentrations of 0, 10, 30, 100 or 3000 ppm (equal to 0, 1.2, 3.8, 12 and 510 mg/kg bw per day for males, 0, 1.4, 4.1, 14 and 560 mg/kg bw per day for females), the NOAEL was 100 ppm (equal to 12 mg/kg bw per day), based on decreased body weight as well as decreased erythrocyte and brain cholinesterase activity at 3000 ppm (equal to 510 mg/kg bw per day) (Suzuki et al., 1978).

In a 104-week toxicity study, mice were exposed to tolclofos-methyl at dietary concentrations of 0, 10, 50, 250 or 1000 ppm (equal to 0, 1.3, 6.5, 32 and 134 mg/kg bw per day in males, 0, 1.3, 6.8, 34 and 137 mg/kg bw per day in females). The NOAEL was identified at 50 ppm (equal to 6.5 mg/kg bw per day), based on reduced brain and erythrocyte cholinesterase activity and an increase in kidney weights at 250 ppm (equal to 32 mg/kg bw per day; Satoh et al., 1983). The carcinogenicity NOAEL was 1000 ppm (equal to 134 mg/kg bw per day), the highest dose tested.

In rats exposed to tolclofos-methyl for 28 weeks at dietary concentrations of 0, 10, 30, 1000, 3000 or 10 000 ppm (equal to 0, 16, 51, 164 and 540 mg/kg bw per day for males, 0, 18, 65, 184, and 623 mg/kg bw/day for females), the NOAEL was 1000 ppm (equal to 51 mg/kg bw per day) on the basis of bile duct proliferation and oval cell proliferation at 3000 ppm (equal to 164 mg/kg bw per day) (Hiromori et al., 1978).

In a two-year chronic and carcinogenicity toxicity study rats were exposed to dietary concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 4.2, 12, and 42 mg/kg bw for males, 0, 4.8, 15, and 49 mg/kg bw for females) for either 122 weeks (males) or 129 weeks (females). A systemic NOAEL could not be identified due to the variability in the cholinesterase activity data. Although an increase in

the incidence of follicular cell carcinomas was noted at 1000 ppm (equal to 42 mg/kg bw per day), in the absence of any indication of thyroid toxicity at higher doses in the remainder of the tolclofos-methyl database this observation was considered to be a spurious finding, based on the weight of the evidence. The carcinogenicity NOAEL was 1000 ppm (equal to 42 mg/kg bw per day), the highest dose tested (Pence et al., 1982).

In a two-year study in rats with concentrations of 0, 100, 300, or 1000 ppm (equivalent to 0, 5, 15, or 50 mg/kg bw per day), the systemic NOAEL was 1000 ppm (equivalent to 50 mg/kg bw per day), the highest dose tested. The carcinogenic NOAEL was 1000 ppm (equivalent to 50 mg/kg bw per day), the highest dose tested (Pence et al., 1985a).

The Meeting concluded that tolclofos-methyl is not carcinogenic in rats or mice.

Tolclofos-methyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that tolclofos-methyl is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that tolclofos-methyl is unlikely to pose a carcinogenic risk to humans.

In a multigeneration toxicity study of rats exposed to dietary concentrations of 0, 100, 300, or 1000 ppm (equal to 0, 6.9, 20.5, and 70.6 mg/kg bw per day for F0 males, 0, 8.9, 26.2, and 90.5 mg/kg bw per day for F0 females) no evidence of parental, reproductive, or offspring toxicity was observed at any dose, The parental, offspring, and reproductive NOAEL was 1000 ppm (equal to 70.6 mg/kg bw per day), the highest dose tested (Pence et al., 1985b).

In a one-generation reproduction toxicity study rats were exposed to dietary concentrations of 0, 2500, 5000, or 10 000 ppm (equal to 0, 173, 338, and 680 mg/kg bw per day for males, 0, 178, 353, or 668 mg/kg bw per day for females). The parental NOAEL was 5000 ppm (equal to 338 mg/kg bw per day) on the basis of body, ovarian, uterine, and liver weight changes at 10 000 ppm (equal to 680 mg/kg bw per day). The reproductive NOAEL was 10 000 ppm (equal to 680 mg/kg bw per day), the highest dose tested. The offspring NOAEL was 2500 ppm (equal to 173 mg/kg bw per day), on the basis of decreased body weight, body weight gain, and food consumption at 5000 ppm (equal to 338 mg/kg bw per day) (Kawabe, 2005).

In a developmental toxicity study in rats, tolclofos-methyl was administered via gavage at doses of 0, 100, 300 or 1000 mg/kg bw per day from GD 6–15. The NOAEL for maternal toxicity was 300 mg/kg bw per day based on decreased body weight gain at 1000 mg/kg bw per day. The embryo/fetal NOAEL was 1000 mg/kg bw per day, the highest dose tested (Morseth, 1987).

In a developmental toxicity study, rabbits were administered 0, 300, 1000 or 3000 mg/kg bw per day tolclofos-methyl via gavage on GDs 6–18. The maternal toxicity NOAEL was 300 mg/kg bw per day on the basis of decreased body weight gain and food consumption at 1000 mg/kg bw per day. The embryo/fetal NOAEL was 3000 mg/kg bw per day, the highest dose tested (Kashima, 1991).

The Meeting concluded that tolclofos-methyl is not teratogenic.

The neurotoxic potential of tolclofos-methyl was evaluated in a series of neurotoxicity studies including a time-to-peak-effect study for cholinesterase activity, acute and subchronic neurotoxicity studies in rats, and a delayed neuropathology study in hens.

In a time-to-peak-effect study designed to investigate the effects of tolclofos-methyl exposure on cholinesterase activity, tolclofos-methyl was administered by gavage at a single dose of 0 or 2000 mg/kg bw. The NOAEL was 2000 mg/kg bw, the highest dose tested (Beck, 2010a, b). As a result of this study, cholinesterase activity was not assessed in a subsequent acute neurotoxicity study.

In an acute neurotoxicity study, rats were given a single oral dose of 0, 200, 700, or 2000 mg/kg bw tolclofos-methyl by gavage. The systemic NOAEL was 200 mg/kg bw on the basis of decreased motor activity at 700 mg/kg bw (Beck, 2010c).

In a subchronic neurotoxicity study tolclofos-methyl was administered to rats in their diet for 90 days at concentrations of 0, 300, 1800, or 10 000 ppm (equal to 0, 20.6, 122, 736 mg/kg bw per day for males, 0, 23.1, 136, and 763 mg/kg bw per day for females). The Meeting noted that at

10 000 ppm erythrocyte cholinesterase activity was slightly reduced from week five onwards, while brain cholinesterase activity was slightly, and inconsistently, reduced at certain time points only. The systemic NOAEL was 1800 ppm (equal to 122 mg/kg bw per day) on the basis of decreased body weight, body weight gain and food utilization, as well as decreases in motor activity at 10 000 ppm (equal to 736 mg/kg bw per day) (Moxon, 2007).

In a delayed neuropathy study, Leghorn hens were administered 0 or 8000 mg/kg bw tolclofos-methyl. Hens treated with tolclofos-methyl had no signs of leg weakness or paralysis and no histopathological changes to their nervous tissues.

The Meeting noted that the small decreases in cholinesterase activity recorded in several studies, particularly in mice, were never associated with the typical signs of the cholinergic syndrome. Even at high single doses, from 1500 mg/kg bw to > 3500 mg/kg bw (the LD_{50} for mice), which caused lethality, such signs were not observed.

Therefore, the Meeting concluded that the clinical observations in these studies are not indicative of specific toxicity to the nervous system but rather a generalized toxic effect, and that the slightly reduced erythrocyte and brain cholinesterase activity observed at doses above the lowest-observed-adverse-effect level (LOAEL) in repeated dose studies is likely not due to direct inhibition by tolclofos-methyl.

The immunotoxic potential of tolclofos-methyl was investigated in an immunotoxicity study with mice exposed to concentrations of 0, 500, 1500, or 4500 ppm (equal to 0, 91, 273, and 811 mg/kg bw per day) for 28 days. The immunotoxicity NOAEL was 4500 ppm (equal to 811 mg/kg bw per day), the highest concentration tested. The systemic NOAEL was 1500 ppm (equal to 273 mg/kg bw per day) on the basis of decreased body weight, body weight gain, and food consumption at 4500 ppm (equal to 811 mg/kg bw per day) (Brennan, 2010).

The Meeting concluded that tolclofos-methyl is not immunotoxic.

Four in vitro assays were conducted to evaluate tolclofos-methyl's potential impact on estrogen activity or pregnane X receptor (PXR) agonism. None of the assays suggested endocrine activity in relation to estrogen or PXR.

Toxicological data on metabolites and/or degradates

No toxicological data specific to the metabolites or degradates identified as residues in crops or livestock (goat) are available. However, all the residues identified (ph-CH_3, TMO-COOH, ph-COOH, TMO, TM-CH_2OH, DM-TM, DM-TM-CH_2OH and TMO-CH_2OH) are also major rat metabolites (> 10%). Hence, the Meeting concluded that the toxicity of these metabolites would be covered by that of tolclofos-methyl.

Microbiological data

No data are available to assess the potential impact of tolclofos-methyl exposure on the microbiome.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. There are no reports of poisoning incidents and no epidemiological studies available for tolclofos-methyl.

The Meeting concluded that the existing database on tolclofos-methyl was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting reaffirmed the ADI for tolclofos-methyl of 0–0.07 mg/kg bw based on a NOAEL of 6.5 mg/kg bw per day based on reduced erythrocyte and brain cholinesterase activity along with increased kidney weights in a two-year study of toxicity and carcinogenicity in mice.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for tolclofos-methyl in view of its low acute oral toxicity and the absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose. The Meeting noted that although brain cholinesterase activity is decreased in mice after 28 weeks of exposure, the oral LD_{50} for mice is > 3500 mg/kg bw, suggesting that acute exposure would not elicit a decrease in cholinesterase activity. Furthermore, the toxic effects reported (for example, decreased motor activity, dyspnea, irregular respiration) were not typical of a cholinergic syndrome and were only noted at doses ≥ 1500 mg/kg bw.

Levels relevant to risk assessment of tolclofos-methyl

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 6.5 mg/kg bw per day	250 ppm, equal to 32 mg/kg bw per day
		Carcinogenicity	1000 ppm, equal to 134 mg/kg bw per day[b]	-
Rat	13-week toxicity study[a]	Toxicity	1000 ppm, equal to 66 mg/kg bw per day	10 000 ppm, equal to 653 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a, c]	Toxicity	1000 ppm, equivalent to 50 mg/kg bw per day[b]	-
		Carcinogenicity	1000 ppm, equivalent to 50 mg/kg bw per day[b]	-
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1000 ppm, equal to 70.6 mg/kg bw per day[b]	-
		Parental toxicity	1000 ppm, equal to 70.6 mg/kg bw per day[b]	-
		Offspring toxicity	1000 ppm, equal to 70.6 mg/kg bw per day[b]	-
	One-generation study of reproductive toxicity[a]	Reproductive toxicity	10 000 ppm, equal to 680 mg/kg bw per day[b]	-
		Parental toxicity	5000 ppm, equal to 338 mg/kg bw per day	10 000 ppm, equal to 680 mg/kg bw per day
		Offspring toxicity	2500 ppm, equal to 173 mg/kg bw per day	5000 ppm, equal to 338 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	300 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	1000 mg/kg bw per day[b]	-
	Acute neurotoxicity study[d]	Toxicity [e]	200 mg/kg bw	700 mg/kg bw
	Subchronic neurotoxicity[a]	Toxicity[e]	1800 ppm, equal to 122 mg/kg bw per day	10 000 ppm, equal to 736 mg/kg bw per day
	Immunotoxicity study[a]	Immunotoxicity	4500 ppm, equal to 811 mg/kg bw per day[b]	
Rabbit	Developmental toxicity study[d]	Maternal toxicity	300 mg/kg bw per day	1000 mg/kg bw per day
		Embryo and fetal toxicity	3000 mg/kg bw per day[b]	

Species	Study	Effect	NOAEL	LOAEL
Dog	26-week toxicity study[a]	Toxicity	600 ppm, equal to 21 mg/kg bw per day	2000 ppm, equal to 59 mg/kg bw per day
	One-year study of toxicity[a]	Toxicity	400 ppm, equal to 11 mg/kg bw per day	2000 ppm, equal to 59 mg/kg bw per day

[a] Dietary administration

[b] Highest dose tested

[c] Three studies combined

[d] Gavage administration

[e] Generalized toxicity not associated with neurotoxicity

Acceptable daily intake (ADI), applies to tolclofos-methyl, ph-CH_3, TMO-COOH, ph-COOH, TMO, TM-CH_2OH, DM-TM, DM-TM-CH_2OH and TMO-CH_2OH, expressed as tolclofos-methyl

0–0.07 mg/kg bw

Acute reference dose (ARfD)

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological studies of human exposure.

Critical endpoints for setting guidance values for exposure to tolclofos-methyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; > 75% at 5 mg/kg bw (mouse and rat)
Dermal absorption	No data
Distribution	Extensive; highest concentration found in the kidney
Potential for accumulation	Low
Rate and extent of excretion	Rapid; largely complete within the first 24 h after dose administration
Metabolism in animals	Converted primarily to Ph-CH_3 (12%) and Ph-COOH (29%) in rats and Ph-COOH (12%), Ph-CO-glycine (13%, unique to mice), and DM-TMO-COOH (12%) in mice
Toxicologically significant compounds in animals and plants	Tolclofos-methyl
Acute toxicity	
Mouse LD_{50}, oral	≥ 3500 mg/kg bw
Rat LD_{50}, oral	> 5000 mg/kg bw
Rat LD_{50}, dermal	> 5000 mg/kg bw
Rat LC_{50}, inhalation	> 3.32 mg/L after 4 h exposure
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Slightly irritating
Guinea pig, dermal sensitization	Sensitizer (Magnusson & Kligman assay)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight gain (dog)
Lowest relevant oral NOAEL	11 mg/kg bw per day
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rabbit; highest dose tested)
Lowest relevant inhalation NOAEC	No data

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced erythrocyte and brain cholinesterase activity and increased kidney weights
Lowest relevant NOAEL	6.5 mg/kg bw per day (mouse)
Carcinogenicity	Not carcinogenic in rat or mouse[a]
Genotoxicity	Not genotoxic[a]
Reproductive toxicity	
Target/critical effect	Decreased body, thymus, kidney, brain, ovarian, uterine, seminal vesicles, epididymal, and liver weights
Lowest relevant parental NOAEL	338 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	173 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	680 mg/kg bw per day (rat; highest dose tested)
Developmental toxicity	
Target/critical effect	No embryo/fetal effects; decreased body weight gains in maternal animals (rats and rabbits)
Lowest relevant maternal NOAEL	300 mg/kg bw per day (rats and rabbits)
Lowest relevant embryo/fetal NOAEL	1000 mg/kg per day (rat; highest dose tested)
Neurotoxicity	
Acute neurotoxicity NOAEL	Not neurotoxic
Subchronic neurotoxicity NOAEL	Not neurotoxic
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	
Immunotoxicity NOAEL	811 mg/kg bw per day (rat; highest dose tested)
Human data	No poisoning incidents or adverse effects have been reported as part of the medical surveillance data collection

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety Factor
ADI	0–0.07 mg/kg bw[a]	Two-year study of toxicity and carcinogenicity (mouse)	100
ARfD	Unnecessary		

[a] Applies to tolclofos-methyl, ph-CH_3, TMO-COOH, ph-COOH, TMO, TM-CH_2OH, DM-TM, DM-TM-CH_2OH and TMO-CH_2OH, expressed as tolclofos-methyl

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Historical control data

In addition to the references cited in the text the following communications detailing laboratory historical control data were referred to in preparing this monograph.

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TRIFLUMURON

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Explanation

Triflumuron is the ISO-approved common name for 2-chloro-*N*-[[4-(trifluoromethoxy)phenyl] carbamoyl]benzamide (IUPAC), for which the Chemical Abstract Service number is 64628-44-0.

Triflumuron is a synthetic insecticide from the active ingredient group of chitin biosynthesis inhibitors (chitin inhibitors) type 0. Triflumuron acts primarily as a feeding poison for biting and sucking pests. It disturbs the chitin biosynthesis of insects, particularly in immature life stages. It is used in a wide range of crops, including apple, pear, cabbage, citrus, cotton, potato and tea. It is also used as a veterinary drug.

Triflumuron has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

The majority of the studies were conducted prior to the implementation of good laboratory practice (GLP) regulation; GLP-compliant studies are identified in the monograph. Many studies were not conducted in accordance with national or international test guidelines since at the time the studies were performed no particular guideline had been agreed. However, the Meeting considered that overall the database was adequate for the risk assessment.

A search of the open literature did not reveal any relevant information additional to that submitted by the sponsor.

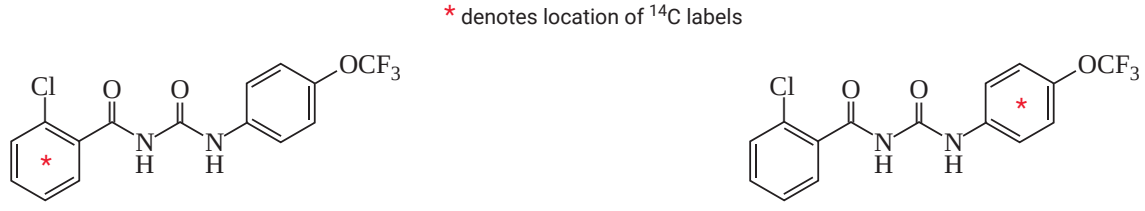
Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

In a GLP-compliant study, distribution of triflumuron was investigated in male Wistar (Hsd/Cpb:WU) rats. Six male rats received a single oral gavage dose of ^{14}C-triflumuron ([4-trifluoromethoxy-phenyl-UL-^{14}C] triflumuron; purity > 98%) in canola oil at 3 mg/kg bw, and one control animal was dosed with the non-labelled compound. After dosing, urine from all animals and faeces from three animals were collected. The animals were terminated 1, 4, 8, 24, 48 and 72 h after dosing. The control animal was terminated 4 h after dosing. Liquid samples were analysed by liquid scintillation counting. All solid samples were weighed and combusted in an oxygen atmosphere using the "Oxidiser 307/387" (Packard Instruments). For whole body autoradiography, the animals were embedded in carboxymethylcellulose (CMC), sectioned and scanned by image analyser. The radioactivity in organs and structures was quantified by radiolumography.

Figure 1. Radiolabelling positions of triflumuron



[2-chlorophenyl-UL-^{14}C] triflumuron

[4-trifluoromethoxy-phenyl-UL-^{14}C] triflumuron

The most intense blackening, with large overexposed regions, reflecting the concentration of radioactivity, was found in the contents of stomach, small and large intestines, 1 h after oral administration. Increased concentrations compared with those in the blood were also detected in the liver, kidney, adrenals, infraorbital gland, brain and spinal cord, as well as in all fatty tissues (brown fat, perirenal, intestinal, epididymal and subcutaneous fat). The concentration in fatty tissues increased at 24 h, but the highest concentration of radioactivity was still detected in the intestine. Up to 72 h after dosing, the radioactivity

declined but the distribution pattern did not change, so higher concentrations were still found in the large intestine followed by fatty tissues, blood, liver, small intestine, kidney and nasal mucosa. For most organs and tissues, maximum concentrations were detected around 4–8 h post administration. With the exception of blood, a steady decline was observed thereafter. In blood, the values increased moderately up to 0.22 µg/g until 72 h after dosing, which might be an indication of possible binding of the parent compound and/or metabolites to blood components. Maximum concentrations in fatty tissues were detected at 8 h and 24 h (0.09–3.74 µg/g). Concentrations remained similar or slightly increased in the kidney and liver up to 72 h indicating that metabolism (liver) and excretion (kidneys) was continuing at that time point. Renal excretion via urine started 8–24 h after dosing and reached only 13.5% of the administered radioactivity after 72 h. This poor renal excretion rate reflects the low concentrations found in the renal medulla and cortex with maximum values of 0.43 µg/g and 0.27 µg/g, respectively. Most of the radioactivity was excreted via the faeces. Equivalent concentrations in the glandular organs like adrenals, thyroid, or testes at maximum concentration (C_{max}) were not higher than 1 µg/g. In these and all other organs and tissues, the values dropped by several orders of magnitude during the whole test period until 72 h post dosing. There was no sign of significant accumulation. At this time, the ratio of the equivalent concentrations in the organs and tissues compared to blood (= 1.0) were in the range 0.02 to 0.69.

Orally administered triflumuron was rapidly absorbed, distributed and excreted predominantly in faeces. There was no sign of significant accumulation triflumuron in the body (Koester, 2002).

In a GLP-compliant study, radiolabelled triflumuron ([4-trifluoromethoxy]aniline-UL-^{14}C; purity 99%) was administered to groups of male Wistar-Hanover rats (four per dose) as a single gavage dose at 1.98 mg/kg bw (low-dose male) or 317.7 mg/kg bw (high-dose male). A single oral dose of 3.93 mg/kg bw was administered to four female Wistar-Hanover rats (low-dose female). Another group of four male Wistar-Hanover rats received unlabelled triflumuron at 3.74 mg/kg bw per day for 14 days by gavage followed by a single oral dose of [^{14}C]-triflumuron at 3.74 mg/kg bw (multiple low-dose). Another group of four male bile duct-cannulated Wistar-Hanover rats were dosed with [^{14}C]-triflumuron at 2.59 mg/kg bw and four jugular-cannulated male Wistar-Hanover rats were dosed orally at a rate of 1.98 mg/kg bw and blood collected via a cannula in the jugular vein to determine plasma concentrations as a function of time after dosing. Urine and faeces were collected at various time points. Bile samples were collected at 1, 2, 3, 4, 6, 8, 12, 24, 48 and 72 hours post treatment. In the plasma curve experiment, whole blood samples were collected at 5, 10, 20, and 40 minutes and 1, 1.5, 2, 4, 6, 8, 12, 24, 32, 48 and 72 hours post treatment.

The total average radioactivity recovered ranged from 95% to 105% of the administered dose. Excreta and tissue samples were not collected in the plasma curve experiment. In the low-dose male, low-dose female and mutiple low-dose male rats, blood and residual carcass each contained 2% to 3% of the administered dose and all other tissues contained < 1% of the dose (Table 1). In the high dose male rats, all tissues contained < 1% of the administered dose; the lower percent is possibly due to the absorption of triflumuron reaching a plateau at some dose below the high dose rate. In the bile-cannulated male rats, the residual carcass, including blood, contained 4% of the administered dose. The total radioactivity in trapped expired air over 48 h was < 1% of the administered dose in low-dose male rats.

Table 1. Excretion of [4-trifluoromethoxy-phenyl-UL-^{14}C] triflumuron by rats

		Average radioactive recovery (% of dose)				
Matrix	**Time point (h)**	**Single low-dose; Male**	**Single low-dose; Female**	**Single high-dose; Male**	**Multiple low-dose; Male**	**Bile duct-cannulated; Male**
Urine	6	< 1	2	< 1	4	3
	12	21	8	1	30	8
	24	16	12	2	16	13
	48	11	10	5	10	7
	72	3	4	< 1	4	1
	96	-	1	-	-	-
	120	-	1	-	-	-
	144	-	1	-	-	-
	168	-	< 1	-	-	-
	Total	51	39	8	64	32
Faeces	24	35	23	69	24	18
	48	8	19	21	9	4
	72	2	5	2	3	1
	96	-	3	-	-	-
	120	-	1	-	-	-
	144	-	< 1	-	-	-
	168	-	< 1	-	-	-
	Total	45	51	92	36	23
Bile	1	-	-	-	-	1
	2	-	-	-	-	1
	3	-	-	-	-	1
	4	-	-	-	-	2
	6	-	-	-	-	3
	8	-	-	-	-	3
	12	-	-	-	-	6
	24	-	-	-	-	14
	48	-	-	-	-	9
	72	-	-	-	-	1
	Total	-	-	-	-	41

- No sample collected

Source: Krolski & Nguyen, 2003

In the low-dose male rats approximately 37% of the administered dose was excreted via urine and 35% via the faeces during the first 24 h, and after 72 h, a total of 96% of the dose had been excreted. For female rats at the low dose, an average of 22% and 23% of the administered dose was excreted during the first 24 h via urine and faeces, respectively, and at termination after 168 h, a total of 90% of the dose had been excreted. Following a single high oral dose to male rats, an average of only 3% of the administered dose was excreted via urine whereas 69% was excreted via the faeces during the first 24 h, and after 72 h, a total of 100% of the dose had been excreted. Following multiple low oral doses to male rats, an average of 50% of the administered dose was excreted via urine and 24% was excreted via the faeces during the first 24 h, and after 72 h 100% of the dose had been excreted. In bile duct-cannulated male rats, following a single oral dose, 31%, 24% and 18% of the administered dose was excreted

during the first 24 h via bile, urine and faeces, respectively, and at termination after 72 h a total of 96% of the dose had been excreted. The residues found in the carcass accounted for 1% to 5% of the total administered dose.

In the low-dose male rats, the average concentration of radioactivity was highest in the blood (1.071 ppm), spleen (0.316 ppm), fat (0.254 ppm), and lung (0.219 ppm). The average total radioactivity residue (TRR) in the remaining tissues and carcass ranged from 0.016 ppm to 0.183 ppm. In the low dose female rats the concentration of radioactivity was highest in the blood (2.344 ppm), spleen (0.725 ppm)and lung (0.545 ppm). In the high-dose male rats, the average TRRs in the remaining tissues and carcass ranged from 0.013 ppm to 0.359 ppm. The concentration of radioactivity was highest in the blood (43.44 ppm), spleen (11.92 ppm), fat (8.48 ppm), and lung (7.53 ppm). The average TRRs in the remaining tissues and carcass ranged from 0.57 ppm to 5.33 ppm. In the multiple low-dose male rats, the concentration of radioactivity was highest in the blood (3.08 ppm), spleen (1.04 ppm), fat (0.64 ppm), and lung (0.64 ppm). The average TRRs in the remaining tissues and carcass ranged from 0.05 ppm to 0.51 ppm. In the male rats from the plasma kinetic study (given 1.98 mg/kg bw), the concentration in whole blood rose to 1.508 ppm at 48 h post treatment before beginning to decline. The plasma concentration, however, was only 0.112 ppm by 12 h post treatment and had decreased to 0.018 ppm at 72 h after dosing (Krolski & Nguyen, 2003).

In a study that was not GLP-compliant, absorption, distribution, excretion and metabolism was investigated in rats using [chlorophenyl-UL-^{14}C]-triflumuron and [(trifluoromethoxy)aniline-UL-^{14}C]-triflumuron. A preliminary study was conducted following administration of a single oral dose of [2-chlorophenyl-UL-^{14}C]-triflumuron or [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron at 5 mg/kg bw to male and female Wistar rats (2/sex per dose) to test whether a significant amount of the dose was excreted in expired air. In the main study, [2-chlorophenyl-UL-^{14}C]-triflumuron was administered to Wistar rats (four males and four females at each dose level) as a single oral low dose (Group B, 5 mg/kg bw), a single oral high dose (Group D, 500 mg/kg bw) or a single radiolabelled dose following pre-treatment with non-radiolabelled triflumuron (Group C, 20 days × 5 mg/kg bw). Another group of three male and four female rats was administered a single oral low dose of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron (Group B′, 5 mg/kg bw). Urine and faeces from these rats were collected at 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after dosing. Animals were terminated at 168 h post treatment, and bone, brain, gonads, blood, heart, liver, kidney, muscle, spleen, lung, fat and carcass were collected for radioassay.

In the preliminary study, the highest levels of radiolabel in the blood were present during the first hour after dosing (1.42 ppm). These levels decreased rapidly to an average of 0.23 ppm after two hours. A minor secondary peak was observed at 48 h after dosing (0.35 ppm). For both the chlorophenyl label and the trifluoromethoxyaniline label, radioalabel was excreted in significant amounts via both urine and faeces. There were no obvious differences in the excretion patterns between male and female rats. No radiolabel was found as carbon dioxide expired by these rats. A significant amount of radiolabel (10 to 33%) was found remaining in the carcass four days after dosing. The levels were found to be highest in liver, kidney, gonads and fat ranging from 0.1 ppm to 7.7 ppm.

Urinary excretion of radiolabel was essentially complete by 96 h after dosing. As shown in Table 2, Group B rats (low-dose, chlorophenyl label) excreted an average of 23% of the radiolabel in urine. In Group C (multiple low-dose, chlorophenyl label) the amount of radiolabel found in the urine increased to 39%. Group D (high-dose, chlorophenyl label) excreted less radiolabel in the urine, (15%) probably because a lower percentage of the high-dose was absorbed. Group B′ rats (low-dose, trifluoromethoxyaniline label) excreted an average of 41% of the radiolabel in urine.

Faecal excretion was more rapid than urinary excretion as it was substantially complete within 48 h following treatment. Rats in Group B (low-dose, chlorophenyl label) excreted an average of 77% of the radiolabel in faeces. Group C (multiple low-dose, chlorophenyl label) excreted somewhat less (only 60%) in the faeces. In Group D (high-dose, chlorophenyl label), 85% was excreted in the faeces. Group B′ (low-dose, trifluoromethoxyaniline label) excreted 56% in the faeces. There was no indication in any of the groups tested of differences in excretion patterns between male and female rats.

Table 2. Distribution of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron and [2-chlorophenyl-UL-^{14}C]-triflumuron in rats following oral dosing

	Average radioactive recovery (% of the administered dose)							
	^{14}C-Trifluoromethoxy phenyl label (Group B′)		^{14}C-Chlorophenyl label (Group B)		^{14}C-Chlorophenyl label (Group D)		^{14}C-Chlorophenyl label (Group C)	
Matrix	Single low-dose male	Single low-dose female	Single low-dose male	Single low-dose female	Single high-dose male	Single high-dose female	Multiple low-dose male	Multiple low-dose female
Urine	45.7	36.7	25.1	20.2	14.8	14.5	40.0	37.7
Faeces	51.3	59.8	74.3	79.2	85.0	85.2	59.4	61.4
Carcass	3.0	3.5	0.3	0.2	0.3	0.3	0.5	0.5
Cage wash	-	-	0.4	0.4	-	-	0.2	0.4
Total	100.0	100	100	100	100	100	100	100
Recovery of radiolabel (%)	96.3	107.7	86.9	116.5	102.6	103.1	102.6	103.1
Mean recovery for whole Group	102.8		101.7		96.4		102.8	

- No sample collected

Source: Sietsema, 1985

Tissue residues were lowest in Group B, ranging from 0.004 ppm (bone) to 0.06 ppm (kidney) (Table 3). Tissue residues were similar in Group C, ranging from 0.004 ppm (bone) to 0.100 ppm (kidney and fat). Group D residues were highest, ranging from 0.30 ppm (carcass) to 2.62 ppm (kidney), but were not proportionately higher than Groups B or C considering the 100 times higher dose level. Group B′ residues were some 2–20 times higher than the Group B residues, ranging from 0.03 ppm (carcass) to 2.39 ppm (blood). The proportion of the residue in each tissue was approximately the same in each group, the general trend being kidney > liver > fat > spleen > lung > heart > gonads > brain > bone. Blood was an exception, in that residues from Group B′ were much higher (2.4 ppm) than would have been predicted based on the tissue residues in this group.

Table 3. Distribution of radioactive residues in rat tissues seven days after administration of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron or [2-chlorophenyl-UL-^{14}C]-triflumuron

	Average concentration of radioactive residue in tissues (mg/kg)											
	^{14}C-Trifluoromethoxy phenyl label (Group B′)			^{14}C-Chlorophenyl label (Group B)			^{14}C-Chlorophenyl label (Group D)			^{14}C-Chlorophenyl label (Group C)		
	Single low dose			Single low dose			Single high dose			Multiple low dose		
Matrix	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F
Bone	0.061	0.054	0.057	0.003	0.004	0.004	0.255	0.436	0.346	0.003	0.006	0.004
Brain	0.038	0.035	0.037	0.004	0.004	0.004	0.262	0.379	0.397	0.003	0.006	0.005
Muscle	0.021	0.046	0.035	0.005	0.008	0.006	0.323	0.357	0.397	0.007	0.010	0.009
Lung	0.339	0.451	0.403	0.015	0.014	0.014	0.523	0.702	0.614	0.011	0.019	0.015
Heart	0.211	0.275	0.248	0.011	0.013	0.012	0.552	0.433	0.562	0.011	0.017	0.014
Spleen	0.437	0.621	0.542	0.095	0.015	0.012	0.397	0.884	0.640	0.012	0.029	0.021
Gonads	0.049	0.080	0.067	0.004	0.014	0.009	0.278	0.705	0.491	0.029	0.018	0.023
Kidney	0.201	0.230	0.217	0.042	0.077	0.060	1.509	3.012	2.262	0.061	0.140	0.100
Liver	0.165	0.205	0.188	0.035	0.053	0.004	1.104	1.844	1.474	0.058	0.077	0.067
Fat	0.174	0.119	0.142	0.053	0.064	0.058	1.097	2.239	1.668	0.089	0.111	0.100
Blood	2.388	2.397	2.393	0.019	0.018	0.019	0.520	0.779	0.649	0.016	0.029	0.002
Carcass	0.034	0.031	0.032	0.007	0.006	0.007	0.258	0.337	0.298	0.015	0.011	0.013

Source: Sietsema, 1985

In summary, orally administered triflumaron was poorly absorbed and excreted rapidly in four days. Faeces contained almost exclusively intact triflumuron. Urine, on the other hand, contained no parent compound. There was no indication of volatile components. Tissue residues ranged from 0.004 ppm to 0.06 ppm at seven days after dosing with 5 mg/kg bw, being highest in kidney > liver > fat > spleen> lung > heart > gonads > muscle > brain > bone. After dosing at 500 mg/kg bw (Group D), tissue residues ranged from 1 to 2.3 ppm in the same order. The low residue concentrations in tissues and rapid excretion leads to the conclusion that no accumulation of triflumeron occurs in the rat (Sietsema, 1985).

In a study, not GLP-compliant, disposition of triflumuron was evaluated in male Sprague Dawley rats. A single oral gavage dose of [2-chlorophenyl-UL-^{14}C]-triflumuron at 5 mg/kg bw was administered to three male rats. Urine was collected at intervals of 0–8 h and 8–24 h, and faeces collected over 0–24 h. Rats were terminated after 8, 24 or 48 h.

Based on total radioactivity eliminated within 24 h, approximately 53% was found in urine and 47% was recovered from faeces. The metabolites in urine were assigned to 2-chlorobenzoic acid (M02, approximately 6%) and 2-chlorohippuric acid (M03, approximately 37%). The remaining 10% of the renally eliminated radioactivity was distributed among several mostly high polar metabolites.

In faeces, chloroform extracts contained approximately 28% of the eliminated radioactivity, of which 24% was parent triflumuron. None of the unassigned faecal metabolites contained more than 2% of the excreted radioactivity. The radioactivity that was non-extractable with chloroform (approximately 19%) was not further investigated.

The tentative results of the study led to the conclusion that the administered radioactivity was mainly excreted via urine and faeces in near equal amounts (Ecker & Karl, 1983).

In a study, not GLP-compliant, triflumuron uniformly labelled with ^{14}C in the 2-chlorbenzoyl moiety was administered once, orally to male rats (strain not specified) at dose levels of 5, 2, and 0.2 mg/kg bw, intraduodenally at a dose level of 0.2 mg/kg bw, and intravenously at a dose level of 2 mg/kg bw. Additionally, female rats were dosed orally at a dose level of 2 mg/kg bw. Data on absorption, distribution and excretion were obtained directly from whole body autoradiographic assays and from time-dependent determinations of radioactivity in the excreta, body fluids as well as from tissues and organs. A group size of typically five rats was used per experiment and per investigation time point.

The pharmacokinetic parameters following oral and intravenous dose are shown in Table 4. After intravenous administration, radioactivity was rapidly distributed in five minutes. An apparent distribution volume of V_c = 0.48 L/kg, and a dose-normalized area under the curve (AUC) of 7.1 h were obtained from the analysis of the plasma concentration curve. Following oral administration of 2 mg/kg bw in males formulated as a micellar solution in Cremophor suspension, absorption commenced immediately after administration, but continued slowly. In males distribution half-life $t_{½a} \approx 3$ h, that is ca 95% of the total quantity absorbed had been taken up from the gastrointestinal tract after 12 h. The maximum plasma concentration (P_{max} = 0.24) was reached after 4.9 hours. Elimination was biphasic with half-lives of 3 h and 13 h. The dose-normalized AUC was found to be 3.8 h. After administration of the same dose (2 mg/kg bw) in tragacanth suspension to male rats, the absorption phase was 3.7 h. The time of the maximum plasma level (P_{max} = 0.23) was found to be 5.6 h after administration. The AUC, calculated to be 4.0 h, was somewhat higher than the value obtained for the male reference group. In females at 2 mg/kg bw, the absorption half-life was very short at 1.3 h.

Table 4. Pharmacokinetic parameters derived from plasma curve analysis following administration of ^{14}C-triflumuron to rats

Dose		Intravenous	Orally administered		
		2.0 mg/kg bw Males	2.0 mg/kg bw Males	2.0 mg/kg bw Females	2.0 mg/kg bw in tragacanth Males
Absorption half-life, $t_{½a}$ (h)		-	3.0	1.3	3.7
Distribution/elimination half-lives (h)	phase 1: $t_{½\lambda1}$	0.081	3.0	13	3.7
	phase 2: $t_{½\lambda2}$	7.5	13	-	31
AUC (h)		7.1	3.8	4.7	4.0
Distribution volume, V_c (L/kg)		0.48	-	-	-
Lag time of absorption, t_{lag} (h)		-	0	0.05	0.17
Total clearance, *CL* (mL/minute)		0.47	0.86	0.70	0.82
Renal clearance, CL_R (mL/minute)		0.25	0.48	0.35	0.53
Maximum relative plasma concentration, P_{max}		-	0.24	0.19	0.23
Time at which P_{max} occurs, t_{max} (h)		-	4.9	5.0	5.6
Mean hold time, <*T*> (h)		11	15	21	19
Volume of distribution, V_{SS} (L/kg)		1.5	3.8	4.4	4.5

AUC Total area under the plasma radioactivity curve normalized for the dose and extrapolated from time zero to infinity

t_{lag} Lag time of absorption; the delay in time between administration and the onset of absorption

CL Total clearance of plasma radioactivity assuming a complete absorption process

CL_R Renal clearance of radioactivity

P_{max} Maximum relative concentration observed in plasma following extravascular administration

V_{SS} Apparent volume of distribution at steady state based on radioactivity concentration in plasma

Source: Klein, Weber & Suwelack, 1983

The radioactivity levels (*P*, that is, normalized to the administered dose) in the individual tissues and organs of male rats were investigated during the period from one hour to ten days following oral administration of 5 mg/kg in tragacanth suspension. The maximum amount of radioactivity (73% of dose) was found in the animals excluding radioactivity in the gastrointestinal tract (GIT) 4 h after oral administration of ^{14}C-triflumuron. This value decreased to 15% of the dose after 24 h and to 0.2% of the dose by day 10. About 36% of the administered radioactivity was detected in the GIT, including its contents, after 4 h. This value dropped to 0.01% after 10 days. The tissue distribution after oral administration was determined in male rats during the period from 1 h to 10 days after dosing, and yielded maximum relative concentrations in all organs tested (except for the liver and whole body) 4 h after dosing at 5 mg/kg bw as a tragacanth suspension. At this time, markedly increased levels were found in fat ($P = 6.34$), the adrenal gland ($P = 1.67$) as well as in the kidney (excretory organ; $P = 2.1$) and the liver (metabolizing organ; $P = 1.2$). After 4 h, the maximum for the whole animal body (excluding GIT) was $P = 0.77$, which was considered a representative value for the sum of all tissues and organs. The maximum relative concentrations in the skin, liver, kidney, and adrenal gland were about three times higher than this value, the relative concentration in the fat after 4 h being eight times higher than in the animal, excluding the GIT. The erythrocyte concentration dropped very slowly after its maximum at 4 h ($P = 0.22$), down to $P = 0.013$ ten days after administration. This value was six times higher than that obtained for the whole body minus GIT. The changes in the relative concentrations of the tissues were characterized by an absorption phase lasting for up to four hours after administration, and by a subsequent rapid elimination lasting until ca 72 h. Thereafter a slow terminal elimination phase followed for up to 10 days after administration. Erythrocytes had a notably long elimination half-life (ca 17 days) associated with a relatively high radioactivity level at the end of the investigation. Half-life in spleen and heart was also relatively long (212 h and 125 h respectively), but at a markedly lower

concentration level with a mean $P_{10d} = 0.0033$. The large AUC in the case of fatty tissue (122 h) was mainly caused by the transiently increased enrichment. Five minutes after intravenous administration of 2 mg/kg bw, radioactivity was found in numerous organs with the highest levels found in the liver, kidney, adrenal cortex, and the mucous of the stomach. Four hours after oral administration of 5 mg/kg bw, a different relative radioactivity distribution pattern was seen; the highest concentrations were found in the GIT, urinary bladder and Cowper's gland. After 24 h concentrations of radioactivity were reduced with the same distribution pattern, except for the fatty tissue where very high concentrations were detected. High concentrations were also found in the liver and in the kidney. After 48 h radioactivity had decreased further without a change in the distribution pattern compared to the 24 h pattern. The increased concentrations found in the liver and in the GIT were considered indicative of a long lasting enterohepatic recirculation.

Elimination via expired air was negligible, amounting to less than 0.5% in male rats after oral dosing at 5 mg/kg bw, demonstrating that the chlorophenyl labelling position was stable with regard to the formation of volatile fragments. On average, 95% of the recovered radioactivity was excreted in urine and faeces. Renal excretion amounted to 49–61% and excretion via faeces was between 36% and 43% of the recovered radioactivity for male or female animals, regardless of dose, route of administration or type of administered formulation (Table 5). Overall, excretion did not reveal a sex-related dependency. The mean renal/faecal excretion quotient for all experiments was approximately 1.4.

Table 5. Excretion and distribution of total radioactivity and radioactively labelled residues under different experimental conditions

Dose (mg/kg bw)	Number and sex of animals	Excreted activity (% of dose)				Residual activity (% of dose)		F_B	End of experiment (h)
		Urine	Faeces	Bile	CO_2	Body excluding GIT	GIT		
5.0 p.o.	4 M	54.4	38.5	–	< 0.5	1.49	0.65	1.053	48
2.0 i.v.	5 M	51.9	42.2	–	–	2.70	1.34	1.019	48
2.0 p.o.	5 M	54.0	39.9	–	–	2.00	0.89	1.034	48
2.0 p.o.	5 F	46.2	37.8	–	–	8.42	2.42	1.054	48
2.0 p.o.	5 M	59.3	34.7	–	–	1.11	0.65	1.034	48
0.2 p.o.	5 M	57.4	40.3	–	–	2.91	1.21	0.982	48
0.2 i.d.	5 M	43.2	3.55	52.6	–	1.69	0.24	0.987	48

p.o. Orally i.v. Intravenously i.d. Intraduodenally Source: Klein, Weber & Suwelack, 1983

F_B (factor of balance) = 100% of radioactivity recovered

In fall study types, at 48 h after administration between 1.6% and 8.9% of the radioactivity was present in the body minus GIT. In male rats 2.8% of the radioactivity was recovered at 48 h after intravenous administration, which was slightly higher than that after oral dosing of male rats at 2.0 mg/kg bw (2.1%). However, residues in the body (minus GIT) were four-fold higher (8.9%) in females compared to males dosed at the same level. The residual radioactivity in male animals dosed orally at 0.2 mg/kg body weight was 2.9% of the balanced radioactivity, which was significantly higher than that obtained for the males treated at the higher dose level of 2.0 mg/ kg bw.

Renal excretion was relatively slow after oral administration. Only 50% was excreted after 12 h, whereas 90% was excreted after 24 h. In female rats, excretion was even slower after oral administration of 2 mg/kg bw, where 50% of the total radioactivity was only excreted after 17 h, and about 90% after 32 h. After 48 h excretion was not yet totally complete.

Bile duct-cannulated rats excreted 52.6% of the total administered radioactivity in the bile fluid within 48 h of intraduodenal administration. About 43% was excreted in the urine and about 4% in the faeces. A comparison of the amounts of radioactivity excreted via urine and faeces by the intact animals and by animals with bile fistulas showed substantial enterohepatic recirculation of the active ingredient and/or its radioactive metabolites (Klein, Weber & Suwelack, 1983).

In the just discussed [2-chlorophenyl-UL-^{14}C]-triflumuron had a relatively long half-life in erythrocytes of about 17 days. In the light of this finding, a study, not GLP-compliant, was conducted to investigate:

- characterization of the erythrocyte-binding compounds,
- identification of the binding site of radioactivity within the erythrocytes,
- binding of triflumuron and its metabolites formed in vitro, to human erythrocytes in vitro.

Following application of a single oral dose of 5 mg/kg bw [2-chlorophenyl-UL-^{14}C]-triflumuron, most of the erythrocyte-bound radioactivity was found in the globin fraction (76.2%) six days post application. Only a very small portion of the erythrocyte radioactivity was measured in the haem fraction (1.9%). The extractable portion exhibited the same behaviour when analysed by thin layer chromatography (TLC) as 2-chlorobenzoic acid (M02) which is the principal renal metabolite. Incubation of this metabolite, which was synthesized in vitro, in the 9000 × *g* supernatant of a rat liver homogenate, with rat or human whole blood, showed that the metabolite failed to bind to globin (as observed under in vivo conditions). However, another metabolite with somewhat less polar behaviour bound to the globin of both human and rat erythrocytes; confirmation of binding of this metabolite to globin was not however obtained when the compound was administered orally to rats (Klein, 1984).

1.2 Biotransformation

The urine, faeces and bile samples collected from the Krolski & Nguyen study (2003) were subjected to metabolic profiling. A total of 17 components were detected in urine from the various dose groups (Table 6). Triflumuron was present at low levels (1–2% of dose) in urine. The main metabolites in urine were identified as 2-hydroxy-4-(trifluoromethoxy)aniline (M09) and 3-hydroxy-4-(trifluoromethoxy)aniline (M10) and their sulfate conjugates (M16 and M17, respectively). Trifluoromethoxyoxalanilide (M19) and SIR 8514-3-hydroxyaniline (M06) were identified as minor metabolites only.

Table 6. Distribution of metabolites in urine following administration of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron to rats

Peak	Triflumuron metabolite ID	Single low-dose female	Single high-dose male	Multiple low-dose male	Bile duct-cannulated male
U1		2	< 1	< 1	< 1
U2		2	< 1	1	< 1
U3		1	< 1	1	< 1
U4		2	< 1	< 1	1
U5		< 1	< 1	1	1
U6		1	< 1	< 1	ND
U7		< 1	< 1	< 1	< 1
U8	Sulfate conjugates[a] (M16, M17)	14	3	28	15
U9	4-Trifluoromethoxy-oxalanilide (M19)	1	< 1	4	< 1
U10		ND	ND	< 1	ND
U11	Phenols[b] (M09, M10)	14	3	22	10
U12		ND	ND	< 1	3
U13		ND	ND	< 1	ND
U14		ND	ND	< 1	ND
U15		ND	ND	< 1	< 1

(Table 6 continues on next page)

Peak	Triflumuron metabolite ID	Single low-dose female	Single high-dose male	Multiple low-dose male	Bile duct-cannulated male
U16	Triflumuron	< 1	< 1	1	2
U17	SIR 8514-3-hydroxyaniline (M06)	< 1	< 1	ND	< 1
Total percent dose in urine		39[c]	11[c]	62[c]	36[c]
Total percent dose identified		29	6	55	27

ND Component was not detected in this experiment

[a] Peak a mixture of 2-hydroxy-4-(trifluoromethoxy)aniline sulfate (M16) + 3-hydroxy-4-(trifluoromethoxy)aniline sulfate (M17)

[b] Peak was a mixture of 2-hydroxy-4-(trifluoromethoxy)aniline (M09) + 3-hydroxy-4-(trifluoromethoxy)aniline (M10)

[c] All values < 1 were assigned to a value of 0.05% for determining the total dose in urine

Source: Sietsema, 1985

A total of five components were observed in faecal extracts with the majority of the residue (19– 91% of the administered dose) being unchanged triflumuron (Table 7). Faeces peak F2, accounting for 2% of the dose in the low-dose males and 5% of the dose in the low-dose females, was identified as the *O*-glucuronide of hydroxy-4-trifluoromethoxy-aniline (M18).

Table 7. Distribution of metabolites in faeces following administration of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron to rats

Peak	Triflumuron metabolite ID	Radioactive recovery (% of dose)				
		Single low-dose male	Single low-dose female	Single high-dose male	Multiple low-dose male	Bile duct-cannulated male
F1		ND	2	ND	ND	ND
F2	*O*-glucuronide of hydroxy-4-(trifluoromethoxy)aniline (M18)	2	5	ND	ND	ND
F3		ND	2	ND	ND	ND
F4		ND	2	ND	ND	ND
F5	Triflumuron	30	20	91	19	20
Total percent dose in faeces extract		32	31	91	19	20
Total percent dose identified		32	25	91	19	20

ND Component was not detected in this experiment

Source: Sietsema, 1985

A total of 26 components were observed in bile from the various time points. Since the largest portion of the dose was found in the 24 h bile sample (14% on average), individual components were isolated from composite 24 h bile for identification. Triflumuron was present at low levels (< 1% of dose) in bile. Metabolites identified were 4-trifluoromethoxyphenyl urea (M08), SIR 8514-3-hydroxyaniline (M06), SIR 8514-2-hydroxyaniline (M26; an isomer of M06) and the conjugates SIR 8514-hydroxy glucuronide (M28) and SIR 8514-cysteine aniline (M27).

Table 8. Distribution of metabolites in bile following administration of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron to bile duct-cannulated male rats

Peak	Triflumuron metabolite ID	Radioactive recovery (% of dose)
B1		< 1
B2		2
B3		2
B4		< 1
B5	SIR 8514-hydroxy glucuronide (M28)	6
B6		1
B7		1
B8		3
B9		2
B10		2
B11		1
B12		< 1
B13		< 1
B14		< 1
B15	SIR 8513-cysteine aniline (M27; tentatively identified)	6
B16		< 1
B17	4-Trifluoromethoxyphenyl urea (M08)	3
B18		1
B19		< 1
B20		1
B21	SIR 8514-3-hydroxyaniline (M06)	2
B22		1
B23		2
B24		1
B25	SIR 8514-2-hydroxyaniline (M26, isomer of M06)	2
B26	Triflumuron	< 1
Total percent dose characterized		39
Total percent dose identified		13

Source: Sietsema, 1985

The proposed metabolic pathway for triflumuron in rats is shown in Fig. 2. The major detoxification pathway proceeds initially through hydrolysis of the urea to yield 4-(trifluoromethoxy)aniline. Minor metabolic pathways include hydrolysis of the 2-chlorobenzamide and direct hydroxylation of the trifluoromethoxyaniline ring prior to excretion. These results are similar to those previously reported, while additionally identifying the major triflumuron metabolites of 2-hydroxyaniline, 3-hydroxyaniline, 2-hydroxyaniline sulfate, and 3-hydroxyaniline sulfate (Krolski & Nguyen, 2003).

Urine, faeces and tissues collected from Sietsema (1985) were subjected to metabolic identification. No parent triflumuron was found in the urine of any group. Urine from the groups treated with chlorophenyl-labelled compound contained 2-chlorobenzoic acid (M02) and 2-chlorohippuric acid (M03) as the major metabolites, accounting for the majority of the urinary activity in Groups B, C, and D.

Urine from the group treated with trifluoromethoxyaniline-labelled compound, contained two major polar components (TB1 and B1) in approximately equal proportions, which could be easily hydrolysed by hydrochloric acid to form 2-hydroxy-4-trifluoro-methoxyaniline (M09). Due to the easy hydrolysis of the metabolites, it seemed reasonable to suspect the corresponding sulfate or glucuronic acid conjugates, but both compounds were unreactive towards glucuronidase or sulfatase treatment.

However, two isomers of hydroxy-4-(trifluoromethoxy)aniline sulfate (M16, M17) were identified in a corresponding rat study also performed with [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron (see Krolski & Nguyen, 2003). Thus, metabolite TB1 or B1 was possibly identical with metabolite M16.

Faeces contained mainly intact triflumuron ranging from 38% to 79% of the administered dose, suggesting incomplete gastrointestinal absorption and very low biliary excretion after oral dosing. Samples from the chlorophenyl label low-dose groups contained a small amount of *O*-methyl-(2-chlorobenzoyl) carbamate (MCBC), which was considered an artefact of storage. In faeces from the single oral dose with the trifluoromethoxyphenyl label, small extractable amounts of 4-trifluoromethoxyphenyl urea (M08; 2% of the administered dose) and 4-(trifluoromethoxy)aniline (M07, trace quantities) were found in addition to unaltered triflumuron. Unextractable residues in faeces were released by mild acid hydrolysis to form 2-chlorobenzoic acid (M02) and lesser amounts of 2-chlorobenzamide (M01) in addition to several unidentified products.

Liver, kidney, and fat were the only tissues that contained sufficient radiolabel at termination (seven days after dosing) for analysis. Tissues were pooled according to sex for each group. Extractable residue in fat was almost entirely triflumuron (94.8% to 98.3%). Triflumuron accounted for 9.3% to 54.6% of the extractable residue in liver and kidney. Liver and kidney also contained a component eluting in the vicinity of 2-chlorobenzamide, but quantitation was not possible due to other closely eluting peaks. Further characterization of tissue residues was not pursued due to the low residue levels present.

Table 9. Distribution of radioactivity among metabolites in urine and faeces after administration of [4-trifluoromethoxy-phenyl-UL-^{14}C]-triflumuron or [2-chlorophenyl-UL-^{14}C]-triflumuron

	Radioactive recovery (% recovered)			
	Urine	**Faeces**	**Cage rinse and carcass**	**Total**
^{14}C-Chlorophenyl label: Group B – single oral low dose (5 mg/kg bw)				
Triflumuron	ND	65.8	ND	65.8
2-Chlorobenzoic acid (M02)	13.2	ND	ND	13.2
2-Chlorohippuric acid (M03)	5.3	ND	ND	5.3
Carbamoyl chlorobenzamide (M29) §	ND	1.4	ND	1.4
Unidentified	4.0	2.7	0.6	7.3
Unextracted	< 0.1	7.0	-	7.0
Total	22.5	76.9	0.6	100.0
^{14}C-Chlorophenyl label: Group C – multiple oral low dose (5 mg/kg bw)				
Triflumuron	ND	44.5	ND	44.5
2-Chlorobenzoic acid (M02)	22.2	ND	ND	22.2
2-Chlorohippuric acid (M03)	9.2	ND	ND	9.2
Carbamoyl chlorobenzamide (M29) §	ND	3.4	ND	3.4
Unidentified	7.3	2.9	0.8	11.0
Unextracted	0.1	9.6	-	9.7
Total	38.8	60.4	0.8	100.0
^{14}C-Chlorophenyl label: Group D – single oral high dose (500 mg/kg bw)				
Triflumuron	ND	78.7	ND	78.7
2-Chlorobenzoic acid (M02)	7.9	ND	ND	7.9
2-Chlorohippuric acid (M03)	3.3	ND	ND	3.3
Carbamoyl chlorobenzamide (M29) §	ND	ND	ND	-
Unidentified	3.3	3.8	0.3	7.4
Unextracted	0.1	2.6	-	2.7
Total	14.6	85.1	0.3	100.0

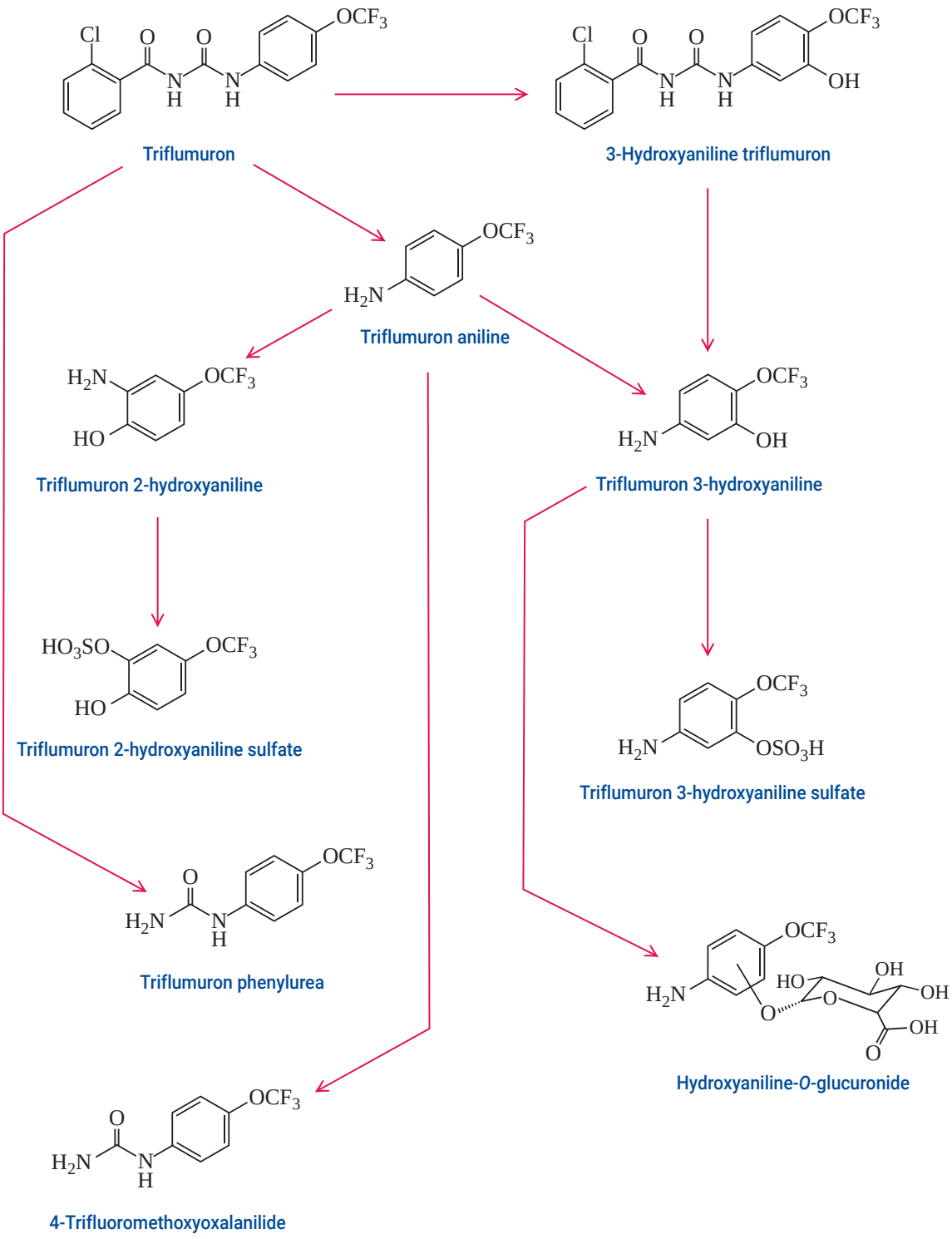
	Radioactive recovery (% recovered)			
	Urine	**Faeces**	**Cage rinse and carcass**	**Total**
^{14}C-Trifluoromethoxyphenyl label: Group B' – single oral low dose (5 mg/kg bw)				
Triflumuron	ND	38.4	ND	38.4
Unknown TB1	12.9	ND	ND	12.9
Unknown B1	14.9	ND	ND	14.9
4-Trifluoromethoxyphenyl urea (M08)	ND	2.4	ND	2.4
4-(Trifluoromethoxy)aniline (M07)	ND	traces	ND	traces
Unidentified	12.6	3.7	3.2	19.2
Unextracted	0.2	11.7	-	11.9
Total	40.6	56.2	3.2	100.0

ND Not detected Source: Sietsema, 1985

§ carbamoyl chlorobenzamide (M29) was considered to be an artefact of the work-up procedure

In summary, the metabolism of triflumuron included cleavage to form 2-chlorobenzoic acid (M02) and 4-trifluoromethoxy-phenyl urea (M08). Also found was 2-chlorobenzoic acid, conjugated with glycine as 2-chlorohippuric acid (M03). Metabolite M08, 4-trifluoromethoxyphenyl urea, was further hydrolysed to 4-(trifluoromethoxy)aniline (M07). Acid hydrolysis of urinary metabolites released 2-hydroxy-4-(trifluoromethoxy)aniline (M09; Sietsema, 1985).

Figure 2. Metabolic pathway of triflumuron in rat



2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with triflumuron (including skin and eye irritation and sensitization studies) are summarized in Table 10 below

Table 10. Acute toxicity of triflumuron

Route	Species	Strain	Sex	Vehicle	Purity	LD_{50} or LC_{50}[§]	Reference
Oral	Mouse	NMRI	M & F	Cremophor EL /water	98.9%	> 5000	Flucke & Kimmerle, 1977
Oral	Rat	Wistar	M & F	Cremophor EL /water	98.9%	> 5000	Flucke & Kimmerle, 1977
Oral	Rat	Not specified	M	Cremophor EL /water	Not specified	> 5000	Mihail, 1981
Oral	Rat	Wistar	M & F	Deionized water	99.1%	> 5000	Johnson, 2002a
Oral	Rat 1and 5 day single dose	Sprague Dawley	M & F	0.5% Carboxymethyl cellulose in deionized water	99.3%	NOAEL 500 mg/kg bw	Wahle, 2005
Oral	Dog	Beagle	F	Cremophor EL /water	98.9%	> 1000	Flucke & Kimmerle, 1977
Oral	Blackhead sheep		M & F	0.5% tylose	96.7%	> 10000	Hoffman, 1981
Dermal	Rat	Wistar	M & F	Cremophor EL /water	98.9%	> 5000	Flucke & Kimmerle (1977)
Dermal	Rat	Wistar	M & F	Deionized water	99.1%	> 5000	Johnson, 2002b
Inhalation	Mouse	NMRI	M	DMSO: PEG[¶] 1:1	98.9%	> 0.17	Flucke & Kimmerle, 1977
Inhalation	Rat	Wistar	M & F (1 × 1 h exposure)	DMSO: PEG[¶] 1:1	98.9%	> 0.15	Flucke & Kimmerle, 1977
Inhalation	Rat	Wistar	M & F (1 × 4 h exposure)	DMSO: PEG[¶] 1:1	98.9%	> 0.12	Flucke & Kimmerle, 1977
Inhalation	Rat	Wistar	M & F (5 × 4 h exposure)	DMSO: PEG[¶] 1:1	98.9%	> 0.13	Flucke & Kimmerle, 1977
Inhalation	Rat	Sprague Dawley	M & F (1 × 4 h exposure)	dust	94.0%	> 1.55	Sangha, 1981
Inhalation	Rat	Wistar	M & F (1 × 4 h exposure)	dust	99.1%	> 5.03	Pauluhn, 2002

Route	Species	Strain	Sex	Vehicle	Purity	LD_{50} or LC_{50}[§]	Reference
Inhalation	Hamster	Syrian golden	M	DMSO: PEG[¶] 1:1	98.9%	> 0.13	Flucke & Kimmerle, 1977
Skin Irritation	Rabbit	New Zealand White	Unspecified	Distilled water	98.9%	Not irritating	Flucke & Kimmerle, 1977
Skin Irritation	Rabbit	New Zealand White	M & F	Distilled water	99.1%	Not irritating	Merkel, 2002a
Eye irritation	Rabbit	New Zealand White	Unspecified	Distilled water	98.9%	Not irritating	Flucke & Kimmerle, 1977
Eye irritation	Rabbit	New Zealand White	M & F	Distilled water	99.1%	Not irritating	Merkel, 2002b
Skin sensitization[#]	Guinea pig	Albino	M	DMSO	94.0%	Not sensitizing	Hixson 1982,
Skin sensitization[#]	Guinea pig	Harlan Hsd Poc:DH	F	Polyethylene glycol 400	99.7%	Not sensitizing	Stropp, 1997
Intraperitoneal	Rat	Wistar	M & F	Cremophor EL /water	98.9%	> 5000	Flucke & Kimmerle, 1977
Subcutaneous	Mouse	NMRI	M & F	Cremophor EL /water	98.9%	> 5000	Flucke & Kimmerle, 1977
Combination administration							
Oral	Rat	Wistar (TNO/W74)	female	Cremophor EL /water	95.2%	> 5000	Flucke (1980)
Oral	Rat	Wistar	female	Cremophor EL /water	Not stated	> 5000	Heimann (1982)

[§] LD_{50} measured in mg/kg bw; LC50 measured in mg/L

[¶] Polyethylene glycol

[#] Magnusson and Kligman test

(a) Oral administration

Mouse

In a non-GLP acute oral toxicity study, NMRI mice (15/sex per dose) were administered triflumuron (purity 98.9%) via gavage in Cremophor EL/distilled water at 500, 1000, 2500 and 5000 mg/kg bw. Treated mice were observed for seven days.

There were no mortalities in the study. All treated mice at 1000 mg/kg bw and above showed clinical signs characterized by depression of general condition (fatigue) and breathing disorders. All signs had disappeared in 48 h.

Under the study conditions utilized, the acute oral median lethal dose (LD_{50}) for mice was > 5000 mg/kg bw (Flucke & Kimmerle, 1977).

Rat

Study 1

In a non-GLP acute oral toxicity study, Wistar (15/sex per dose) fasted rats were administered triflumuron (purity 98.9%) via gavage in Cremophor EL/distilled water at 500, 1000, 2500 and 5000 mg/kg bw. Treated rats were observed for seven days. Additional 15 non-fasted rats of both sexes were administered triflumuron at 1000, 2500 and 5000 mg/kg bw using the same procedure.

There were no mortalities in the study. All non-fasted treated rats at 2500 mg/kg bw and above showed clinical signs characterized by depression of general condition (fatigue) and breathing disorders. All fasted treated rats at 1000 mg/kg bw and above showed clinical signs characterized by depression of general condition (fatigue) and breathing disorders. All signs disappeared within 48 h.

Under the study conditions utilized, the acute oral LD_{50} for rats (fasted and non-fasted) was > 5000 mg/kg bw (Flucke & Kimmerle, 1977).

Study 2

In a non-GLP acute oral toxicity study, fasted male rats (10/dose) were administered triflumuron (purity not specified) via gavage in Cremophor EL/distilled water at 5000 mg/kg bw. Treated rats were observed for seven days.

No mortality was observed. No clinical signs were reported, however, all treated animals were reported as having clinical signs of toxicity.

Under the study conditions utilized, the acute oral LD_{50} for male rats was > 5000 mg/kg bw (Mihail, 1981).

Study 3

In a GLP acute oral toxicity study, Wistar rats (6/sex per dose) were administered triflumuron (purity 99.1%) by gavage, formulated in deionized water to fasted male and female CD-1 rats at 5000 mg/kg bw. Control animals were treated with deionized water only. Rats were observed for 14 days and clinical observations were performed twice daily for first three days and then once daily. Body weights were measured weekly. At termination rats were killed by carbon dioxide asphyxiation and underwent a gross necropsy.

No mortality was observed at any dose level. There were no compound-related effects on body weights. There were no clinical signs related to the test compound at any dose level. No treatment-related macroscopic findings were reported.

Under the study conditions utilized, the acute oral LD_{50} for rats was > 5000 mg/kg bw (Johnson, 2002a).

Dog

In a non-GLP acute oral toxicity study, fasted female Beagle dogs (two per dose) were administered triflumuron (purity 98.9%) via gavage in Cremophor EL/distilled water at 500 and 1000 mg/kg bw. Treated dogs were observed for seven days.

No mortality was observed. Treated dogs at 1000 mg/kg bw excreted loose faeces during 1 to 2 days post dosing.

Under the study conditions utilized, the acute oral LD_{50} for dogs was > 1000 mg/kg bw (Flucke & Kimmerle, 1977).

Black headed sheep

In a non-GLP acute oral toxicity study, Black headed sheep (1/sex per dose) were administered triflumuron (purity 96.7%) once by oral gavage at doses of 1000, 2500, 5000 and 1000 mg/kg bw in 0.5% tylose. Treated animals were observed for 14 days.

There were no mortalities or clinicals signs of toxicity during the study. Treated animals gained body weight as expected.

Under the study conditions utilized, the acute oral LD_{50} for Black headed sheep was > 10000 mg/kg bw (Hoffmann 1981).

(b) Dermal application

Rat

Study1
In a non-GLP acute dermal toxicity study, Wistar rats (five per sex) were dermally treated with triflumuron (purity 98.9%) at 5000 mg/kg bw. Triflumuron paste was prepared with a few drops of Cremophor EL for a contact time of 24 h to the intact dorsal skin. The treated skin areas were covered with aluminium foil and wrapped in an adhesive plaster sleeve. Upon removal of the occlusive dressings, the test compound was removed from the treated skin areas by washing with soap and water. The rats were kept under observation for up to seven days.

No mortality occurred. Treated rats did not show any variations from normal in their physical appearance or behaviour.

Under the study conditions utilized, the acute dermal LD_{50} to rats was > 5000 mg/kg bw (Flucke & Kimmerle 1977).

Study2
In a GLP acute dermal toxicity study, Wistar rats (6/sex per dose) were dermally treated with triflumuron (purity 99.1%) at 5000 mg/kg bw for 24 h. Triflumuron was moistened with deionized water and applied directly onto an area of the back of the animal, representing approximately 10% of the body surface area. After approximately 24 h post dosing the bandages and gauze were removed and the dose site was wiped using paper towels dampened with tap water to remove as much of the residual test substance as feasible without damaging the skin. Animals were terminated 14 days after dosing. Both controls and treated rats were treated in an identical manner with the exception that the control group did not receive test substance. Detailed clinical observations were performed at least twice daily for three days and once daily thereafter and on weekends. Body weights were measured weekly. At termination rats were killed by carbon dioxide asphyxiation and underwent a gross necropsy.

All animals survived until scheduled termination. There were no compound-related effects on body weight or compound-related clinical signs at the dose tested. There were no gross observational findings at necropsy.

Under the study conditions utilized, the acute dermal LD_{50} for rats was > 5000 mg/kg bw (Johnson, 2002a).

(c) Exposure by inhalation

Mouse

In a non-GLP acute inhalation toxicity study, four NMRI male mice were exposed to triflumuron (purity 98.9%) dissolved in a mixture of equal volumes of dimethyl sulfoxide and polyethylene glycol 400 and administered by dynamic flow inhalation chamber (head/nose exposure). The mice were exposed to the test material for 4 h at a dose level of 166 mg/m^3. The concentration of triflumuron in the chamber air inhaled by the experimental animals was measured by spectrophotometry at 250 nm. The treated mice were observed for seven days.

No mortality occurred during the study. No clinical signs of toxicity were observed.

Under the study conditions utilized, acute inhalation median lethal dose (LC_{50}) for male mice was > 166 mg/m^3 or > 0.17 mg/L (Flucke & Kimmerle 1977).

Rat

Study1
In a non-GLP acute inhalation toxicity study, Wistar rats were exposed to triflumuron (purity 98.9%) dissolved in a mixture of equal volumes of dimethyl sulfoxide (DMSO) and polyethylene glycol 400 and administered by dynamic flow inhalation chambers (head/nose exposure). Table 11 describes the experimental set-up and results. The concentration of triflumuron in the chamber air inhaled by the experimental animals was measured by spectrophotometry at 250 nm.

Table 11. Results of inhalation exposure to triflumuron on Wistar rats

	Dose (mg/m^3 air) [analytical concentration]	Exposure time (hours)	Toxicological results			LC_{50} [mg/m^3 air]
			Number dead	Number with toxic signs	Number of animals used	
Rat, male	150	1	0	0	10	> 150*
	39	4	0	0	10	
	91	4	0	0	10	> 119*
	119	4	0	10	10	
	74	5 × 4	0	0	10	> 133**
	133	5 × 4	0	10	10	
Rat, female	150	1	0	0	10	> 150*
	39	4	0	0	10	
	91	4	0	0	10	> 119*
	119	4	0	10	10	
	74	5 × 4	0	0	10	> 133**
	133	5 × 4	0	10	10	

* Post-treatment observation period seven days

** Post-treatment observation period 14 days

No mortality was observed. Inhalation of an aerosol at the highest concentrations led to transient (up to two hours after the end of exposure) non-specific disturbances of animals' general condition.

Under the study conditions utilized, the acute inhalation LC_{50} in rats was > 119 mg/m^3 (Flucke & Kimmerle, 1977).

Study2

In a GLP acute inhalation study, Sprague Dawley rats (10/sex per dose) were exposed under dynamic conditions, by their heads only, to a concentration of 1550 mg/m^3 of dust of triflumuron (purity 94.0%) for four hours. Control rats received only air. Rats were observed for mortality and signs of toxicity during exposure, at 0.5, 1 and 4 h post-exposure, and twice daily thereafter for 14 days. Individual body weights were recorded prior to exposure and on days 2, 3, 4, 7 and 14. A complete necropsy was performed on each rat and all gross abnormalities observed were recorded. Tissues of lung, liver and kidney were excised and fixed in 10% buffered formalin for histopathological processing and examination.

The mass median aerodynamic diameter (MMAD) was calculated to be 5.8 µm (average of three samples). The data indicates that 50% of the particle mass was below 6 µm and was respirable. No mortality was observed. Runny noses were observed in two males and one female rat. No significant differences in the mean body weights of control and exposed groups were observed. Macroscopic examination did not reveal treatment-related findings. No histopathological lesions were related to the exposure.

Under the study conditions utilized, the four-hour acute inhalation LC_{50} was > 1550 mg/m^3 (Sangha, 1981).

Study3

In a GLP acute inhalation study, Wistar rats (5/sex per dose) were exposed by nose-only method to a concentration of 2108 or 5030 mg/m^3 of dust of triflumuron (purity 99.1%) for four hours. Control rats received only air. Rats were observed for mortality and signs of toxicity several times on the day of exposure and at least once per day thereafter. Individual body weights were recorded prior to exposure and on days 3, 4, 7 and 14. Rectal temperatures were measured at termination. A complete necropsy was performed on each rat and all gross abnormalities observed were recorded.

The MMAD was 3.7 µm with a geometric standard deviation (GSD) of about 2 at the low dose of 2108 mg/m^3 and an MMAD of 6.6 µm with GSD of about 2 at the high dose of 5030 mg/m^3.

At 5030 mg/m^3, piloerection was observed in one male and nostrils with red encrustations in one female. There were no significant differences in the mean body weights of control and treated groups. At 5030 mg/m^3, isolated dark-red foci in lungs in 2/5 male rats were observed at necropsy.

Under the study conditions utilized, the four-hour acute inhalation LC_{50} was > 5030 mg/m^3 (Pauluhn, 2002).

Hamster

In a non-GLP acute inhalation toxicity study, Syrian golden hamster (four males) were exposed to triflumuron (purity 98.9%) dissolved in a mixture of equal volumes of DMSO and polyethylene glycol. This was administered once by dynamic flow inhalation chamber (head/nose exposure). The hamsters were exposed to test material for four hours at a dose level of 166 mg/m^3. The concentration of triflumuron in the chamber air inhaled by the experimental animals was measured by spectrophotometry at 250 nm. The treated hamsters were observed for seven days.

No mortality occurred during the study. No clinical signs of toxicity were observed.

Under the conditions of this study, acute inhalation LC_{50} for male hamster was > 166 mg/m^3 or 0.17 mg/L (Flucke and Kimmerle 1977).

(d) Dermal irritation

Study 1
In a non-GLP skin irritation study, New Zealand White rabbits (six rabbits, unspecified sex) were treated once dermally on intact and abraded skin with triflumuron (purity 98.9%) in distilled water according to the method recommended by the US Department of Agriculture (Federal Register 38 (187) 27019 (1973). No other details were given.

No redness (erythema) or swelling (oedema) was observed on the treated skin.

The results of the study indicate that triflumuron was nonirritating to the rabbit's skin (Flucke & Kimmerle, 1977).

Study 2
In a GLP primary skin irritation study, New Zealand White rabbits (one male, two females) were treated once, dermally on intact skin with triflumuron (purity 99.1%). Prior to application, the test substance was moistened with distilled water to achieve a dry paste by preparing a 70% w/w mixture. The desired amount of test substance (0.5 g, representing 0.71 g of the test mixture) was placed on a 1 inch × 1 inch, four-ply gauze pad and applied to one 6 cm^2 intact dose site on each animal. The pad and entire trunk of each animal were then wrapped with semi-occlusive 3 inch micropore tape, to avoid dislocation of the pad. Elizabethan collars were placed on each rabbit, and they were returned to their designated cages. After 4 h of exposure to the test substance the pads and collars were removed and the test sites gently wiped with water and a clean towel to remove any residual test substance. Individual dose sites were scored according to the Draize scoring system at approximately 1, 24, 48 and 72 h after patch removal.

All animals appeared active and healthy. There were no signs of gross toxicity, dermal irritation or abnormal behaviour.

Under the conditions of this study, triflumuron is non-irritating to the skin of rabbits (Merkel, 2002a).

(e) Ocular irritation

Study 1
In a non-GLP eye irritation study, triflumuron (purity 98.9%) was administered once into the conjunctival sac of five New Zealand White rabbits (unspecified sex) for five minutes and 24 h according to the method recommended by the U.S. Department of Health, Education and Welfare (Federal Register 37 (83) 8534 (1972). No other detail was given.

Slight redness in the conjunctiva was observed at 1 and 24 h post instillation.

The results of the study indicate that triflumuron was non-irritating to the eyes of rabbits (Flucke & Kimmerle,1977).

Study 2
In a GLP primary eye irritation study, triflumuron (purity 99.1%) was administered, once into the conjunctival sac of 3 New Zealand White rabbit eyes (one male and two females) at a volume of 0.1 mL. Ocular irritation was evaluated according to Draize method at 1, 24, 48 and 72 h post instillation.

All animals appeared active and healthy. No corneal opacity or iritis was observed during the study. One hour following test substance instillation, all three treated eyes exhibited conjunctivitis. The overall incidence and severity of irritation decreased with time. All animals were free of ocular irritation within 48 h. The maximum mean total score of triflumuron according to the Draize method of scoring was 4.0 .

Under the conditions of this study, triflumuron was minimally irritating to the eye of rabbits (Merkel, 2002b).

(f) Dermal sensitization

Study 1
In a GLP acute sensitization study, triflumuron (purity 94%) was tested for dermal sensitization potential on male albino Guinea pigs in the maximization test according to Magnusson & Kligman. The test substance was formulated in DMSO containing 10% material. The concentration for intradermal induction was 1%, whereas a 10% formulation was used for topical induction and a 5% level for the challenge.

No significant differences were observed in the solvent control group between treated and control sides. Similarly, no significant differences were observed in Guinea pigs treated with triflumuron. In contrast, significant differences between treated and control sides of Guinea pigs treated with penicillin G, used as a positive control, were observed on both days 23 and 24. This indicates that the method and the test animals were sufficiently sensitive to detect a sensitizing material.

Under the conditions of this study, triflumuron was not sensitizing to the skin of Guinea pigs (Hixson, 1982).

Study 2
In a GLP acute sensitization study, triflumuron (purity 99.7) was tested for dermal sensitization potential on female Harlan Guinea pigs in the maximization test according to Magnusson & Kligman. The test substance was formulated in polyethylene glycol 400. The concentration for intradermal induction was 2.5%, whereas a 50% formulation was used for topical induction and a 50% level for the challenge.

The challenge using a 50% test substance formulation led to slight skin effects (Grade 1) in 1/20 animals in the treatment group after 48 h and in none of the animals in the treatment group after 72 h. In the control group none of the animals showed skin effects after 48 h or 72 h.

Under the conditions of this study, triflumuron was not sensitizing to the skin of Guinea pigs (Stropp, 1997).

(g) Intraperitoneal administration

Rats

In a non-GLP study, triflumuron (purity 98.9%) emulsified in distilled water and Cremophor EL was administered intraperitoneally to male and female Wistar rats (15/sex per dose) at 500, 1000, 2500 and 5000 mg/kg bw in males and 250, 500, 1000, 2500 and 5000 mg/kg bw in females. The post-treatment observation period lasted seven days.

No mortality occurred. There were signs of depression of general condition (fatigue) and breathing disorders; signs lasted up to four days in both sexes. These signs were observed at 1000 mg/kg bw and above in males and at 500 mg/kg bw and above in females.

Under the study conditions utilized, the acute intraperitoneal LD_{50} in rats was > 5000 mg/kg bw (Flucke & Kimmerle, 1977).

(h) Subcutaneous administration

In a non-GLP study, triflumuron (purity 98.9%) emulsified in distilled water and Cremophor EL was administered subcutaneously to male and female NMRI mice (15/sex per dose) at 500, 1000, 2500 and 5000 mg/kg bw in males and females. The post-treatment observation period lasted seven days.

No mortality occurred. At 1000 mg/kg bw and above, disturbances of general health condition were observed.

Under the study conditions utilized, the acute subcutaneous LD_{50} in mice was > 5000 mg/kg bw (Flucke & Kimmerle, 1977).

(i) Potentiation/combination acute toxicity

Study 1

In a non-GLP acute oral toxicity study (combination toxicity study), triflumuron (purity 95.2%), formulated in distilled water and Cremophor EL, was administered to fasted female Wistar rats (strain TNO/W74) by single oral application. In this study, NTN 9306 (sulprofos; purity ca 89.1%) was used for combination administration via gavage. The individual active ingredients were applied in combination as equitoxic doses in percentage ratio to their LD_{50} values as previously determined. The LD_{50} of triflumuron is greater than the maximum applicable dose of 5000 mg/kg bw so the latter maximum dose was used to calculate the composition of the active ingredient combination. The LD_{50} value for NTN 9306 was 65 mg/kg bw. The combination doses were 500, 1000, 2500, 3500, 4500 and 5000 mg/kg bw (containing 1.28% NTN 9306 and 98.72% triflumuron). Ten female rats were used per dose. Animals were observed for 14 days.

There were mortalities; 1, 4, 6 and 9 treated rats died within four days post dosing at 2500, 3500, 4500 and 5000 mg/kg bw, respectively. The observed LD_{50} was 3720 (3179–3826) mg/kg bw. The expected LD_{50} ranged from 2536 to 4060 mg/kg bw depending up on the assumed triflumuron LD_{50} value.

Results show that there was no evidence of a more than additive (potentiating) effect under the described experimental conditions (Flucke, 1980).

Study 2

In a non-GLP acute oral toxicity study (combination toxicity study), triflumuron (purity not stated) formulated in distilled water and Cremophor EL was administered to fasted female Wistar rats by single oral application. In this study, FCR 1272 (α-cyano-3′-phenoxy-4′-fluorobenzyl-2,2-dimethyl-3-dichlorvinyl-*cis*/*trans*-cyclopropane carboxylate; purity not stated) was used for combination administration via gavage. The individual active ingredients were applied in combination as equitoxic doses in percentage ratio to their LD_{50} values as previously determined. The LD_{50} for FCR 1272 was 14.3 (12.8–15.9) mg/kg bw. The combination doses were 500, 1000, 2500 and 5000 mg/kg bw (containing 0.28% FCR 1272 and 99.72% triflumuron). Ten female rats were used per dose. Animals were observed for 14 days.

The toxic signs shown by rats treated with the active ingredient combination consisted of disturbed behaviour, accelerated respiration, reduced motility, unco-ordinated movements, stretched and spastic gait, staggering and salivation. At 5000 mg/kg bw one rat died. The observed LD_{50} was > 5000 mg/kg bw. The expected LD_{50} of combination was 2526 mg/kg bw.

The results show that there was no evidence of a more than additive (potentiating) effect under the described experimental conditions (Heimann, 1982).

(j) Single-dose toxicity study to derive an ARfD

In a toxicity study, Sprague Dawley rats (20/sex per dose) were administered triflumuron (purity 99.3%) via a single gavage dose at 0, 10, 70 or 500 mg/kg bw in 0.5% (w/v) methyl cellulose in deionized water in a volume of 10 ml/kg bw. The analytically measured concentrations were 0, 9.8, 67 and 466 mg/kg bw at 0, 10, 70 and 500 mg/kg bw, respectively. At 24 h after dosing, the first 10 surviving animals/sex per dose were terminated. All remaining animals were terminated on day 5 post dose. A general assessment of all animals with respect to moribundity and mortality was carried out at least

once daily during the in-life phase of the study. Individual body weights were determined on day 0, prior to dosing, and then again just prior to necropsy. Food consumption was determined over the five-day in-life interval (for those animals killed on day 5 only). Selected haematological parameters were evaluated at termination. All animals placed on study were subject to a postmortem examination, which included: [1] documenting and saving all gross lesions; [2] weighing designated organs; [3] collecting representative tissue specimens for histopathologic evaluation (adrenal, brain, kidney, liver, and spleen).

Analytical data indicated that triflumuron was homogeneously distributed in the dosing solution and was stable for eight days at room temperature. Mean analytical concentrations were 93–98% of the nominal concentrations. No animals were found dead or were terminated in extremis during the course of the study. No treatment-related clinical signs of toxicity was observed. Body weight and body weight gain remained unaffected in both sexes at all doses tested. Food consumptions was unaffected by the treatment (assessed for day 5 animals). Evaluation of the haematological parameters from blood collected at 1 and 5 days post dosing provided no indication of a triflumuron-induced change in either sex at any dose tested. No treatment-related effects were observed on organ weights, macroscopic or microscopic examinations.

Under the conditions of the study, the no-observed-adverse-effect level (NOAEL) was 500 mg/kg bw; the highest dose tested (Wahle, 2005).

Short-term studies of toxicity

(a) Oral administration

Rats

Study 1

In a non-GLP 28-day oral toxicity study, Wistar rats (20/sex per dose) were administered triflumuron (purity 98.9%) once daily via gavage at a dose of 0, 30, 100 and 300 mg/kg bw per day for 28 consecutive days. Cremophore EL in distilled water was used as a vehicle. Half of the animals in each group were terminated and examined at the end of the administration phase, and the other half at the conclusion of the four-week observation period. The rats were inspected daily for their physical appearance, behavioural patterns and posture throughout the study. All the rats were weighed at the start of each study week and prior to termination. At the end of the 28-day treatment period and at the end of the four-week post-treatment observation period, laboratory blood tests (haematological and clinical chemistry parameters) and urinalyses were performed on five male and five female rats of each group. Gross pathological examination was conducted at termination including on the recovery group. Selected organs were removed, weighed and prepared for histopathological examinations.

The appearance, behaviour, body weight, body weight gains and mortality were unaffected in all groups. In the female rats at 300 mg/kg bw per day, the erythrocyte counts were significantly lower (10%) and the thrombocyte counts (19%) and reticulocyte counts (46%) were higher than in the controls. No treatment-related changes in clinical chemistry parameters, urinary parameters or organ weights were observed. Gross pathology revealed darker coloured spleens in all male and female rats at 300 mg/kg bw per day. Histopathological examination of the spleen revealed minimally to moderately increased extramedullary haematopoiesis (effect was reversible) in the red splenic pulp of female rats at 300 mg/kg bw per day.

Under the conditions of the study, the NOAEL was 100 mg/kg bw per day based on decreases in erythrocytes and elevated reticulocytes and thrombocytes counts and extramedullary haematopoiesis (splenic) seen in females at the lowest-observed-adverse-effect level (LOAEL) of 300 mg/kg bw per day (Flucke & Schilde, 1978b).

Study 2

In a non-GLP 90-day toxicity study, Wistar rats (20/sex per dose) were administered triflumuron (purity 96.7%) via their diet at concentrations of 0, 50, 500 and 5000 ppm (equal to 0, 3.6, 35.5, and 349.2 mg/kg bw per day for males, 0, 4.5, 47.0 and 448.7 mg/kg bw per day for females) for three months. The rats were inspected daily. The following examinations were performed during the study: body weight, food and test compound intake, haematology, clinical chemistry, urinalysis, gross necropsy and histopathology.

Male rats in the 5000 ppm group were subject to severe spasms lasting about 15–30 minutes during the entire study period, and the spasms sometimes occurred several times daily. After the spasms had subsided, a number of these rats had physical head injuries and bled at the nose. During the entire study period, seven (four male and three female) rats in the 5000 ppm group died. At 5000 ppm, statistically significantly ($p < 0.01$) lower body weights (10–15%) were observed in both sexes at various time points. Female rats in the 500 and 5000 ppm groups had slightly, but significantly lower body weights (about 7%) up to the sixth study week, when compared with the control. Above 50 ppm female rats showed a slight (up to 5%, occasionally statistically significant) retardation in body weight development throughout the study period. This was not considered to be toxicologically significant. Food consumption was reduced in 5000 ppm group male rats. Haematological findings are described in Table 12. At 5000 ppm in male rats: erythrocytes, haemoglobin (Hb), haematocrit (Ht) were reduced; reticulocytes increased; in differential count, segmented neutrophils increased and lymphocytes decreased. In female rats: erythrocytes, leukocytes, Hb, Ht, mean corpuscular Hb concentration (MCHC) were reduced; reticulocytes, mean corpuscular Hb (MCH) and mean corpuscular volume (MCV) increased. At 500 ppm in male rats: Hb, Ht were reduced; leukocytes increased. At 500 ppm in females: erythrocytes, Hb, Ht, MCH, MCHC were reduced; MCV increased. At 50 ppm in females, the MCHC was reduced by 3% after one month but was unchanged after three months; this was not considered to be toxicologically significant. In males at 5000 ppm, statistically significantly ($p < 0.01$) lower total protein concentration was observed. In females at 5000 ppm, glutamate dehydrogenase was significantly reduced, bilirubin and cholesterol were statistically significantly ($p < 0.05$) higher than in controls. No treatment-related effects were observed on urinary parameters measured at one month and termination. Macroscopic findings revealed enlarged and swollen spleen of male rats at 5000 ppm. Relative liver, spleen, and adrenal weights increased in both sexes and relative kidney weights (males only) at 5000 ppm. At 500 ppm relative spleen weights were increased in males and relative liver and spleen weights were increased in female rats.

Table 12. Summary of haematological findings (average)

	Males				Females			
Dose (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Haemoglobin, Hb (g/L)								
1 month	145	146	146	135**	149	146	139*	133**
3 months	157	156	144*	141**	154	149	141*	133*
Haematocrit, Ht (L/L)								
1 month	0.47	0.48	0.47	0.45*	0.48	0.49	0.49	0.49
3 months	0.51	0.51	0.47*	0.46**	0.51	0.49	0.47**	0.45**
Erythrocyte count (10^{12}/L)								
1 month	6.98	7.08	7.03	6.52	7.26	7.53	7.46	6.46**
3 months	8.6	8.71	8.15	7.51**	8.16	7.89	7.19**	6.55**
Leucocyte count (10^9/L)								
1 month	7.8	7.6	10.6*	13.1**	6.7	7.0	7.4	12.6**
3 months	9.3	10.8	10.0	10.9	8.2	9.4	7.8	11.3*
Mean corpuscular volume, MCV (fL)								
1 month	67	68	67	69	67	65	66	76**
3 months	59	59	57	62	63	62	65*	69**
Mean corpuscular Hb content, MCHC (g/L)								
1 month	310	306	310	301	307	298**	280**	273**
3 months	310	305	308	304	302	303	299	294

	Males				Females			
Dose (ppm)	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Mean corpuscular Hb, MCH (pg)								
1 month	20.8	20.7	20.7	20.7	20.5	19.3	18.6**	20.6
3 months	18.3	17.9	17.6	18.8	18.9	18.8	19.5	20.4*
Reticulocytes (0/00)								
1 month	24	25	24	58**	17	17	18	51**
3 months	20	16	27	37**	19	19	24	73**
Relative organ weights								
Spleen (mg/100g)	172	179	204**	238**	196	196	246**	341**
% of control value	-	[1.04%]	[118%]	[138.4%]	-	[0%]	[125.5%]	[174%]
Liver (mg/100g)	3480	3554	3534	3807*	3301	3548*	3622**	4136**
% of control value	-	[102.2%]	[101.6%]	[109.4%]	-	[107.5%]	[109.7]	[125,3%]
Kidney (mg/100g)	604	602	606	686**	646	672	687	692
% of control value	-	[100%]	[100%]	[113.6%]	-	[104.0%]	[106.3%]	[107.1%]

* $p < 0.05$ ** $p < 0.01$

Kroetlinger & Kaliner, 1981

Histopathological findings at 5000 ppm, revealed follicular aplasia of the spleen, increased iron content, hyperaemia of splenic pulp sinusoid content and extramedullary haematopoiesis. There was increased blood cell content in the bone marrow. Increased iron content in Kupffer cells of the liver was also observed. At 500 ppm, increased iron content, hyperaemia of splenic pulp sinusoid content and extramedullary haematopoiesis were observed. In addition, increased blood cell content in the bone marrow and was seen. Increased iron content in Kupffer cells of the liver was also noted.

The NOAEL was 50 ppm (equal to 3.6 mg/kg bw per day) based on relative liver and spleen weight increase and slight declines in Hb and erythrocytes seen at the LOAEL of 500 ppm (equal to 35.5 mg/kg bw per day) (Kroetlinger & Kaliner, 1981).

Study 3

In a non-GLP, 90-day toxicity study, Wistar rats (20/sex per dose) were administered triflumuron (purity 94.9%) via their diet at concentrations of 0, 5, 15 and 45 ppm (equal to 0, 0.34, 1.02 and 3.12 mg/kg bw per day for males, 0, 0.39, 1.18 and 3.63 mg/kg bw per day for females) for three months. The animals were inspected twice daily, but only once daily on weekends/public holidays. The following examinations were performed during the study: body weight, food and test compound intake, haematology, clinical chemistry, urinalysis, gross necropsy and histopathology. In addition, fluoride levels were determined in teeth and bones to assess whether any free fluoride was released from triflumuron.

The appearance, behaviour, food consumption, body weight development and mortality were unaffected at doses up to and including 45 ppm. Haematology showed no indications of blood damage in any dose group. The plasma glucose and cholesterol levels were in the normal range in all dose groups. Clinical chemistry findings and urinalysis gave no indication in any dose group of injury to the liver, kidneys or other organs examined. Gross pathological and histopathological findings gave no indications of injury in any dose group to the liver, kidneys or other organs examined. No treatment-related changes in the fluoride levels of the teeth or bones were revealed in any dose group.

The NOAEL was 45 ppm (equal to 3.12 mg/kg bw per day) the highest dose tested (Kroetlinger & Vogel, 1983).

Study 4

I five rats/sex per dose at one month and three months. At termination gross necropsy was performed on all animals and selected organs were removed, weighed and preserved for histopathological examinations. In addition, fluoride levels were determined in teeth and bones.

The appearance and behaviour in treated groups were comparable to controls. During the entire study period one male rat died in each of the 200 and 2000 ppm groups. One male rat in each of the 0 and 2000 ppm dose groups also died when blood samples were taken after one month. One animal also died as a result of the treatment at 2000 ppm. Body weight development was unaffected by the treatment. Slightly reduced feed consumption was observed in female rats at 2000 ppm. Haematological results are presented in Table 13. At 200 and 2000 ppm, male and female rats had significantly lower erythrocyte counts and Hb values and significantly higher reticulocyte counts, MCH and MCV values in comparison to the controls after three months. The Ht values recorded at these dose levels were also significantly lower, with the exception of males after 2000 ppm. In addition, the females in the 2000 ppm group exhibited lower MCHC values. Lower erythrocyte counts and Hb values and higher reticulocyte counts were also recorded after 20 ppm, but they were only significant in the case of the females The leucocyte counts were significantly higher in both sexes at 2000 ppm. Microscopic examinations revealed increased incidences of normoblasts (basophile, polychromatic, oxyphile) and signs of polychromasia, anisocytosis and Heinz inclusion bodies or Howell–Jolly bodies in both sexes in the 200 and 2000 ppm dose groups. After 20 ppm one oxyphile normoblast was found in one male.

Table 13. Summary of haematological findings (average)

	Males				Females			
Dose (ppm)	**0**	**20**	**200**	**2000**	**0**	**20**	**200**	**2000**
Haemoglobin, Hb (g/L)								
1 month	152	149	152	145	160	144**	127**	131**
3 months	157	153	143**	145*	153	140*	132**	130**
Haematocrit, Ht (L/L)								
1 month	0.47	0.46	0.47	0.44	0.50	0.44**	0.40**	0.40**
3 months	0.48	0.47	0.44*	0.46	0.47	0.44	0.42**	0.42**
Erythrocyte count (10^{12}/L)								
1 month	7.66	7.45	6.65**	6.27**	6.78	7.34*	6.52	5.29**
3 months	8.96	8.51	7.74**	6.84**	8.12	7.24**	6.47**	5.84**
Leucocyte count (10^9/L)								
1 month	8.9	8.3	9.6	21.1**	10.3	9.7	8.9	54.6**
3 months	8.8	7.4	10.0	15.9**	5.9	5.4	5.7	20.9**
Mean corpuscular volume, MCV (fL)								
1 month	61	62	71**	71**	74	60**	61**	76
3 months	54	55	57*	67**	58	61	66**	72**
Mean corpuscular Hb content, MCHC (g/L)								
1 month	324	322	323	327	320	325	318	323
3 months	324	325	321	316	322	318	311	311
Mean corpuscular Hb, MCH (pg)								
1 month	19.9	20.1	22.9**	23.2**	23.6	19.6**	19.5**	24.7
3 months	17.5	18	18.5*	21.2**	18.8	19.4	20.4**	22.3**
Reticulocytes (0/00)								
1 month	24	31	28	169**	18	20	25*	503**
3 months	15	17	39	50	15	17	28	73

* $p < 0.05$ ** $p < 0.01$

The bilirubin value was slightly higher in both sexes in the 2000 ppm group, and in the males in the 200 ppm group. Urinalysis did not reveal any variations between control rats and treated groups. The fluorine concentrations in teeth and bones were statistically significantly higher (< p0.01) in both sexes

at 2000 ppm. Necropsy of all the rats at end of study did not provide any indications of substance-induced alterations in the treated groups. At 2000 ppm, relative liver, spleen and kidney weights were increased in males and relative spleen weights increased in females. At 200 ppm, relative spleen weights were increased in both sexes. At 2000 ppm histopathological examination revealed pronounced fluoride accumulation in the teeth and bones, necrogenic hepatic cell changes (small demarcated necrotic foci and single-cell necroses) pronounced liver siderosis and increased extramedullary haematopoiesis with sinus dilatation in the spleen. At 200 ppm histopathological examination revealed necrogenic hepatic cell changes (small demarcated necrotic foci and single-cell necroses) pronounced liver siderosis and an increase in the red spleen pulpa with sinus dilation.

The NOAEL was 20 ppm (equal to 1.34 mg/kg bw per day) based on decreases in erythrocytes, haemoglobin, haematocrit, MCH, MCV and increased in reticulocytes (females only) seen at the LOAEL of 200 ppm (equal to 13.85 mg/kg bw per day) (Kroetlinger & Janda, 1984).

Dog

Study 1

In a non-GLP, 90-day toxicity study, groups of four male and four female Beagle dogs were administered triflumuron (purity 95.1%) at concentrations of 0, 100, 500 or 2500 ppm in their diet (equal to 0, 3.21, 17.3 or 85.2 mg/kg bw per day for males, 0, 3.66, 17.1 or 87.7 mg/kg bw per day for females) for a period of 13 weeks. All the dogs were inspected daily for their physical appearance and behavioural patterns; these inspections were made several times each day. Body weights were measured weekly. Ophthalmoscopic examination and urinalysis was performed prior to initiation of the study, at week 7 and at week 13. Body temperatures, pulse rates and reflexes were evaluated periodically. Haematological and clinical parameters were evaluated prior to initiation of the study, at week 6 and at week 12. Gross pathological examination was conducted at termination. Selected organs were removed, weighed and prepared for histopathological examinations.

No treatment-related effects were observed on pulse rate, reflexes, body temperature, clinical signs of toxicity, urinalysis, ophthalmoscopic examinations, clinical chemistry parameters, mortality, body weight, body weight gain or food consumption. One dog at 500 ppm died as the result of strangulation in week 9. At 500 and 2500 ppm, Hb and erythrocytes were reduced (combined sexes), however, statistical significance was not achieved. Methaemoglobin (MetHb) was increased significantly (combined sexes) at 500 ppm (2.6% compared with 1.19% prior to study initiation) and at 2500 ppm (3.66% compared with 1.19% prior to study initiation). In compensation of the anaemic damage, reticulocytes at 2500 ppm and thrombocytes at 500 and 2500 ppm were increased. Macroscopic examination revealed spleen alterations (dark colour, hard consistency and increased blood content) at 500 and 2500 ppm and thymus atrophy at 2500 ppm. The weight of the spleen in the 2500 ppm dogs (absolute and relative) was statistically significantly higher than in the controls ($p < 0.01$). The weight of the thymus in the 2500 ppm dogs (absolute and relative) also differed statistically significantly from that of the controls ($p < 0.05$ and $p < 0.02$, respectively). Histopathological examination revealed, increases in bone marrow cells and extramedullary erythropoiesis in the spleen at 500 and 2500 ppm.

The NOAEL was 100 ppm (equal to 3.21 mg/kg bw per day) based on decreased in Hb and erythrocytes, increased in MetHb, reticulocytes and thrombocytes and histological findings (increase in bone marrow cells and extramedullary erythropoiesis in the spleen) at 500 ppm (equal to 17.1 mg/kg bw per day) (Hoffmann and Groening, 1980).

Study 2

In a non-GLP, 12-month toxicity study, groups of six male and six female Beagle dogs were administered triflumuron (purity 95.3%) at concentrations of 0, 40, 200 or 1000 ppm in their diet (equal to 0, 1.42, 7.1 or 35.3 mg/kg bw per day for males, 0, 1.50, 7.3 or 37.9 mg/kg bw per day for females) for a period of 12 months. All the dogs were inspected daily for their physical appearance and behavioural patterns. Body weights were measured weekly. Food consumption was measured daily. Ophthalmoscopic examination, haematology, clinical chemistry and urinalysis were performed two weeks prior to initiation of the study, at weeks 6, 13, 26, 39 and 52. Body temperatures, pulse rates and reflexes were evaluated periodically. Gross pathological examination was conducted at termination. Selected organs were removed, weighed and prepared for histopathological examination.

No differences between the control and dose groups with regard to the appearance, behaviour, body temperature, pulse frequency, food and water intake, body weight development, mortality or the neurological and ophthalmoscopic examinations, were present at doses up to and including 1000 ppm. No treatment-related effects were observed on clinical chemistry or urinary parameters examined at doses up to and including 1000 ppm. Changes in haematological parameters for both sexes combined are shown in Table 14. At 200 and 1000 ppm, Hb, Ht and erythrocytes were reduced, MCV increased and MCH decreased. In compensation of the anaemic damage, reticulocytes and thrombocytes increased. Howell–Jolly and Heinz bodies increased. Target cells were seen from week 39 onwards and occasionally polychromatic and acidophilic normoblasts as well as anulocytes. At 1000 ppm, in some dogs, pronounced polychromatophilia, anisocytosis and poikilocytosis were found. At 40 ppm there was tendency towards a dose-related increase in reticulocytes in males only, however, statistical significance was not achieved. The reticulocyte counts in males at 40 ppm were 4.3, 3.7, 2.8, 6.8, 8.7 and 10.0% at weeks 0, 6, 13, 26, 39 and 52, respectively. The reticulocyte counts in concurrent control dogs were 2.8, 3.0, 1.0, 4.7, 5.03, and 5.3% at week 0, 6, 13, 26, 39 and 52, respectively. They were outside the historical control range. The increase in reticulocytes is considered a compensatory change, adaptive and reversible on cessation of exposure. In addition, no real organ dysfunction could be observed supporting the adaptive character of these effects. No clear indicators of anaemia like statistically significant reduction of Hb concentration, red blood cell count and Ht could be discerned.

Table 14. Summary of haematological findings (both sexes combined)

Dose (ppm)	0	40	200	1000
Haemoglobin, Hb (g/L)	149.1	144.1	↓ 143.9*	↓ 124.7***
Erythrocytes (10^{12}/L)	6.462	6.157	↓ 6.161*/**	↓ 5.152***
Mean corpuscular volume, MCV (fL)	70.03	72.34	↑ 72.33**/***	↑ 77.37***
Mean corp. Hb content, MCHC (g/L)	329.79	323.59	↓ 322.61*/***	↓ 312.97 ***
Thrombocytes (10^9/L)	280.8	328.3	↑ 341.24*/**/***	↑ 434.7***
Reticulocytes (0/00)	5.5	7.4	↑ 18.9 **/***	↑ 59.9 ***
Bilirubin (µmol/L)	3.62	3.79	↑ 4.23 §	↑ 5.67*/***
Methaemoglobin (%)	-	-	[WSF]	↑ */***
Liver weight; absolute (g)	373.8	365.2	389.6 [WSF]	↑405.6 § [Male]
Spleen weight; absolute (g)	36.3	46.3	↑ 56.0 ***	↑ 74.8***

§ Tendentious

WSF Without special finding

↑ = elevated ↓ = reduced

* $p \leq 0.05$ ** $p \leq 0.02$ *** $p \leq 0.01$

At 200 and 1000 ppm, macroscopic finding revealed grey/brown–bluish discolorations in kidneys and dark discoloured urines. There appeared increased congestion and dark discoloured spleens. Red–brown discoloration of the distal end of the femur was observed in individual animals. At 40 ppm, there was an increase in congestion and dark discoloured spleens. Both the absolute and the relative spleen weights of the 1000 ppm group dogs were substantially greater than control ($p < 0.01$). The difference was less marked for the dogs in the 200 ppm group ($p < 0.02$). Spleen weights of the dogs in the 40 ppm group was also greater than controls, but the difference was not statistically significant. At 200 and 1000 ppm histopathological examination revealed pronounced pigment accumulation in the liver, kidneys and spleen. Reactive, hypercellular bone marrow and foci of extramedullary erythropoiesis in the spleen were observed. At 40 ppm, very minimal increased pigmentation in the liver, kidney and spleen were observed.

The NOAEL was 40 ppm (equal to 1.42 mg/kg bw per day) based on haematological changes (increased reticulocytes 18.9% against 5.5 % in controls), increase in absolute spleen weight (154% of the controls), and pigmentation in liver, kidney and spleen seen at the LOAEL of 200 ppm (equal to 7.1 mg/kg bw per day) (Hoffmann & Schilde, 1989a).

Study 3
In a non-GLP, 12-month toxicity study, groups of six male and six female Beagle dogs were administered triflumuron (purity 94.6%) in their diet at concentrations of 0 or 20 ppm (equivalent 0.72 mg/kg bw per day) for a period of 12 months. All the dogs were inspected daily for their physical appearance and behavioural patterns. Body weights were measured weekly. Food consumption was measured daily. Ophthalmoscopic examination, haematology, clinical chemistry and urinalysis was performed two weeks prior to initiation of the study, then at weeks 6, 13, 26, 39 and 52. Body temperatures, pulse rates and reflexes were evaluated periodically. Gross pathological examination was conducted at termination. Selected organs were removed, weighed and prepared for histopathological examination.

No treatment-related effects were observed on any of the parameters evaluated in the study.

The NOAEL was 20 ppm (equivalent to 0.72 mg/kg bw per day) the highest dose tested (Hoffmann & Schilde, 1989b).

(b) Dermal application

Rabbit

Study 1
In a non-GLP dermal toxicity study, groups of six male and six female New Zealand White rabbits received triflumuron (purity 98.9%) in Cremophor EL/distilled water on the shaved back skin (intact and abraded) at a dose of 0, 50 or 250 mg/kg bw per day, seven hours per day, five days per week, for three weeks. At the end of each 7 h hour contact time, the treated skin areas were washed with soap and water. Animals were observed daily for clinical signs/mortality; signs of skin irritation were assessed daily. Body weights were recorded once weekly. Haematological, clinical chemistry and urinary parameters were measured prior to initiation of the study and at the end of the study. The animals were killed at the end of the study, their organs weighed and subjected to gross and microscopic examination.

No treatment-related clinical signs were observed, nor treatment-related effects on mortality, skin irritation, body weight, body weight gain, food consumption, clinical chemistry, urinalysis or organ weights. At the end of the experiment, the erythrocyte counts, Hb levels and Ht values in the 250 mg/kg bw per day males and females were somewhat lower than those measured in the controls and before treatment initiation. This finding applied both to rabbits with intact skin and those with abraded skin. At 250 mg/kg bw per day, spleens were enlarged, dark-coloured and heavier in 4/12 animals. Histopathological examination revealed medium-grade acute splenic congestion at 250 ppm.

The NOAEL was 50 mg/kg bw per day based on reduced erythrocyte, haemoglobin and haematocrit levels, and macroscopic and microscopic findings seen at the LOAEL of 250 mg/kg bw per day (Flucke & Schilde, 1978a).

Study 2
In a GLP dermal toxicity study, groups of five male and five female New Zealand White rabbits received triflumuron (purity 99.5%) in physiological saline solution with added Cremophor EL on the shaved back skin at doses of 0, 100, 300 or 1000 mg/kg bw per day, six hours per day, five days per week, for three weeks. Five additional animals per sex were used to monitor the reversibility of the treatment (a two-week recovery phase after concluding the application) in the control and the highest dose groups. Animals were observed daily for clinical signs/mortality; signs for skin irritation were assessed daily. Body weights and food consumption were recorded once weekly. Haematology, clinical chemistry and urinalyses were performed in all animals prior to initiation and termination of the study. A number of organs were weighed after termination and subjected to histopathological examination which was performed in all animals. Liver pieces were removed for special enzyme measurements.

No treatment-related clinical signs were observed, nor treatment-related effects on mortality, skin irritation, body weight, body weight gain, food consumption, clinical chemistry, urinalysis or organ weights. At 1000 mg/kg bw per day, depressed erythrocyte counts, reduced Ht and lower MCHC (males only) and elevated reticulocyte counts were observed. A slight effect on the red blood cells of the 1000 mg/kg bw per day males (depressed erythrocyte counts and Ht) was still present at the end of the recovery phase. At 300 and 1000 mg/kg bw per day swollen spleens were observed. Histopathological

examination revealed engorgement and pronounced haemosiderosis in the spleen at 300 and 1000 mg/kg bw per day. These effects on spleen were reversible during the 14-day recovery phase.

The NOAEL was 100 mg/kg bw per day based on engorgement and pronounced haemosiderosis in the spleen seen at the LOAEL of 300 mg/kg bw per day (Kroetlinger & Snodgrass, 1990).

(c) Inhalation

Study1

In a non-GLP inhalation toxicity study, male and female Wistar rats (10/sex per dose) were exposed daily for six hours, 15 times over three weeks in three test series (I, II and III) to the following analytically determined concentrations of triflumuron (purity 97.4%–99.2%), present as an easily respirable aerosol in the breathing air of the animals (nose-only exposure). For technical reasons triflumuron was formulated in a mixture of DMSO/Lutrol® (1:1).

Control group	(I) and (II)	0 mg/m^3 air	
	(III)	20 000 µL DMSO/Lutrol®/m^3 air	
Concentration group	(I)	1	9 mg triflumuron/m^3 air
	(I)	2	29 mg triflumuron/m^3 air
	(I)	3	92 mg triflumuron/m^3 air
	(II)	1	3 mg triflumuron/m^3 air
	(II)	2	8 mg triflumuron/m^3 air
	(III)	1	9.3 mg triflumuron/m^3 air
	(III)	2	94.3 mg triflumuron/m^3 air.

Laboratory examinations were performed at the end of the experiment. At termination, gross findings were recorded and organs weighed. A number of organs were examined histopathologically.

The particles administered for inhalation at all concentrations and in the solvent control group were largely within the range that could easily be inhaled by the rat (always > 98% of all particles). In test series I the control animals mainly tolerated exposure to the formulation agents without clinical signs occurring. Only one female rat showed signs of behavioural and breathing disorders after days 3 and 4 of the exposure. This animal died on day 5 of exposure. In test series II and III there were no effects of treatment on behaviour or appearance. In test series I, only the female animals of the highest concentration (92 mg/m^3) exhibited a slightly lower body weight compared to the control animals after the first five exposures (that is after the first test week). However, the body weights of these animals corresponded to the body weights of the control animals during the later course of the test. No treatment-related effects on body weights were observed in test series II and test series III. No treatment-related effects were observed in haematological or clinical chemistry parameters, urinalysis or organ weights. Only in test series I was there histological evidence of inflammatory changes to the respiratory tract. These findings were not reproducible in test series II or III and were thus considered incidental.

The no-observed-adverse-effect concentration (NOAEC) was 94.3 mg/m^3, the highest dose tested (Thyssen, 1979).

Study2

In a non-GLP inhalation toxicity study, groups of 10 male and 10 female rats were exposed to aerosols of aqueous formulation of triflumuron 065 EC 059B at analytically determined preparation concentrations of 71, 282 and 1692 mg/m^3 air for 15 × 6 hours on 15 consecutive workdays in a dynamic inhalation device (nose-only type exposure). These levels were equivalent to active ingredient concentrations of 4.2, 18.05 and 108.29 mg/m^3 air. Groups of 10 additional animals per sex were exposed to pure air as a negative control or to air with 1536 mg/m^3 of triflumuron I 000EC 060 (formulation without active ingredient) as a solvent control. During the three weeks of exposure, body weights, signs and mortality were recorded daily. At the end of the study clinical chemistry, haematology, urine, gross pathological and histopathological examinations were performed.

Approximately 99% of the particles were in the size range 0.4–3.3 μm, that is well within the inhalable range for small mammals (0–5 μm). Nonspecific behavioural disorders (inactivity, ungroomed hair/coat) were observed throughout the study in the rats in the high concentration group. One female in the 71 mg/m^3 died during the weekend. No other mortalities were observed. Body weights and body weight gains were unaffected by the treatment. At 1692 mg/m^3, elevated reticulocyte and thrombocyte counts, depressed erythrocytes, Hb and Ht were observed in both sexes. There was a decrease in MCHC in the females. At 282 mg/m^3, elevated reticulocyte counts, depressed Hb and Ht were observed in both sexes. At 282 and 1692 mg/m^3, the plasma urea levels were decreased in both sexes. No variations from the physiological norm were seen in the rat urine from any group. At 1692 mg/m^3, increases in absolute lung, liver, spleen and kidney weights were observed in both sexes. At 282 mg/m^3 increases in absolute kidney weights was observed in females only. The kidneys in all rats sacrificed at the end of the experiment had lightly spotted or mottled surfaces. Dark-coloured spleens were seen in male rats of the high-concentration group and in female rats of the medium- and high-concentration groups. No effects were noted in histopathological examinations.

The NOAEC was 71 mg/m^3 (equivalent to 4.5 mg triflumuron/m^3) based on elevated reticulocyte counts, depressed hemoglobin and hematocrit, decreases in plasma urea levels and increased absolute kidney weights, seen only in females at the LOAEC of 282 mg/m^3 (equivalent to 18.05 mg triflumuron/m^3) (Thyssen & Mohr, 1981).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a non-GLP study of carcinogenicity, groups of 50 male and 50 female BOR:CFW1 mice received triflumuron (purity 94.9%) via diet for 24 months at a concentration of 0, 20, 200 or 2000 ppm (equal to 0, 5.19, 49.0 and 523.3 mg/kg bw per day in males, 0, 6.68, 67.9 and 691.6 mg/kg bw/day in the females). In addition, 10 mice/dose per sex were employed for determination of the haematological and clinical chemistry parameters at 6, 12, and 18 months. After blood samples had been obtained, the mice were terminated and necropsied. All animals were examined twice a day (once a day on weekends and holidays). The body weights of the mice were determined either weekly (on weeks 1–14 and 88–end of study) or every three weeks (on weeks 15–87). Weekly feed consumption was determined by weighing back the unconsumed feed. Necropsy and gross examination were performed on the mice that died or were terminated when moribund during the experiment. After 24 months mice were terminated and necropsy and gross examination performed. The weights of the following organs were determined: brain, heart, testes, liver, lungs, spleen, kidneys and ovaries. Histopathological examination was conducted on all animals.

No treatment-related clinical signs of toxicity were observed at doses up to and including 2000 ppm. Mortality and feed consumption were unaffected by the treatment. At the 2000 ppm dose level, there was a transitory delay in the body weight development of male mice in comparison to controls. There was no indication of treatment-related damage to the blood or haemopoietic tissues of males or females in the 20 ppm groups. Haematological findings that were observed at 12 and 24 months are summarized below:

Findings after 12 months

200 ppm	males	Haemoglobin and haematocrit values reduced ($p < 0.01$); reticulocytes increased (32% vs 28% control)
2000 ppm	males	Erythrocytes and haematocrit reduced ($p < 0.01$); leukocytes ($p < 0.01$), reticulocytes (78% vs 28% control); MCH and MCHC values increased ($p < 0.01$)
2000 ppm	females	Leukocytes and MCV values increased ($p < 0.01$); increased reticulocytes (47% vs 41% control)

(*continued on following page*)

Findings after 24 months

200 ppm	males	Thrombocytes reduced ($p < 0.05$); Heinz bodies increased (81% vs 3% controls)
200 ppm	females	Reticulocytes (34% vs 23% controls); Heinz bodies (125 vs 3 control); MCV and MCH values increased ($p < 0.01$)
2000 ppm	males	Reticulocytes (85% vs 28% controls); Heinz bodies (371 vs 3 controls); MCV and MCHC increased ($p < 0.01$)
2000 ppm	females	Erythrocyte values reduced ($p < 0.05$); reticulocytes (54% vs 23% controls), Heinz bodies (397 vs 3 controls); MCV, MCH and MCHC values increased ($p < 0.01$)

Heinz body formation was evaluated at 18 and 24 months only. No toxicologically relevant shifts in the leukocyte differential count were observed.

Table 15. Haematological changes in mice at 24 months (terminal kill)

	Males				Females			
Dose (ppm)	**0**	**20**	**200**	**2000**	**0**	**20**	**200**	**2000**
Leucocytes (10^9/L)	7.1	6.3	5.6	11.8	5.8	7.3	5.5	7.4
Erythrocytes (10^{12}/L)	7.86	8.95*	8.82	7.27	8.01	7.61	7.68	7.16*
Haemoglobin, Hb(G/L)	130	147	158*	144	148	133	148	150
Mean corp. volume, MCV (fL)	57	57	58	63**	57	58	59*	63**
Thrombocytes (10^9/L)	1244	978	908	960	752	767	642	747
Reticulocytes 0/00	28	26	35	85**	23	28	34**	54**
Haematocrit, Ht (L/L)	0.45	0.51	0.51	0.45	0.46	0.44	0.45	0.45
Mean corpuscular Hb, MCH (pg)	16.6	16.5	17.0	28.8**	17.5	17.5	18.5**	21.8**
Mean corp. Hb content, MCHC (g/L)	289	288	294	319**	386	384	313	336**

* $p \leq 0.05$ ** $p \leq 0.02$

At 24 months the bilirubin concentration for male mice at 200 ppm and higher was increased in comparison to control. For the females, 2000 ppm resulted in an increased bilirubin concentration at 18 and 24 months in comparison to the control. The fluoride concentration in the bones and teeth of mice treated at dose levels up to and including 200 ppm was comparable to that of control. At 2000 ppm, statistically significant increases in fluoride concentration were found in the teeth and bones of male mice at 12 and 24 months and, in the case of females, in the teeth at 12 months and in the bones at 24 months. The statistically significant increase in fluoride concentration found at 24 months in the case of males of the 20 ppm dose group in comparison to the control is regarded as a random occurrence since it is not dose-related. For female mice in the 2000 ppm dose group, increased numbers of enlarged spleens were observed. At 2000 ppm the absolute and/or relative spleen and liver weights of male and female mice for the most part, were higher than control at 6, 12, and 18 months. At 12 months, increased pigment deposits in comparison to the control were observed in the spleen at dose levels of 200 ppm and in the liver and spleen at 2000 ppm. At 24 months, increased haemosiderin pigment storage in the spleen in comparison to controls was observed in both sexes at 200 ppm and increased haemosiderin pigment storage in spleen, liver and bone marrow plus hyperplasia of the bone marrow in both sexes at 2000 ppm. The number, type, location and distribution of the neoplasms found in the study groups did not provide any indications of carcinogenic effects due to triflumuron.

The NOAEL for systemic toxicity was 20 ppm (equal to 5.19 mg/kg bw per day) based on haematological changes (reduced thrombocyte, Heinz bodies increased in males, and reticulocytes, Heinz bodies, MCV and MCH values increased in females) and histopathological findings (increased haemosiderin pigment storage in the spleen in both sexes) seen at the LOAEL of 200 ppm (equal to 49.0 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 523.3 mg/kg bw per day) the highest dose tested (Suberg & Kroetlinger, 1984, 1988).

Rat

In a non-GLP study of chronic toxicity/carcinogenicity, groups of 50 male and 50 female Wistar (BOR:WISW) rats received triflumuron (purity 95.3%) via diet for 24 months at a concentrations of 0, 20, 200 or 2000 ppm (equal to 0, 0.82, 8.45 and 86.1mg/kg bw per day in males, 0, 1.11, 11.2 and 110 mg/kg bw/day in the females). In addition, 10 rats/dose per sex were employed for intermediate necropsy after one year. All animals were examined twice a day (once a day on weekends and holidays). Body weights of the rats were determined weekly for the first 27 weeks, and then every two weeks until termination. The weekly feed consumption was determined by weighing back the unconsumed feed. Clinical laboratory tests (haematology, clinical chemistry and urinalysis) were conducted on 10 males and 10 females from each test group at 6, 12, 18, and 24 months after the start of the feeding study. A determination of fluoride in the teeth and bones was conducted on five rats per sex of the 2000 ppm and control groups at 12 months, and on five rats per sex of all groups at the end of the study. Necropsy was performed on all rats that died during the study (or were terminated due to moribund condition), interim terminations and at termination. The weights of the following organs were determined: brain, heart, testes, liver, lungs, spleen, kidneys, adrenals, thyroids and ovaries. Histopathological examination was conducted on all animals.

No differences from the control rats were found in the appearance and behaviour of the rats that received triflumuron. Mortality, body weight, body weight gain, feed consumption, clinical chemistry and urinalysis were not affected by treatment with triflumuron. Haematological effects observed at 12 and 24 months are summarized in Table 16. Briefly, at 12 months at 200 ppm, leukocytes, erythrocytes and Hb values were reduced and MCV increased in males, while erythrocytes, Hb and MCHC values reduced and MCV values increased in females. At 2000 ppm, erythrocytes, Hb, Ht and MCHC values were reduced and leukocytes, reticulocytes, MCV and MCH values increased in males, while erythrocytes, Hb,Ht, MCHC and monocyte values were reduced and leukocytes, reticulocytes, thrombocytes and MCV and MCH values increased in females.

At 24 months and 200 ppm, reticulocytes were increased in males and leukocytes, erythrocytes, Hb and Ht values reduced in females. At 2000 ppm, erythrocytes, Hb and Ht values were reduced and thrombocytes, reticulocytes, MCV and MCH values increased in males, while erythrocytes, Hb, Ht and MCHC values reduced and leukocytes, reticulocytes, MCV and MCH values increased in females. At 12 months, both sexes of the 2000 ppm group had higher fluoride concentrations in the teeth and bones at both test times, except for male rats at 12 months. Higher concentrations were also found for the 200 ppm dose group, but only in the bones. No significant differences were found following administration of 20 ppm.

Table 16. Summary of haematological findings

	Males				Females			
Dose (ppm)	**0**	**20**	**200**	**2000**	**0**	**20**	**200**	**2000**
12 months								
Leucocytes (10^9/L)	8.4	7.4	7.2*	10.4	5.9	5.8	5.9	7.9**
Erythrocytes (10^{12}/L)	8.68	8.53	8.14**	7.39**	7.77	7.71	7.35**	6.15**
Haemoglobin, Hb(G/L)	158	153	153	141**	151	145**	147*	128**
Mean corp. volume, MCV (fL)	58	58	61*	64**	59	59	64**	67**
Thrombocytes (10^9/L)	885	873	827	823	816	839	872	1041**
Reticulocytes 0/00	17	20	19	51**	18	17	20	87**
Haematocrit, Ht (L/L)	0.50	0.49	0.50	0.47*	0.46	0,45	0.47	0.41**
Mean corpuscular Hb, MCH (pg)	18.2	17.9	18.8	19.1*	19.4	18.8	20.0	20.8*
Mean corp. Hb content, MCHC (g/L)	315	310	308	298**	327	319	312**	310**

(*continued on following page*)

	Males				Females			
Dose (ppm)	0	20	200	2000	0	20	200	2000
24 months								
Leucocytes (10^9/L)	10.2	9.4	9.0	8.9	5.4	4.7	4.3*	9.4*
Erythrocytes (10^{12}/L)	8.53	8.42	8.43	7.53**	8.13	7.91	7.23**	6.15**
Haemoglobin, Hb(G/L)	167	169	168	156**	161	157	148**	137**
Mean corp. volume, MCV (fL)	61	62	62	65**	61	61	63	72**
Thrombocytes (10^9/L)	772	818	760	918**	819	789	773	799
Reticulocytes 0/00	16	23	21**	37**	21	16*	17	70*
Haematocrit, Ht (L/L)	0.52	0.52	0.52	0.49*	0.50	0.48*	0.46**	0.44**
Mean corpuscular Hb, MCH (pg)	19.6	20.2	20.0	20.7**	19.8	19.9	20.5	22.4**
Mean corp. Hb content, MCHC (g/L)	322	326	323	320	323	327	323	312**

* $p \leq 0.05$ ** $p \leq 0.02$

For rats in the 2000 ppm dose group, enlarged spleens were observed at both examination times. Both males and females of all dose groups had statistically significantly higher spleen weights (absolute and relative to body weight) at 12 months (relative weight change not significant for 20 ppm females). In the absence of any other changes, the effects on spleen weight at 20 ppm were not considered toxicologically relevant. At the end of the experiment the organ weights in the groups up to and including 200 ppm showed no relevant and/or dose-related differences from controls. At the end of the study both male and female rats in the 2000 ppm dose group had higher spleen weights, absolute and relative to body weight. In addition, higher lung, kidney, and ovary weights were found for females only at 2000 ppm after 24 months. Increased deposits of pigment (haemosiderin) were detected in the spleen and liver of rats in the 2000 ppm dose group. Haemopoiesis in the spleen was increased. The number, type, location and distribution of neoplasms found in the study groups did not provide any indication of carcinogenic effects due to triflumuron.

The NOAEL for systemic toxicity was 20 ppm (equal to 0.82 mg/kg bw per day) based on haematological effects (reticulocytes increased in males; leukocytes, erythrocytes, Hb and Ht values reduced in females), higher fluoride levels in bones, and increases in spleen weight seen at the LOAEL of 200 ppm (equal to 8.45 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 86.1 mg/kg bw per day) the highest dose tested (Kroetlinger & Patton, 1984; Kroetlinger, 1988).

2.4 Genotoxicity

The results from studies of genotoxicity due to triflumuron are summarized in Table 17.

Table 17. Summary of genotoxicity in vitro

Test system	Test object	Concentration	Purity (%)	Result	Reference
In vitro					
Point mutation assays					
Salmonella microsome test	*S. typhimurium* TA98, TA100, TA1535, TA1537	Up to 2500 µg/plate ± S9 mix in DMSO	95.1	negative	Herbold, 1979[§]
	S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	Up to 10 000 µg/plate ± S9 mix in DMSO	99.6	negative	Lawlor & Wagner, 1988
	S. typhimurium TA98, TA100, TA1535, TA1537	Up to 5000 µg/plate ± S9 mix in DMSO	99.3	negative	Herbold, 1991

Test system	Test object	Concentration	Purity (%)	Result	Reference
Reverse mutation assay	*Saccharomyces cerevisiae* S138, S211α	Up to 10 000 µg/mL ± S 9 mix in DMSO	99.8	negative	Hoorn, 1983a
Mitotic crossing over and gene conversion assay	*Saccharomyces cerevisiae* D7	33.3–10 000 µg/mL ± S 9 mix in DMSO	99.8	negative	Hoorn, 1983b
CHO/HGPRT assay	Chinese hamster ovary cells	Up to 300 µg/mL ± S 9 mix in DMSO	99.04	negative	Harbell, 1988
	Chinese hamster ovary cells	Up to 100 µg/mL ± S 9 mix in DMSO	99.5	negative	Lehn, 1989
DNA Damage					
Pol test	*E. coli*	Up to 2500 µg/plate ± S 9 mix in DMSO	99.8	negative	Herbold, 1983[§]
Unscheduled DNA synthesis assay	rat primary hepatocytes	Up to 1000 µg/mL in DMSO	99.04	negative	Curren, 1988
Chromosome damage					
Sister chromatid exchange assay	Chinese hamster ovary cells	up to 50 µg/mL ± S 9 mix in DMSO	99.04	negative	Putman, 1988
Chromosome aberration assay in human lymphocytes	human lymphocytes	10–80 µg/mL ± S 9 mix in DMSO	99.3	negative	Heidemann 1992
In vivo					
Chromosome damage					
Micronucleus test	NMRI mice (male/female)	2 × 200 mg/kg bw 2 × 400 mg/kg bw in 0.5% Cremophor	95.1	negative	Herbold, 1978a[§]
Dominant lethal test	NMRI mice (male)	400 mg/kg bw in 0.5% Cremophor	95.1	negative	Herbold, 1978b[§]

DMSO Dimethyl sulfoxide

[§] Non-GLP study

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a non-GLP multigeneration reproduction study, groups of 10 male and 20 female Wistar rats (BOR:WISW) were given diets containing triflumuron (purity 95.3%) at nominal doses of 0, 20, 200 or 2000 ppm (equivalent to 0, 1.32, 13.2 or 132 mg/kg bw per day) for three generations (two litters per generation). The rats were weighed before and after mating in a weekly rhythm. F0 animals were weighed weekly during first mating and every three days during second mating. F1b and F2b rats were weighed every three days during both matings. Each litter was weighed immediately after birth, on the fifth day after birth, after reduction to ten pups per birth, and after 1, 2, 3 and 4 weeks. The rats which died during the study were dissected to establish cause of death. The four-week-old pups in the F3b generation and their parents (F2b generation) were anaesthetized with ether, killed by exsanguination and then autopsied. Selected organs were examined macroscopically. Histopathological examination was conducted on the selected organs.

The rats in the 20–2000 ppm dose groups did not differ in appearance or behaviour from the controls during the study period. Mortality and body weights and body weight gains were unaffected by the treatment. No dose-related differences in fertility, viability, lactation, litter size, birth weight or ratio of males to females were observed between treated and untreated animals at doses up to and including 2000 ppm. No increase in malformations was observed in any generation. Organ weights were unaffected by the treatment. No treatment-related effects were detected macroscopically or microscopically.

The NOAEL for parental systemic toxicity, reproductive toxicity and offspring toxicity was 2000 ppm (equivalent to 132 mg/kg bw per day) the highest dose tested (Loeser, Eiben & Janda, 1983: Eiben, 2003).

(b) Developmental toxicity

Rat

In a developmental toxicity study (non-GLP-compliant), Long–Evans rats (25 per dose) received triflumuron (purity 91.7%) once daily by gavage at 0, 10, 30 or 100 mg/kg bw per day in 0.5% aqueous Cremophor emulsion during gestation days (GDs) 6–15. Fetuses were delivered by caesarean section on GD 20.

No treatment-related clinical signs of toxicity were observed at doses up to and including 100 mg/kg bw per day. There was no mortality during the study. Body weight and body weight gain were unaffected by the treatment. No treatment-related embryotoxicity or teratogenic effects were observed in any treated groups. No treatment-related effects were observed on implantation rate, litter size, resorption rate, mean fetal and placental weight, nor on malformations.

The NOAEL for maternal and embryotoxicity was 100 mg/kg bw per day; the highest dose tested (Schlueter, 1981).

In a GLP-compliant developmental toxicity study, Wistar rats (25 per dose) received triflumuron (purity 99.4%) once daily by gavage at 0, 100, 300 or 1000 mg/kg bw per day in 0.5% aqueous Cremophor EL emulsion during GDs 6–15. In addition, satellite groups of five rats each were treated at 0 or 1000 mg/kg bw per day. Blood samples were obtained from these rats on GD 16 for haematological examinations and spleens were removed and weighed. Fetuses were delivered by caesarean section on GD 20.

No changes in the appearance and behaviour of the dams that could be regarded as a result of treatment were found during the daily observations. None of the animals died during the test period. Body weight gain and feed consumption were comparable in all groups. There were significant reductions in Hb, erythrocyte counts and Ht of rats treated at 1000 mg/kg bw per day; the increase in spleen weight was not significant. Triflumuron doses up to and including 1000 mg/kg bw per day had no effect on pregnancy rates, number of corpora lutea, number of live fetuses per litter, number of resorptions per litter, mean fetus weight and placental weight per litter, crown-rump length, number of stunted fetuses per litter or number of fetuses with malformations per litter. There was a slight treatment-related increase in the number of fetuses with slight skeletal changes (primarily delayed ossifications) in the 1000 mg/kg bw per day group. No treatment-related findings were noted on macroscopic examination.

Table 18. Summary of fetal effects in rat developmental toxicity study

Dose (mg/kg bw per day)	Mean weight (g)	Mean placental weight (g)	Fetuses with minor variations	Fetuses with malformations
Controls	3.55 ± 0.20	0.60 ± 0.06	1.88 ± 1.51	0.13 ± 0.34
100	3.54 ± 0.25	0.57 ± 0.05	2.56 ± 1.66	0.16 ± 0.47
300	3.49 ± 0.29	0.58 ± 0.05	1.96 ± 1.63	0.21 ± 0.66
1000	3.53 ± 0.24	0.60 ± 0.06	2.88 ± 1.86*	0.24 ± 0.83

* $p = < 0.05$ Source: Renhof, 1987a

Table 18 above summarizes the significant finding for this study. It was not possible to identify a NOAEL for maternal toxicity as no haematological measurements were conducted at 100 and 300 mg/kg per day and effects were seen at 1000 mg/kg bw per day The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day based on slight skeletal variations (delayed ossifications) seen at the LOAEL of 1000 mg/kg bw per day (Renhof, 1987a).

Rabbit

Study1

In a developmental toxicity study (not GLP-compliant), Himalayan rabbits (15 per dose) received triflumuron (purity 91.7%) once daily by gavage at 0, 10, 30 or 100 mg/kg bw per day in 0.5% aqueous Cremophor emulsion during GDs 6–18. Fetuses were delivered by caesarean section on GD 29.

Haematological analysis was not conducted in this study. No treatment-related clinical signs of toxicity were observed at doses up to and including 100 mg/kg bw per day. There was no mortality in the study. Body weight and body weight gain were unaffected by the treatment. No treatment-related embryotoxicity or teratogenic effects were observed in any treated group. Triflumuron doses up to and including 100 mg/kg bw per day had no effects on implantation, litter size, resorption, fetus weight, placental weight, incidence of fetuses with slight alterations of bone development, or malformations.

Under the conditions of the study, the NOAEL for maternal and embryo/fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Schlueter, 1981).

Study2

In a developmental toxicity study (GLP-compliant), Himalayan rabbits (20 per dose) received triflumuron (purity 99.4–99.5%) once daily by gavage at 0, 100, 300 or 1000 mg/kg bw per day in 0.5% aqueous Cremophor EL emulsion during GDs 6–18. In addition, satellite groups of five rabbits each were treated at 0 or 1000 mg/kg bw per day. Blood samples were obtained from these rabbits on GD 19 for haematological examinations and their spleens were removed and weighed. Fetuses were delivered by caesarean section on GD 29.

No changes in the appearance and behaviour of the dams that could be regarded as a result of treatment were found during daily observations. One animal from the 1000 mg/kg bw per day group died. According to the study report the animal had a bloody nose presumably following a fight with an animal in an adjacent cage. There were no other deaths in the study. Body weight gain and feed consumption were comparable in all groups. There were significant reductions in the Hb, erythrocyte counts, Ht and elevation of the leukocytes in rabbits treated with 1000 mg/kg bw per day and their spleen weights were increased. There was a significant increase in the resorption (late resorptions) in the 1000 mg/kg bw per day group. The number of resorptions was 0.7 ± 0.9, 1.5 ± 2.5**, 1.3 ± 1.3 and 2.5 ± 3.1*** at 0, 100, 300 and 1000 mg/kg bw per day, respectively (*$p = < 0.05$, **$p = < 0.025$ and ***$p = < 0.001$).Triflumuron doses up to and including 1000 mg/kg bw per day had no effect on pregnancy rates, number of corpora lutea, number of live fetuses per litter, mean fetus weight and placental weight per litter, crown-rump length, number of stunted fetuses per litter, or number of fetuses with malformations per litter.

It was not possible to identify a NOAEL for maternal toxicity as no haematological measurements were conducted at 100 and 300 mg/kg per day and effects were seen at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day based on increased resorption rate (late) seen at the LOAEL of 1000 mg/kg bw per day (Renhof, 1987b).

2.6 Special studies

(a) Methaemoglobin formation

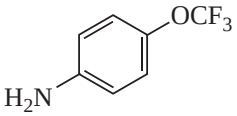
In order to determine whether methaemoglobin formation in erythrocytes following ingestion of triflumuron (SIR 8514) occurs, the test article (SIR 8514 batch 1616001/78) was formulated in distilled water and Cremophor EL at an application volume of 5 mL/kg bw and then administered orally to two fasted cats at a dose of 500 mg/kg bw. The concentration of methaemoglobin in the blood was measured at 3 h, 5 h and 24 h post administration.

The results show that the test article did not have a methaemoglobin-forming effect following administration of a sufficient dose, that is 500 mg/kg bw (Mihail, 1979).

(b) Studies on metabolite *p*-aminotrifluoroanisol (M07)

The structure of *p*-aminotrifluoroanisol (also known as trifluoromethyl-4-aminophenyl ether; 4-(trifluoromethoxy)aniline, KLU 2996 B, M07, AE F069069 and TNO/W 74) is shown in Fig. 3.

Figure 3. Structure of p-aminotrifluoroanisol



Rat

In a non-GLP acute oral toxicity study, fasted Wistar TNO/W 74 (15/sex per dose) rats were administered *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) once by gavage, emulsified in polyethylene glycol 400, at 0, 1, 10, 25, 75, 100, 150 or 250 mg/kg bw in males and at 0, 1, 10, 25, 50, 75, or 100 mg/kg bw in females. Treated rats were observed for 14 days.

Signs apparent in the test animals, observed in all treatment groups, were apathy, laboured breathing, cyanosis and spastic gait (staggering and lying on side/prostration in the higher doses). The signs occurred within one hour following treatment and lasted four to seven days. Mortalities in males were 5, 8, 13 and 15, at 75, 100, 150 and 250 mg/bw, respectively. Mortalities in females were 4, 9, 14 and 15, at 50, 75, 100, and 100 mg/bw, respectively.

Necropsy of the animals that died revealed a clay to rusty brown coloring of the internal organs (most strongly defined in the lung). There were isolated cases of emphysema of the lung or confluent ulceration of the mucous membrane of the glandular stomach. Apparent among the rats killed at the end of the post-treatment observation period were dark, swollen spleens with a clear follicle structure.

The acute oral LD_{50} of *p*-aminotrifluoroanisol to male rats was 102 mg/kg bw (range 83–128 mg/kg bw); for females this was 63 mg/kg bw (range 54–74 mg/kg bw) (Mihail & Thyssen, 1980).

Dermal

In a non-GLP acute dermal toxicity study, Wistar TNO/W 74 rats (10/sex per dose, except females at 100 mg/kg bw) were dermally treated with *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) at 25, 50, 75, 100 and 250 mg/kg bw for males and 25, 50, 75 or 100 mg/kg bw for females. The test substance was applied concentrated on the intact dorsal skin. The treated skin areas were covered with occlusive dressing for 24 h. Upon removal of the occlusive dressings the test compound was removed from the treated skin areas by washing with soap and water. The rats were kept under observation for up to 14 days.

Dermal applicaion of the test article caused similar signs to the oral treatment: apathy, shallow, faster breathing, cyanosis, lying on side/prostration. The time at which the signs occurred was short for this type of treatment (12–35 minutes). Mortalities in males were 2, 7, 9 and 10, at 50, 75, 100, or 250 mg/kg bw, respectively. Mortalities in females were 8, 10 and 5, at 50, 75 or 100 mg/kg bw, respectively. Gross necropsy of the rats which died revealed discolored lungs (clay-coloured) and isolated cases of emphysema of the lung and slightly enlarged thymus glands. There were no findings in animals terminated at the end of the study.

The acute dermal LD_{50} of *p*-aminotrifluoroanisol to male rats was 64 mg/kg bw (range 52–79 mg/kg) and the range 25–50 mg/kg bw given for female rats (Mihail & Thyssen, 1980).

Inhalation

In a non-GLP acute inhalation toxicity study, Wistar TNO/W 74 fasted rats (5/sex per dose) were exposed once to *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) via inhalation via dynamic flow inhalation chamber (head/nose exposure) at 200 L air/hour passed through approximately 50 g of the test article. The rats were held in the inhalation chamber for 10 minutes, 30 minutes or for one hour. The post-treatment observation period lasted up to 15 days.

There was no mortality in rats exposed to the test article for 10 or 30 minutes. After 1 h of exposure to the test material two males and four females died within threevdays. Immediately after exposure, all rats showed behavioural signs such as apathy, ungroomed fur, and piloerections. Following longer exposure (> 30 minutes) the animals showed signs of staggering, later sedation and were lying on their sides or prostrated. Behavioural and respiratory disorders were observed up to day 14 of the observation period. The remaining signs could only be observed during the first day of the observation period. The visible mucosa of the eyes and nose were irritated. At necropsy the rats that died revealed dark brownish-coloured lungs. The lungs showed signs of mottled changes. There were some cases of emphysema, and in some cases the lungs had a solid, liver-like consistency. A small amount of fluid was present in the thoracic cavity. The liver showed signs of mottled changes. At termination, in a small number (3/24) there were still slight emphysematous lung changes present. The LC_{50} of the test material was determined in the study described below (Mihail & Thyssen, 1980).

In a non-GLP acute inhalation toxicity study, Wistar TNO/W 74 rats (10/sex per dose) were exposed once to *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) via inhalation via dynamic flow inhalation chamber (head/nose exposure; exposure to vapours only) at 1–3 L air/minute passed through a sintered wash bottle. The rats were exposed to vapour for 4 h at 241, 519, 860, 950 and 1185 mg/m^3. Treated animals were observed for 21 days.

The skin of the animals was bluish colored for up to five days. Behavioural and respiratory disorders were observed for up to 16 days following exposure. Mortalities in males were 3 and 7 at 950 and 1185 mg/m^3 respectively. Mortalities in females were 1, 9 and 8 at 860, 950 and 1185 mg/m^3, respectively. The following findings were made on necropsy of the rats that had died:

- lungs; dark brown, mottled changes, emphysema;
- liver; mottled changes; spleen: pale discoloration; contents of small intestine; reddish slime;
- kidneys; pale discoloration;
- fluid in the thoracic cavity.

At termination slight emphysema of the lungs was observed and in some cases pale pink coloured lungs with slight emphysematous changes were also observed.

The 4 hour inhalation LC_{50} was 950–1185 mg/m^3 in male rats and 860–950 mg/m^3 in female rats (Mihail & Thyssen, 1980).

Skin irritation

In a non-GLP skin irritation study, New Zealand White rabbits (two rabbits, unspecified sex) were exposed to 0.5 g of the test substance *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) on fur-free ears for 8–24 h. The material was held in contact with the ear by means of an adhesive dressing. The post-treatment observation period was seven days.

No skin changes were observed after the dressings were removed or during the post-treatment observation period.

Under the conditions of the study, *p*-aminotrifluoroanisol was non-irritating to the rabbit's skin (Mihail & Thyssen, 1980).

Eye irritation

In a non-GLP eye irritation study, 50 mg *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) was administered once into the conjunctival sac of two New Zealand White rabbits (unspecified sex). The observation period was seven days.

The conjunctivas were moderately reddened and swollen for up to 24 h after application. The irises were slightly reddened and swollen and corneas slightly opaque. Slight redness of the conjunctiva was observed only during observation days 2 and 3. After this, no further changes were observed in the treated eyes.

p-Aminotrifluoroanisol was moderately irritating to the eyes of rabbits (Mihail & Thyssen, 1980).

Special study; effect of p-aminotrifluoroanisol on cats

In a non-GLP study, purebred domestic cats (unspecified sex; two cats per dose) were administered *p*-aminotrifluoroanisol (M07, chemically pure; batch not stated) via a single gavage dose in polyethylene glycol 400 at 0.5, 1.0 or 2.5 mg/kg bw. Treated cats were observed for 14 days. Blood samples were taken prior to treatment and at various time points after dosing for determination of MetHb. Cats were presumably selected because of their known sensitivity to MetHb-inducing agents.

Slight cyanosis of both cats was observed in the 2.5 mg/mg dose group. The levels of MetHb found are described in Table 19. The results show that the test article had a MetHb-forming effect from a dose of 1.0 mg/kg bw, as the value of 13.3% MetHb (one cat, 3 h value) can clearly be termed pathological. Methaemoglobin concentrations were back within the physiological range 24–48 h after treatment.

Table 19. Methaemoglobin levels in p-aminotrifluoroanisol-treated cats at various time points

Dose (mg/kg bw)	Time of blood sampling				
	Before treatment	3 h	5–6 h	24 h	48 h
	Methaemoglobin concentration determined (%)				
0.5	1.0	3.5	3.2	1.9	2.2
	1.7	5.3	2.9	2.4	2.2
1.0	2.7	3.9	2.5	1.4	1.7
	2.2	13.3	8.7	0.0	2.4
2.5	1.7	59.0	50.0	3.0	3.6
	1.5	48.2	34.1	4.3	2.4

Under conditions of the study, *p*-aminotrifluoroanisol oral exposure showed methaemoglobin formation at a single gavage dose of 1 mg/kg bw and above (Mihail & Thyssen, 1980).

Methaemoglobin formation in cats

Study 1

In a non-GLP single dose oral toxicity study, fasted purebred domestic cats (two males) were administered single gavage doses of *p*-aminotrifluoroanisol (Batch no. 1616001/78; purity not stated) formulated in distilled water and Cremophor EL at a dose level of 500 mg/kg bw. Determination of MetHb concentration was conducted prior to dosing and then 3, 5 and 24 h post dosing.

No methemoglobin was detected at any time point.

The metabolite *p*-aminotrifluoroanisol did not have a methemoglobin-forming effect following administration of a 500 mg/kg bw to male domestic cats (Mihail, 1979).

Study 2

In a non-GLP short-term oral toxicity study, fasted purebred domestic cats (four females) were administered daily gavage doses (five days per week) for two weeks of 0.1 mg/kg bw per day of *p*-aminotrifluoroanisol (M07; purity 99.2%) formulated in polyethylene glycol 400. Another group of five female cats received the test material on consecutive days. On dosage days they were administered eight aliquots of 2.5 mg/kg bw, giving a total of 20 mg/kg bw (averaged to 10 mg/kg bw per day). Determinations were made of blood count, reticulocyte count, number of Heinz bodies, bilirubin and MetHb concentrations.

p-Aminotrifluoroanisol administered as 10 × 0.1 mg/kg bw, did not show any haemotoxic effects or MetHb formation in cats. Administering 8 × 2.5 mg/kg bw doses of *p*-aminotrifluoroanisol caused MetHb formation; 36.86% 3 h after eight doses compared with 0.88% before administration of the test material. There were additional signs of a toxic decomposition of the blood pigment leading to increased numbers of Heinz bodies. The haemotoxic effect resulted in an increase in the reticulocyte count in the blood, a sign of increased blood regeneration (Mihail & Machemer, 1982).

Study 3

In a GLP-compliant study, Wistar Rj:WI (IOPS HAN) rats (20/sex per dose) were administered *p*-aminotrifluoroanisol (AE F069069; purity 97.7%), an animal metabolite of triflumuron. The test substance was administered as a single oral gavage dose at concentrations of 0, 0.5, 2 and 10 mg/kg bw per day. Ten animals/sex per dose were designated for interim termination 24 h post dosing on study day 2 (SD 2), the other 10 rats being designated for final termination five days post dosing (SD 6). Animals were observed for mortality and clinical signs frequently after dosing on first day and daily thereafter. Haematology parameters including MetHb level were determined at 1 h and 24 h post dose for animals designated for interim sacrifice and on SD 6 for animals designated for final termination. All animals were necropsied, selected organs weighed and a range of tissues taken, fixed and examined microscopically.

Up to a dose of 10 mg/kg per day there were no mortalities observed during the study and no effect on body weight parameters. At haematological evaluation no Heinz bodies were found either the control or high-dose groups in either sex throughout the study. No treatment-related clinical signs of toxicity were observed at 0.5 mg/kg bw per day. At 2 mg/kg bw per day, treatment-related clinical signs consisted of cyanosis, dyspnoea and reduced motor activity, the peak-effect time being one hour after treatment. These signs were totally reversible: no clinical signs were observed after two hours in males and three hours in females. At 10 mg/kg bw per day, treatment-related clinical signs consisted of cyanosis, dyspnoea and reduced motor activity (signs of hypoxia commonly encountered with aniline derivates), the peak-effect time being at one hour after dosing; all clinical signs disappeared within 24 h. Body weight and body weight gain remained unaffected in either sex at all doses tested. Food consumption was measured in groups terminated on SD 6 only. In females at 10 mg/kg bw per day, food consumption was slightly reduced, by 8.9%.

One hour after dosing (SD 1), higher MetHb levels were noted at 10 and 2 mg/kg per day in both sexes. In these groups a specific chocolate brown colouration of the blood was observed. At 2 mg/kg bw MetHb levels were 9.6% in males and 19.7% in females. At the high dose of 10 mg/kg bw MetHb concentration was 44.8% in males and 57.7% in females. Other red blood cell parameters were not affected in either sex at this time point at 2 and 10 mg/kg bw.

Twenty-four hours after dosing (SD 2), at 10 mg/kg in females, mean MetHb level (0.9%) was statistically significantly higher when compared to controls. However, as all the individual values were within the range of the MetHb level obtained for the control females of the study, it was considered not to be toxicologically relevant. The change in MetHb was judged to be fully reversible by 24 h after a single treatment. At 10 mg/kg in females, slightly higher mean absolute reticulocyte (+21%, $p \leq 0.05$) and platelet (+31%, $p \leq 0.01$) counts were observed when compared to the control group.

Five days after dosing (SD 6) at 10 mg/kg in females, a lower mean erythrocyte count (−12%, $p \leq 0.01$), Hb concentration (−14%, $p \leq 0.01$) and Ht (−11%, $p \leq 0.01$) were seen when compared with controls. These changes were associated with markedly higher mean absolute reticulocyte counts (+105%, $p \leq 0.01$). Mean platelet counts were also slightly higher (+27%, $p \leq 0.01$). In addition, slightly higher mean absolute reticulocyte counts were noted at 10 mg/kg in males (+24%, $p \leq 0.01$) and at 2 mg/kg in females (+23%, not statistically significant). Twenty-four hours after dosing (SD 2), at 10 mg/kg, mean total leucocyte and absolute neutrophil counts were higher in both sexes. Five days after dosing (SD 6), at 2 mg/kg and 10 mg/kg, mean total leucocyte and absolute lymphocyte counts were lower in males. In view of the variation of individual values in the treated groups (within the normal control range, except one value for absolute lymphocytes in the 2 mg/kg male group on SD 6) and the inconsistency of the results, all the changes noted in white blood cells were considered not to be treatment-related. Throughout the study no Heinz bodies were found in either control or high-dose groups, in either of the sexes.

At 10 mg/kg in females, mean absolute and relative spleen weights were statistically significantly higher compared to the controls. At 0.5 and 2 mg/kg, there was no treatment-related effect. At 2 mg/kg in females, mean spleen:brain weight ratio was statistically significantly higher compared to the controls. Five days after dosing (SD 6), macroscopic examinations revealed enlarged spleens in 6/10 females at 10 mg/kg bw. Twenty-four hours after dosing (SD 2) at 10 mg/kg, a slightly higher severity grade of diffuse extramedullary haematopoiesis was noted in the spleen in females. Five days

after dosing (SD 6) at 10 mg/kg, a higher severity grade of diffuse extramedullary hematopoiesis and hemosiderin pigment were noted in the spleen in both sexes. At 2 mg/kg a slightly higher severity grade of diffuse extramedullary haematopoiesis was noted in the spleen in a few females.

The NOAEL was 0.5 mg/kg bw for rats treated with *p*-aminotrifluoroanisol based on clinical signs, chocolate brown colouration of the blood, higher methemoglobin levels in both sexes, increase in both absolute and relative spleen weight in females and a slightly higher severity grade of diffuse extramedullary haematopoiesis in the spleen in females, seen at the LOAEL of 2 mg/kg bw (Blanck, 2009).

Mutagenicity test results for 4-(trifluoromethoxy)aniline

The results of mutagenicity studies on p-aminotrifluoroanisol are shown in Table 20.

Table 20. Results of mutagenicity studies for p-aminotrifluoroanisol (KLU 2996 B)

Test system	Test object	Concentration	Purity (%)	Result	Reference
In vitro					
Point mutation assays					
Salmonella microsome test	*S. typhimurium,* TA98, TA100, TA1535, TA1537	up to 12 500 µg/plate ± S 9 mix in DMSO	99.4	negative	Herbold, 1984
DNA damage					
Pol test	*E. coli*	up to 10 000 µg/plate ± S 9 mix in DMSO	99.4	negative	Herbold, 1985a
In vivo					
Micronucleus test	NMRI mice (5 male, 5 female)	0.5 mg/kg bw in peanut oil	95.1	negative	Herbold, 1985b

DMSO Dimethyl sulfoxide

(c) Studies on metabolite *N,N′*-bis-(trifluoromethoxyphenyl) urea

Acute oral toxicity of N,N'-bis-(trifluoromethoxyphenyl) urea in rats

In a non-GLP acute oral toxicity study, fasted Wistar rats (WISW SPF/CPB; 10/sex per dose) rats were administered *N,N′*-bis-(trifluoromethoxyphenyl) urea (KRJ 161080; purity 99.9%) once by gavage in DMSO at 0, 25, 50, 60, 75, 100, 125, 150, 200 and 350 mg/kg in males and at 0, 100, 125, 175, 250, 350, 500, 750 and 1000 mg/kg bw in females. At 500 mg/kg bw 20 females were treated instead of the 10 in other dosed groups. Treated rats were observed for 14 days.

Clinical signs of toxicity were observed, such as increased motility, tachypnea, tonic and clonic spasms, periodic tremors. Apathy in the surviving animals was recorded for up to 12 days. Mortalities in females were 2, 5, 4, 6, 16, 8, and 9 at 125, 175, 250, 350, 500, 750 and 1000 mg/kg bw, respectively. Mortalities in males were 1, 1, 4, 5, 7, 7 8, and 9 at 50, 60, 75, 100, 125, 150, 200 and 350 mg/kg bw, respectively.

The acute oral LD_{50} for male rats was 133 mg/kg bw (range 104–171 mg/kg bw) and in females 277 mg/kg bw (range 198–388 mg/kg bw) (Mihail & Machemer, 1982).

Acute oral toxicity of N,N'-bis-(trifluoromethoxyphenyl) urea in cats

In a non-GLP acute oral toxicity study, fasted purebred domestic cats (two male cats per dose) were administered a single dose of *N*,*N'*-bis-(trifluoromethoxyphenyl) urea (KRJ 161080; purity 99.9%) by gavage in DMSO at 100, 250, 500 and 1000 mg/kg bw. Treated cats were observed for 14 days. Blood samples were taken prior to administration, then 24 h and 7 days post dosing for blood counts and bilirubin measurement. Blood samples were also drawn for measurement of MetHb formation at 0, 3, 5, 24 h and at 3 and 7 days after administration.

All treated cats in all dosed groups showed apathy, ungroomed fur, lack of appetite, with vomiting in some cases. There was no mortality. No treatment-related changes in bilirubin or in the formation of MetHb were observed at doses up to and including 1000 mg/kg bw.

The acute oral LD_{50} for domestic cat was > 1000 mg/kg bw and no haemotoxic effects were observed (Mihail & Machemer, 1982).

3. Observations in humans

There is no information indicating or suggesting any health hazards to employees associated with the manufacture or formulation of triflumuron (Hard, 1990).

Occupational medical surveillance of 40 workers exposed to triflumuron during manufacturing was conducted every 1–2 years on a routine basis (laboratory parameters, clinical and technical examinations). Since 1990 no accidents with triflumuron had occurred in workers and no consultation was needed due to contact with triflumuron (Kehrig & Steffens, 2005a). In a separate report, no adverse findings in workers were reported. No triflumuron-related allergenicity could be determined since 1990 in workers exposed to triflumuron during the manufacturing process (Kehrig and Steffens, 2005b).

No medical problems related to handling of triflumuron have been reported to plant or medical department since the start of production in 1994 and until 2018, in total of 50 plant workers (Steffens, 2018).

Comments

Biochemical aspects

Absorption, distribution and excretion of triflumuron [(4-trifluoromethoxy)aniline-UL-^{14}C] was studied in rats at a single gavage dose of 1.98 mg/kg bw (low-dose male), 318 mg/kg bw (high-dose male), unlabelled triflumuron at 3.74 mg/kg bw per day for 14 days, followed by a single oral dose of [^{14}C] triflumuron at 3.74 mg/kg bw by gavage (multiple dose) and 2.59 mg/kg bw in bile-cannulated rats. Oral absorption of triflumuron was estimated to be greater than 77% based on excretion of 41% in bile and 32% in urine, together with 4% in blood and carcass at 48 hours in bile-cannulated rats (Klein, Weber & Suwelack, 1983). The radioactivity was distributed within the body at low concentrations, with the highest levels found in the liver, kidney, spleen, lung and in fatty tissues. No significant differences were seen between the distribution pattern of [chlorophenyl-UL-^{14}C]-triflumuron and that of [(trifluoromethoxy)aniline-UL-^{14}C]-triflumuron (Sietsema, 1985).

Following a single oral administration of 2 mg/kg bw in males, the maximum plasma concentration of radioactivity was reached after 4.9 hours. Elimination was biphasic with half-lives of three and 13 hours in males (Klein, Weber & Suwelack, 1983). Excretion via urine and faeces was essentially complete 96 hours after dosing, with similar amounts excreted in urine and faeces, although female rats excreted slightly less of the radioactivity in urine over a longer period than males (Krolski & Nguyen, 2003).

The half-life in the rat of radiolabelled [2-chlorophenyl-UL-^{14}C]triflumuron following oral dosing was relatively long in erythrocytes (ca 17 days); the radioactivity was primarily present in the globin fraction (76.2%). Triflumuron was rapidly metabolized in rats. A total of 17 components were detected in urine. Unchanged triflumuron was present at only low levels (1–2% of dose) in urine. The main metabolites in urine were identified as 2-hydroxy-4-(trifluoromethoxy)aniline (M09) and 3hydroxy-4-(trifluoromethoxy)aniline (M10) and their sulfate conjugates (M16 and M17 respectively). A total of five components were observed in faecal extracts with the majority of the residue (19% at 3.7 mg/kg bw multiple doses; 91% at 318 mg/kg bw of the administered dose) remaining as unchanged triflumuron. A total of 26 components were observed in bile, with unchanged triflumuron present at only very low levels (< 1% of dose) (Krolski & Nguyen, 2003).

In rats, the major detoxification pathway proceeds initially through hydrolysis of triflumeron's urea moiety to yield 4-(trifluoromethoxy)aniline and chlorobenzoic acid. Minor metabolic pathways include hydrolysis of the 2-chlorobenzamide and direct hydroxylation of the trifluoromethoxyaniline ring prior to excretion. Parent and metabolites may also be conjugated (Krolski & Nguyen, 2003).

Toxicological data

The acute LD_{50} by the oral and dermal routes in the rat was > 5000 mg/kg bw (Flucke & Kimmerle, 1977; Mihaili, 1981; Johnson, 2002a, b). The acute inhalation LC_{50} in rats was > 5.03 mg/L (Pauluhn, 2002). Triflumuron is non-irritating to the skin and eyes of rabbits (Flucke & Kimmerle, 1977; Merkel, 2002a, b). Triflumuron was not sensitizing to the skin of guinea pigs, as determined by the Magnusson and Kligman test (Hixson, 1982; Stropp, 1997). In a single-dose oral toxicity study, rats were administered triflumuron via gavage at a dose of 0, 10, 70 or 500 mg/kg bw. The NOAEL was 500 mg/kg bw, the highest dose tested (Wahle, 2005).

The main target organ of toxicity in short- and long-term studies in mice, rats and dogs was the haematopoetic system (erythrocyte damage). Compensation or regeneration processes (highly active bone marrow, extramedullary haematopoiesis in the spleen and frequent appearance of immature erythrocytes in the peripheral blood) were observed as a result of the erythrocyte damage. Enlarged spleen and haemosiderosis in spleen, liver and kidneys represented secondary effects.

In a 28-day toxicity study in rats, triflumuron was administered once daily via gavage at doses of 0, 30, 100 and 300 mg/kg bw per day. The NOAEL was 100 mg/kg bw per day based on decreases in erythrocytes and elevated reticulocytes and thrombocytes counts, and extramedullary haematopoiesis (splenic) seen in females at the LOAEL of 300 mg/kg bw per day (Flucke & Schilde, 1978).

In a 90-day toxicity study in rats, triflumuron was administered via diet at concentrations of

0, 50, 500 and 5000 ppm (equal to 0, 3.6, 35.5, and 349 mg/kg bw per day for males, 0, 4.5, 47.0 and 449 mg/kg bw per day for females). The NOAEL was 50 ppm (equal to 3.6 mg/kg bw per day) based on increased spleen weight in females, and slight declines in haemoglobin and erythrocytes seen at the LOAEL of 500 ppm (equal to 35.5 mg/kg bw per day) in both sexes (Kroetlinger & Vogel, 1983).

In an another 90-day toxicity study in rats, triflumuron was administered via diet at concentrations of 0, 5, 15 and 45 ppm (equal to 0, 0.34, 1.02 and 3.12 mg/kg bw per day for males, 0, 0.39, 1.18 and 3.63 mg/kg bw per day for females), the NOAEL was 45 ppm (equal to 3.12 mg/kg bw per day), the highest dose tested (Kroetlinger & Vogel, 1983).

In a separate 90-day toxicity study in rats, triflumuron was administered via diet at concentrations of 0, 20, 200 and 2000 ppm (equal to 0, 1.34, 13.9 and 142 mg/kg bw per day for males, 0, 1.52, 15.9 and 149 mg/kg bw per day for females), the NOAEL was 20 ppm (equal to 1.34 mg/kg bw per day) based on decrease in erythrocytes, haemoglobin, haematocrit, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and increase in reticulocytes (females only) seen at the LOAEL of 200 ppm (equal to 13.9 mg/kg bw per day) (Kroetlinger & Janda, 1984).

The overall NOAEL for triflumuron in 90-day toxicity studies in rats was 50 ppm (equal to 3.6 mg/kg bw per day), on the basis of haematological effects (decreased haemoglobin and erythrocytes) seen at 200 ppm (equal to 13.9 mg/kg bw per day).

In a 90-day toxicity study in dogs, triflumuron was administered in the diet at concentrations of 0, 100, 500 or 2500 ppm (equal to 0, 3.21, 17.3 or 85.2 mg/kg bw per day for males, 0, 3.66, 17.1 or 87.7 mg/kg bw per day for females). The NOAEL was 100 ppm (equal to 3.21 mg/kg bw per day) based on decreased haemoglobin and erythrocytes, increased methaemoglobin, reticulocytes and thrombocytes and histological findings (increase in bone marrow cells and extramedullary erythropoiesis in the spleen) at 500 ppm (equal to 17.1 mg/kg bw per day) (Hoffman & Groening, 1980).

In a 12-month toxicity study in dogs, triflumuron was administered at concentrations of 0, 40, 200 or 1000 ppm in the diet (equal to 0, 1.42, 7.1 or 35.3 mg/kg bw per day for males, 0, 1.50, 7.3 or 37.9 mg/kg bw per day for females), the NOAEL was 40 ppm (equal to 1.42 mg/kg bw per day) based on haematological changes (increased reticulocytes), increased absolute spleen weight, pigmentation in liver, kidney and spleen seen at the LOAEL of 200 ppm (equal to 7.1 mg/kg bw per day) (Hoffman & Schilde, 1989a).

In a separate 12-month toxicity study in dogs, triflumuron was administered at concentrations of 0 or 20 ppm in the diet (equivalent to 0 and 0.72 mg/kg bw per day) for a period of 12 months. The NOAEL was 20 ppm (equivalent to 0.72 mg/kg bw per day), the highest dose tested (Hoffman & Schilde, 1989b).

The overall NOAEL for 90-day and 12-month toxicity studies in dogs was 100 ppm (equal to 3.2 mg/kg bw per day). The LOAEL was 200 ppm (equal to 7.1 mg/kg bw per day).

Fluoride levels were elevated in the bones and teeth of rats and mice in chronic studies. No macroscopic or microscopic alterations of the bones or teeth were observed.

In a study of carcinogenicity in mice, triflumuron was administered via diet for 24 months at a concentration of 0, 20, 200 or 2000 ppm (equal to 0, 5.19, 49.0 and 523 mg/kg bw per day in males, 6.68, 67.9 and 692 mg/kg bw/day in females). The NOAEL for systemic toxicity was 20 ppm (equal to 5.19 mg/kg bw per day) based on haematological changes (reduced thrombocytes, increased Heinz bodies in males; reticulocytes, Heinz bodies, MCV and MCH values increased in females) and histopathological findings (increased haemosiderin pigment storage in the spleen in both sexes) seen at the LOAEL of 200 ppm (equal to 49.0 mg/kg bw per day). The NOAEL for carcinogenicity was 2000 ppm (equal to 523 mg/kg bw per day), the highest dose tested (Suberg & Kroetlinger, 1984, 1988).

In a study of chronic carcinogenicity in rats, triflumuron was administered for 24 months via diet at a concentration of 0, 20, 200 or 2000 ppm (equal to 0, 0.82, 8.45 and 86.1 mg/kg bw per day in males, 0, 1.11, 11.2 and 110 mg/kg bw/day in the females). The NOAEL for systemic toxicity was 20 ppm (equal to 0.82 mg/kg bw per day) based on haematological effects (reticulocytes increased in males; leukocytes, erythrocytes, haemoglobin and haematocrit values reduced in females), and increased spleen weights seen at the LOAEL of 200 ppm (equal to 8.45 mg/kg bw per day). The

NOAEL for carcinogenicity was 2000 ppm (equal to 86.1 mg/kg bw per day), the highest dose tested (Kroetlinger & Patton, 1984; Kroetlinger, 1988).

The Meeting concluded that triflumuron is not carcinogenic in mice or rats.

Triflumuron was tested for genotoxicity in a range of in vitro and in vivo assays. Although there were some deficiencies in the studies, no concerns were identified (Curren, 1988; Harbell, 1988; Heidemann, 1992; Herbold, 1978a, b, 1979, 1983; Hoorn, 1983a, b; Lawlor & Wagner, 1988; Lehn, 1989; Putman, 1988).

The Meeting concluded that triflumuron is unlikely to be genotoxic in vivo.

In view of the lack of carcinogenicity in mice and rats, and that it is unlikely to be genotoxic, the Meeting concluded that triflumuron is unlikely to pose a carcinogenic risk to humans from the diet.

In a multigeneration reproduction study in rats, triflumuron was administered via diet at a nominal dose of 0, 20, 200 or 2000 ppm (equivalent to 0, 1.32, 13.2 or 132 mg/kg bw per day) for three generations. The NOAEL for parental systemic toxicity, reproductive toxicity and offspring toxicity was 2000 ppm (equivalent to 132 mg/kg bw per day), the highest dose tested (Loeser, Eiben & Janda, 1983; Eiben, 2003).

In a developmental toxicity study in rats, triflumuron was administered once daily by gavage at 0, 10, 30 or 100 mg/kg bw per day during GD 6–15. The NOAEL for maternal and embryo toxicity was 100 mg/kg bw per day, the highest dose tested (Schluter, 1981).

In a separate developmental toxicity study in rats, triflumuron was administered once daily by gavage at 0, 100, 300 or 1000 mg/kg bw per day during GD 6–15. At 1000 mg/kg bw per day haematological effects were seen in maternal animals, however, a NOAEL for maternal toxicity could not be identified as no haematological measurements were conducted at 100 and 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day based on a slight increase in skeletal variations (delayed ossifications) seen at the LOAEL of 1000 mg/kg bw per day, the highest dose tested (Renhof, 1987a).

In a developmental toxicity study in rabbits, triflumuron was administered once daily by gavage at 0, 10, 30 or 100 mg/kg bw per day during GD 6–18. The NOAEL for maternal and embryo/foetal toxicity was 100 mg/kg bw per day, the highest dose tested (Schlueter, 1981).

In a separate developmental toxicity study in rabbits, triflumuron was administered once daily by gavage at 0, 100, 300 or 1000 mg/kg bw per day during GD 6–18. At 1000 mg/kg bw per day haematological effects were observed in maternal animals, however, a NOAEL for maternal toxicity cannot be identified as no haematological measurements were conducted at 100 and 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day based on increased resorption rate (late) seen at the LOAEL of 1000 mg/kg bw per day (Renhof, 1987b).

The Meeting concluded that triflumuron is not teratogenic.

No neurotoxicity studies are available, however, no evidence of neurotoxicity or neuropathology was observed in any of the studies of systemic toxicity.

The Meeting concluded that triflumuron is unlikely to be neurotoxic.

No evidence of direct immunotoxic effects were observed in the available toxicity studies.

The Meeting concluded that triflumuron is unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

The compound *p*-aminotrifluoroanisole (M07; 4-(trifluoromethoxy)aniline; trifluoromethyl-4-aminophenyl ether; KLU 2996B) is a plant and rat metabolite. It was detected in a rat metabolism study at a trace level.

The LD_{50} of *p*-aminotrifluoroanisole in rats was 63 mg/kg bw (Mihail & Thyssen, 1980). The acute dermal LD_{50} of *p*-aminotrifluoroanisole in rats was 25–50 mg/kg bw (Mihail & Thyssen, 1980). The four hour inhalation LC_{50} in rats was 0.86–0.95 mg/L (Mihail & Thyssen, 1980). It was non-irritating to the skin of rabbits and moderately irritating to the eyes of rabbits (Mihail & Thyssen, 1980). In a limited study, cats were orally administered (gavage) *p*-aminotrifluoroanisole at 0, 0.5, 1.0 and

2.5 mg/kg bw (two cats/dose) and methaemoglobin formation was measured at 0, 3, 6, 24 and 48 hours post dosing. Methaemoglobin formation was observed at a single gavage dose of 1 mg/kg bw and above (Mihail & Thyssen, 1980).

The compound *p*-aminotrifluoroanisole did not have any methaemoglobin-forming effect following single-dose administration of 500 mg/kg bw to male domestic cats (Mihail, 1979).

Following administration of a 0.1 mg/kg bw per day gavage dose (total of 10 doses) no haemotoxic effects or methaemoglobin formation were detected in female domestic cats. After 2.5 mg/kg bw per day gavage dose (total of eight doses) *p*-aminotrifluoroanisole caused haemotoxic effects resulting in methaemoglobin formation and destruction of haemoglobin (Heinz body formation) in female domestic cats (Mihail & Machemer, 1982).

In a toxicity study in rats, *p*-aminotrifluoroanisole was administered as a single oral gavage dose at concentrations of 0, 0.5, 2 and 10 mg/kg bw per day. Animals from one group were killed 24 hours post-dosing and another group at five days post-dosing. The NOAEL of 0.5 mg/kg bw was based on clinical signs, chocolate brown coloration of the blood, higher methaemoglobin levels in both sexes, a slight increase in mean absolute reticulocyte counts in females (not statistically significant), observed one hour post-dosing, and increases in both absolute and relative weight in females and a slightly higher severity grade of diffuse extramedullary haematopoiesis in the spleen of females (termination) seen at the LOAEL of 2 mg/kg bw (Blanck, 2009).

Investigations using the Ames test, an in vitro DNA test and an in vivo micronucleus test indicated *p*-aminotrifluoroanisole was negative for genotoxicity (Herbold, 1984; Herbold 1985a, b).

The metabolite *N*,*N'*-bis(trifluoromethoxyphenyl)urea (technical impurity) has an acute oral LD_{50} to rats of 133 mg/kg bw (Mihail & Machemer, 1982). The acute oral LD_{50} to domestic cats was > 1000 mg/kg bw and no haemotoxic effects were observed (Mihail & Machemer, 1982).

The Meeting concluded that metabolite M02 and metabolite M03 are major rat metabolites and would be covered by the toxicity of the parent compound. No data are available for metabolite M01 and M04 and these metabolites were not detected in rat metabolism studies, therefore the Meeting concluded that the genotoxic TTC value is appropriate for M01 and M04 for dietary exposure assessment. The Meeting also concluded that metabolite M08 would be covered by the toxicity of metabolite M07.

Microbiological data

No data are available.

Human data

Occupational medical surveillance of workers exposed to triflumuron during manufacturing indicate no health hazard to workers (Hard, 1990; Kherig & Steffens, 2005a; Steffens, 2018)

The Meeting concluded that the existing database on triflumuron was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI for triflumuron of 0–0.008 mg/kg bw, based on the NOAEL of 20 ppm (equal to 0.82 mg/kg bw per day) based on haematological effects and increase in spleen weights seen at the LOAEL of 200 ppm (equal to 8.45 mg/kg bw per day), observed in the two-year carcinogenicity study in rats (Kroetlinger & Patton, 1984; Kroetlinger, 1988) and using a safety factor of 100.

The Meeting concluded that it was not necessary to establish an ARfD for triflumuron in view of its low acute oral toxicity, lack of systemic toxicity in a single-dose study at doses up to 500 mg/kg bw, lack of methaemoglobin formation in cats at doses up to 500 mg/kg bw and the absence of any other toxicological effects, including developmental toxicity, that are likely to be elicited by a single dose.

The Meeting established an ADI and ARfD of 0.02 mg/kg bw for 4-(trifluoromethoxy)aniline (M07) on the basis of the NOAEL of 0.5 mg/kg bw based on clinical signs, chocolate brown coloration of the blood, higher methaemoglobin levels in both sexes, and a slight increase in mean absolute

reticulocyte counts in females, observed one hour post-dosing, and increase in both absolute and relative spleen weight in females and a slightly higher severity grade of diffuse extramedullary haematopoiesis in the spleen in females (termination) seen at the LOAEL of 2 mg/kg bw observed in single dose oral (gavage) toxicity study (Blanck, 2009). A safety factor of 25 was used as the effect was C_{max}-dependent.

The Meeting concluded that the ADI and ARfD also cover the toxicity of metabolite M08.

Levels relevant to risk assessment of triflumuron

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equal to 5.19 mg/kg bw per day	200 ppm, equal to 49.0 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 523 mg/kg bw per day[c]	-
Rat	Single-dose studies of toxicity[b]	Toxicity	500 mg/kg bw[c]	-
	90-day studies of toxicity[d]	Toxicity	50 ppm, equal to 3.6 mg/kg bw per day	200 ppm, equal to 13.9 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equal to 0.82 mg/kg bw per day	200 ppm, equal to 8.45 mg/kg bw per day
		Carcinogenicity	2000 ppm, equal to 86.1 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	2000 ppm, equal to 132 mg/kg bw per day[c]	-
		Parental toxicity	2000 ppm, equal to 132 mg/kg bw per day[c]	-
		Offspring toxicity	2000 ppm, equal to 132 mg/kg bw per day[c]	-
	Developmental toxicity study[b]	Maternal toxicity	-	1000 mg/kg bw per day[f]
		Embryo and fetal toxicity	300 mg/kg bw per day	1000 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	-	1000 mg/kg bw per day[f]
		Embryo/fetal toxicity	300 mg/kg bw per day	1000 mg/kg bw per day
Dog	13-week and one-year studies of toxicity[d,e]	Toxicity	100 ppm, equal to 3.2 mg/kg bw per day	200 ppm, equal to 7.1 mg/kg bw per day
Metabolite M07				
Rat	Single dose studyof toxicity	Toxicity	0.5 mg/kg bw	2.0 mg/kg bw

[a] Dietary administration [b] Gavage administration [c] Highest dose tested

[d] Two or more studies combined [e] Capsule administration

[f] NOAEL for maternal toxicity could not be identified as no haematological measurements were measured at lower doses

Acceptable daily intake (ADI), applies to triflumuron, M02, M03, expressed as triflumuron

0–0.008 mg/kg bw

Acute reference dose (ARfD), applies to triflumuron, M02, M03, expressed as triflumuron

Unnecessary

Acceptable daily intake (ADI) applies to 4-(trifluoromethoxy)aniline (M07) and M08, expressed as M07

0–0.02 mg/kg bw

Acute reference dose (ARfD), applies to 4-(trifluoromethoxy)aniline (M07) and M08, expressed as M07

0–0.02 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure. Toxicity studies on plant metabolites and new genotoxicity studies on triflumuron.

Critical end-points for setting guidance values for exposure to triflumuron

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly absorbed and eliminated in urine and faeces, oral absorption ≥ 77% within 48 hours (based on urine, bile and carcass)
Dermal absorption	No data provided
Distribution	Widely distributed (fatty tissue, blood, liver, kidney, lung and spleen)
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Almost completely excreted via urine and faeces within 72 h
Metabolism in animals	In rats, metabolites were formed through hydrolysis followed by subsequent conjugation, or by hydroxylation of the parent compound followed by hydrolysis and/or conjugation
Toxicologically significant compounds in animals and plants	Triflumuron, M07, M08, M01, M02, M03 and M04
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.03 mg/L
Rabbit, dermal irritation	Not irritant
Rabbit, ocular irritation	Not irritant
Guinea pig, dermal sensitization	Not a sensitizer (Magnusson and Kligman test)
Short-term studies of toxicity	
Target/critical effect	Haematopoietic system (reduced erythrocytes count, haemoglobin and haematocrit)
Lowest relevant oral NOAEL	3.6 mg/kg bw per day (rat) 3.21 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	100 mg/kg bw per day
Lowest relevant inhalation NOAEC	0.0045 mg/L (rat, three-week study)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematopoietic system (haemolytic anaemia)
Lowest relevant NOAEL	0.82 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice and rats[a]
Genotoxicity	Unlikely to be genotoxic[a]
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	132 mg/kg bw per day, highest dose tested
Lowest relevant offspring NOAEL	132 mg/kg bw per day, highest dose tested
Lowest relevant reproductive NOAEL	132 mg/kg bw per day, highest dose tested

Developmental toxicity	
Target/critical effect	Delayed ossification (rat) and late resorptions (rabbit)
Lowest relevant maternal NOAEL	None (no assessment of haematological parameters at lower doses)
Lowest relevant embryo/fetal NOAEL	300 mg/kg bw per day (rat, rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No specific studies, no evidence of neurotoxicity in the database
Subchronic neurotoxicity NOAEL	No specific studies, no evidence of neurotoxicity in the database
Developmental neurotoxicity NOAEL	No specific studies, no evidence of developmental neurotoxicity in the database
Immunotoxicity	No data provided

Studies on toxicologically relevant metabolites

Acute toxicity	
4-(trifluoromethoxy)aniline (metabolite M07)	
Rat, LD_{50}, oral	63 mg/kg bw
Rat, LD_{50}, dermal	25–50 mg/kg bw
Rat, LC_{50}, inhalation	0.008–0.009 mg/L
Rabbit, dermal irritation	Not irritant
Rabbit, ocular irritation	Moderately
Genotoxicity	
4-(trifluoromethoxy)aniline (metabolite M07)	Negative for genotoxicity in the Ames test, DNA damage test and in vivo micronucleus test
Human data	No adverse effects in workers

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.008 mg/kg bw[a]	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	Unnecessary		
4-(trifluoromethoxy)aniline (metabolite M07)			
ADI	0.02 mg/kg bw[b]	Single dose study of toxicity (rat)	25
ARfD	0.02 mg/kg bw[b]	Single dose study of toxicity (rat)	25

[a] Applies to triflumuron and M02, M03, expressed as triflumuron

[b] Applies to 4-(trifluoromethoxy)aniline (M07) and M08, expressed as M07

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VALIFENALATE

First draft prepared by
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Explanation

Valifenalate is the ISO-approved common name for methyl-*N*-(isopropoxycarbonyl)-*L*-valyl-(3*RS*)-3-(4-chlorophenyl)-β-alaninate (IUPAC), Chemical Abstracts Service number 283159-90-0. Its structure is shown in Fig. 1.

Valifenalate is a racemic mixture of L-(R)- and L-(S)-valifenalate. It is an antiperonosporic fungicide used to control mildew in many crops including grapes, potatoes and tomatoes. Its pesticidal mode of action is as a cellulose synthase inhibitor.

Valifenalate has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of Codex Committee on Pesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines, unless otherwise stated. A literature search did not identify any toxicological information additional to that submitted for the current assessment.

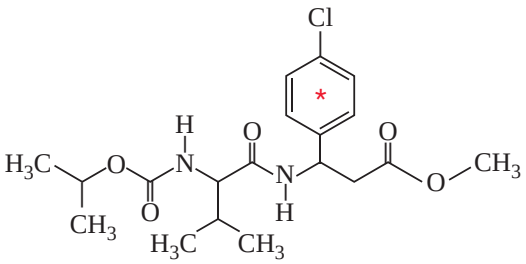
Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

A preliminary study was conducted to provide information on the absorption, distribution and elimination of valifenalate (nonradiolabelled valifenalate, batch number FCF/T/180-00; purity 98.6%. [^{14}C chlorophenyl-valifenalate, batch number 192, radiochemical purity > 97%, specific radioactivity 5.089 MBq/mg). For the preliminary investigation of excretion kinetics, groups of two male and two female Sprague Dawley rats were given a single oral (gavage) dose of 250 mg/kg bw. Urine, faeces, and cage wash were collected at selected time points up to 168 h post dose. Expired air was collected for 24 h post dose. Levels of radioactivity in excreta and tissues were quantified using liquid scintillation counting (LSC). The majority of the radioactivity was excreted in the faeces (88.8% and 72.0% of the total administered dose (AD) for males and females respectively). Urine excretion accounted for a mean of 10.5% for males and 26.3% for females. The amount of radioactivity in the expired air accounted for a small proportion of the administered dose (≤ 0.01%). Radioactivity was excreted relatively rapidly, with up to 95% of the dose recovered within 24 h. The total radioactivity was quantitatively recovered by the end of the collection period (100.1% and 100.5% for males and females, respectively). Levels of radioactivity in tissues at 168 h were below the limit of reliable measurement, consistent with the observed complete excretion of the dose by that time. For the preliminary investigation of blood kinetics, a further two male and two female rats were given a single oral dose of 250 mg/kg bw. Blood samples were collected at intervals up to 72 h and levels of radioactivity were determined by LSC. Mean levels of total radioactivity decreased to 8.3 and 4.2 µg eq/mL at six h for males and females respectively, and were at or near the limit of reliable measurement by 24 h post dose (Cameron & Nong, 2002).

Figure. 1. Structure of valifenalate and position of [^{14}C] radiolabel



* denotes position of [^{14}C] radiolabel

A definitive study was conducted in the rat following single and repeated oral administration of valifenalate (nonradiolabelled, batch number FCF/T/180-00; purity 98.9%. [^{14}C]-valifenalate, batch number 199, radiochemical purity > 97%, specific radioactivity 4.435 MBq/mg) (Kidd & Gedik, 2003). Values are expressed as µg equivalents of [^{14}C]-valifenalate/g tissue and as a proportion of the administered dose. The study was conducted in 10 phases, with each phase conducted in groups of 4 male and 4 female rats. These 10 phases consisted of: mass balance studies with single low (phase 1), single high (phase 2) or repeated low (phase 3) doses; kinetic studies with single low (phase 4), single high (phase 5) or repeated low (phase 6) doses; tissue distribution studies with single low (phase 7), single high (phase 8) or repeated low (phase 9) doses; biliary elimination study with single low dose (phase 10).

In mass balance studies (phases 1, 2 and 3), rats were treated with single low (100 mg/kg bw) or high (1000 mg/kg bw) doses, or repeated low oral doses (14 unlabelled doses followed by a single radiolabelled dose). Urine (up to 72 h post dose), faeces (up to 72 h post dose), expired air (up to 24 h post dose), cage wash (up to 72 h post dose), gastrointestinal tract (plus contents) and carcass (at 72 h) were analyzed for radioactivity. The mean total recovery of the administered radioactivity for these groups ranged from 92.2% to 101.0%. The main route of elimination of total radioactivity for both males and females was in the faeces, however the amount of radioactivity excreted in urine was somewhat higher for females of all three groups than for males. Expired air, collected during the first 24 h of the collection period, accounted for less than 0.06% and 0.11% of AD in males and females respectively. The amount of radioactivity remaining in the carcass accounted for ≤ 0.22% of the total dose. At 72 h

post dose, small amounts of radioactivity were detected in the gastrointestinal tract, with mean values ≤ 1.91% and 0.08% of the dose for males and females, respectively. The excretion of radioactivity was very rapid, with the majority of administered radioactivity recovered within 24 h of dosing. Excretion was complete by approximately 48 h after the final dose.

Table 1. Excretion balance following single low, single high or repeated low doses of valifenalate

	Percentage of total radioactivity (%)					
	SOLD		SOHD		MOLD[a]	
Sample	Male	Female	Male	Female	Male	Female
Urine (0–72 h)	9.5 ± 2.0	41.6 ± 4.1	15.9 ± 1.4	27.0 ± 2.3	8.86 ± 0.7	33.6 ± 5.5
Faeces (0–72 h)	86.8 ± 4.1	50.7 ± 3.0	76.2 ± 4.3	65.4 ± 1.3	82.9 ± 2.8	57.7 ± 5.3
Cage wash (0–72 h)	1.21 ± 0.4	6.04 ± 2.5	1.06 ± 0.5	1.59 ± 0.9	1.67 ± 1.5	4.06 ± 1.8
Expired air (0–24 h)	0.06 ± 0.0	0.05 ± 0.0	0.04 ± 0.01	0.06 ± 0.0	0.06 ± 0.0	0.11 ± 0.1
GIT + contents (72 h)	0.02 ± 0.0	0.01 ± 0.0	1.11 ± 1.1	0.04 ± 0.0	1.91 ± 2.2	0.08 ± 0.1
Carcass (72 h)	0.03 ± 0.0	0.02 ± 0.0	0.11 ± 0.1	0.00 ± 0.0	0.22 ± 0.1	0.19 ± 0.1
Total	97.6 ± 2.7	98.4 ± 1.1	94.4 ± 2.6	94.1 ± 0.9	95.7 ± 1.9	95.8 ± 0.9
Range total recovery	94.6–101.0	97.7–100.0	92.3–98.1	93.0–95.3	94.5–98.6	94.7–96.9

SOLD Single oral low dose SOHD Single oral high dose MOLD Multiple oral low dose

[a] 14-day repeated nonradiolabelled dose, followed by a single radiolabelled dose

GIT Gastrointestinal tract Source: Kidd & Gedik, 2003

In kinetic studies (phases 4, 5 and 6), four males and four females were treated with 100 or 1000 mg/kg bw radiolabelled valifenalate to determine the time–concentration profile in blood after a single oral low and high dose, and 15-day repeated oral dose (14 days unlabelled followed by a radiolabelled dose on day 15). Blood samples were taken for analytical determination at 0.1, 0.2, 0.4, 1, 2, 4, 6, 8, 24, 48 and 72 h following dosing. For low-dose group animals (phase 4) the maximum mean blood concentration (C_{max}) was 12.45 µg/g for males and 9.42 µg/g for females. C_{max} in blood of males was reached at 2 h post dose (T_{max}), whereas in females T_{max} occurred at 1 h post dose. Mean concentrations subsequently declined in a biphasic manner with concentrations decreasing rapidly to 3.39 µg/g (males) and 1.17 µg/g (females) at 8 h post dose. Thereafter, the rate of decline slowed, with low mean concentrations of radioactivity in the whole blood (< 0.01 µg/g for both males and females) observed at 72 h post dose. As T_{max} was 2 h post dose for male rats and 1 h post dose for females, these times were selected as the respective sacrifice times for the animals in phase 7. The elimination half-life ($t_{½}$) was calculated to be 3.93 h in males and 5.46 h in females. The area under the concentration–time curve ($AUC_{0–72\ h}$) values were calculated to be 98.78 h µg g^{-1} in males and 49.20 h µg g^{-1} in females.

In the high-dose group for the kinetic study (phase 5), the C_{max} was 30.24 µg/g for males and 19.86 µg/g for females. *C*max was reached rapidly at 2 h post dose for both male and female animals (T_{max}). Mean blood concentrations then declined in a biphasic manner with concentrations decreasing rapidly to 1.34 µg/g (males) and 0.58 µg/g (females) at 24 h post dose. Thereafter, the rate of decline slowed, with low mean concentrations of radioactivity in the whole blood (< 0.01 µg/g for males and 0.50 µg/g for females) observed at 72 h post dose. As T_{max} was 2 h post dose for both male and female rats, this time was selected as the sacrifice time for the animals in phase 8. The half-life $t_{½}$ was calculated to be 4.42 h in males and 4.50 h in females; the AUC was calculated as 346.14 h × µg g^{-1} in males and 175.12 h × µg g^{-1} in females. AUC values in both sexes at the high-dose level were approximately 3.5-fold higher compared to the low-dose levels. The maximum concentrations of total radioactivity in whole blood following oral administration of [^{14}C]-valifenalate (100 mg/kg bw) after multiple oral administrations of unlabelled valifenalate (phase 6) were 9.14 µg/g in males and 8.20 µg/g in females. In both sexes, the *C*max was reached at 1 h post final dose (T_{max}). Mean levels of radioactivity then decreased rapidly to a level of 0.24 and 0.19 µg/g in males and females respectively, until 24 h post final dose, and thereafter continued to decline at a slower rate. The mean concentrations of total radioactivity at 72 h after the final dose were 0.05 µg/g and 0.04 µg/g in males and females respectively. As T_{max} was 1 h post dose for both male and female rats, this time was selected as the sacrifice time for the animals in phase 9. The half-life $T_{½}$ was calculated to be 5.60 h in males and 6.60 h in females; the AUC was calculated to be 56.76 h × µg g^{-1} in males and 38.72 h × µg g^{-1} in females.

In the tissue distribution studies (phases 7, 8 and 9), groups of four male and four female rats were given a single oral low or high dose and 14-day repeated oral doses of valifenalate, followed by a single radiolabelled dose. The collection time intervals were selected based on the time points representing C_{max} of whole blood concentrations. Following administration of [^{14}C]-valifenalate at 100 mg/kg bw (phase 7), the distribution of total radioactivity was similar in both sexes. The highest concentration of total radioactivity was found in the GI tract with mean values of 930.95 µg/g (84.91% of AD) and 806.45 µg/g (72.84% of AD) in male and female animals respectively. Lower levels of radioactivity were found in the liver (accounting for 7.64% and 7.00% of the administered in males and females respectively), and the kidneys (0.30% and 0.46% respectively). With the exception of whole blood, which contained mean concentrations of 10.20 and 9.71 µg/g, all other tissues contained mean concentrations of less than 6.5 µg/g and accounted for 0.032% of the dose or less in both sexes. The carcasses contained a mean of 3.19 µg/g (males) and 3.06 µg/g (females); the recovery of radioactivity in carcass was 2.48% and 2.45% of the administered dose in males and females respectively.

The highest mean concentration of total radioactivity following administration of valifenalate at the high-dose level (1000 mg/kg bw, phase 8) was observed in the GI tract, with values of 7547.70 (89.64% of the AD) and 9007.40 µg equiv./g (83.76%) in males and females, respectively. Mean concentrations were 74.12 µg equiv./g (males) and 25.31 µg equiv./g (females) for whole blood, 199.18 and 128.44 µg equiv./g for the kidneys, and 351.65 and 269.22 µg equiv./g for the liver. Mean recoveries of radioactivity from liver were 1.52% (males) and 1.18% (females), and from kidneys 0.17% and 0.11% of the AD. Residues in the remaining tissues were lower, with mean values in the range 5.60–36.44 µg equiv./g (≤ 0.02% of the dose) for males and 3.37–16.50 µg equiv./g (≤ 0.01% of the dose) for females. The carcass was an exception: although mean levels of radioactivity were 28.34 and 6.16 µg equiv./g in males and females respectively, the levels of radioactivity accounted for 2.12% and 0.46% of the AD. Distribution of radioactivity was similar in both sexes. The highest mean concentration of total radioactivity following oral administration of [^{14}C]-valifenalate (100 mg/g bw) after multiple oral dosing of unlabelled parent compound (phase 9) was observed in the GI tract. Mean levels of radioactivity in the GI tract were 852.15 µg equiv./g (males) and 806.99 µg equiv./g (females), accounting for 86.75% and 74.61% respectively of the AD. The liver and kidneys contained 222.77 (10.08% of AD) and 55.31 µg equiv./g (0.42%) in males. For females this was 124.48 (5.09% of AD) and 31.53 µg equiv./g (0.26%). With the exception of whole blood, which contained mean concentrations of 11.47 and 5.33 µg equiv./g, all other tissues contained mean concentrations of less than 7.2 µg equiv./g and accounted ≤ 0.03% of the dose. The carcass contained a mean of 3.09 (males) and 1.67 µg equiv./g (females). The recovery of radioactivity in carcass was 2.41% (males) and 1.30% (females) of the AD.

Table 2. Tissue distribution of radioactivity in the low, high and repeated low dose groups of rats

Treatment group	SOLD		SOHD		MOLD	
	Male	Female	Male	Female	Male	Female
Tissue						
Brain (% AD)	0.004	0.006	0.003	0.002	0.002	0.003
Heart (% AD)	0.022	0.020	0.012	0.006	0.019	0.013
Kidneys (% AD)	0.30	0.460	0.168	0.110	0.421	0.264
Liver (% AD)	7.637	7.000	1.519	1.180	10.084	5.090
Lungs (% AD)	0.032	0.032	0.017	0.009	0.029	0.015
Spleen (% AD)	0.007	0.007	0.004	0.002	0.007	0.004
GI tract + contents (% AD)	84.908	72.841	89.640	83.762	86.753	74.611
Carcass (% AD)	2.475	2.449	2.124	0.461	2.405	1.298
Total (% AD)	95.39	82.815	93.487	85.532	99.720	81.297

SOLD Single oral low dose: (100 mg/kg bw, phase 7) SOHD Single oral high dose: (1000 mg/kg bw, phase 8)

MOLD Multiple oral low dose: (100 mg/kg bw per day, phase 9) AD Administered dose

Source: Kidd & Gedik, 2003

In bile elimination studies (phase 10), four male and four female bile duct-cannulated rats were administered a single oral low dose of [^{14}C]-valifenalate. Bile was collected in the periods 0–6, 6–12, 12–24 and at 24–48 h post dose. Urine, faeces and cage wash were also collected at various time points, whereas the carcass and the GI tract were retained separately at the end of the collection period. At 48 h after dosing, the mean recovery of radioactivity was 96.02% and 97.73% in male and female animals respectively. The large amount of radioactivity detected in the bile of both males and females indicated that biliary excretion was an important elimination route. Excretion in faeces accounted for 16.63% and 15.55% of the dose in male and female animals respectively. Total recovery of radioactivity in bile and faeces represented 81.18% and 64.23% of dose in males and females respectively. These values, and those for urine, were consistent with the recovery of radioactivity in faeces and urine observed in phase 1. The excretion of total radioactivity in urine, bile and faeces was very rapid, with the majority of the radioactivity being excreted within 12 h of dosing and a plateau was reached by 24 h. Very small amounts of radioactivity were detected in the GI tract and carcass (Kidd & Gedik, 2003).

Table 3. Excretion of radioactivity in bile duct-cannulated rats (% administered dose)

	Male	Female
Urine	12.84 ± 7.56	30.79 ± 3.05
Faeces	16.63 ± 4.22	15.55 ± 5.22
Bile	64.55 ± 12.80	48.68 ± 6.55
Cage wash	1.48 ± 1.75	2.19 ± 1.40
Gastrointestinal tract	0.16 ± 0.16	0.45 ± 0.44
Carcass	0.36 ± 0.08	0.08 ± 0.07
Total	**96.02 ± 1.19**	**97.73 ± 2.56**

Source: Kidd & Gedik, 2003

1.2 Biotransformation

In a preliminary study, profiling of metabolites of [^{14}C chlorophenyl]-valifenalate in the urine and faeces of male and female rats was carried out on samples from the excretion and tissue distribution study by Cameron & Nong (2002); this was done following a single 250 mg/kg bw oral administration of radiolabelled valifenalate. The study established that excretion after a single oral administration of 250 mg/kg bw was almost complete within 24 h for urine and 48 h for faeces: total excretion amounted to 97.87% and 96.58% in males and females respectively. Faecal samples for each sex were pooled by time interval (0–24 h and 24–48 h), extracted twice with acetone and once with a 1:1 acetone/water mix. The extracts were analyzed for radioactivity content by LSC. The dried faecal residues were oxidized to determine the nonextractable radioactivity content by LSC. Approximately 99% of faecal radioactivity was extractable. Aliquots of pooled urine samples were analyzed directly by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) for distribution of radioactivity. Profile of metabolites in faecal extracts was also obtained by chromatography. Chromatographic analyses of the pooled urine samples showed five metabolites (R2, R3, R4, R5 and R6); no traces of unchanged valifenalate (R1, IR5885) were found in urine. The main urinary metabolite (R2) accounted for 6.29% and 21.83% of the administered dose in male and female rats respectively. Other metabolites accounted in both sexes for < 3% of the dose in urine samples. Pooled faecal extracts contained two metabolites (R2, R3): unchanged valifenalate (R1) was also identified and accounted for 40.76% and 41.33% of the dose in males and females respectively. The main faecal metabolite was R2 (43.44% and 28.10% of the dose), whilst levels of R3 were < 3% and < 2% of the dose in males and females respectively. A comparison of representative urine samples and faecal extracts showed co-chromatography of metabolites between samples and between the two sexes. Identification of the [^{14}C]-compounds was performed by co-chromatographic analyses (both TLC and HPLC) of representative samples with authentic reference standards and identified R1 as valifenalate. R2 was identified by co-chromatography as: RS-β-alanine, *N*-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl) acid or valifenalate acid. The diastereoisomeric ratios of unchanged valifenalate (R1) and R2 in faecal extracts and urine samples were comparable and had not changed significantly (Scacchi,Oriolo & Pizzingrilli, 2002).

In a definitive study, profiling was carried out on the metabolites of [^{14}C]-valifenalate in the urine, faeces, bile, liver and kidneys of male and female rats after its single (100 and 1000 mg/kg bw) and repeated oral administration (100 mg/kg bw per day). Samples used had been generated during the main excretion and tissue distribution study of Kidd & Gedik (2003). Urine and bile samples for each sex up to 24 h were pooled per time interval, and analyzed for radioactivity content by LSC. Aliquots of pooled urine and bile samples were analyzed directly by TLC and HPLC for distribution of radioactivity. Faecal samples of each sex up to 48–72 h were pooled by time interval, extracted twice with acetone and once with 1:1 acetone/water mix. Extracts were analyzed for radioactivity by LSC and the metabolite profile obtained by TLC and HPLC. The dried faecal residues were oxidized to determine the nonextractable radioactivity content by LSC. The nonextractable radioactivity was < 2% of the AD. The livers and kidneys were also pooled by dose and sex, extracted and analyzed in the same way as the faecal samples. In the single oral low-dose group radioactivity in urine was 9.21% and 40.59% for males and females respectively; radioactivity in faeces was 86.23% in males and 50.48% in females; radioactivity in bile was 64.27% in males and 48.01% in females. In the single oral high-dose group, radioactivity in urine was 14.45% and 24.77% in males and females respectively; radioactivity in faeces was 71.13% in males and 64.52% in females. In the repeated oral low-dose group, radioactivity in urine was 8.18% in males and 32.25% in females; radioactivity in faeces was 73.48% in males and 56.46% in females.

Chromatographic analyses showed that valifenalate (R1) was extensively metabolized and six metabolites were demonstrated (R2, R3, R4, R5, R6, R7). The metabolic profiles were similar between the dose groups and sexes, with only some quantitative differences. Six urinary metabolites (R2, R3, R4, R5, R6, R7) were demonstrated by TLC or HPLC; unchanged valifenalate was not identified in urine samples. The main urinary metabolites were R2 and R5 whilst other metabolites were detected at levels of < 1% of the AD, with the exception of R6 in males. Co-chromatography of metabolites was seen between samples and between the two sexes; metabolite R7 was identified in the urine of male rats of the repeated dose groups but was not found in females.

Table 4. Urinary and faecal metabolites of valifenalate

	SOLD		SOHD		MOLD	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
			Urine			
R1, valifenalate (% AD)	ND	ND	ND	ND	ND	ND
R2 (% AD)	2.68	36.25	10.38	21.40	2.20	27.91
R3 (% AD)	0.82	0.54	0.59	0.52	0.73	0.56
R4 (% AD)	0.68	0.75	0.44	0.41	0.69	0.79
R5 (% AD)	3.60	2.07	2.11	1.64	3.15	2.23
R6 (% AD)	1.33	0.85	0.90	0.79	1.32	0.76
R7 (% AD)	0.10	0.13	ND	ND	0.09	ND
			Faeces			
R1, valifenalate (% AD)	5.30	6.17	40.41	9.50	7.80	5.47
R2 (% AD)	73.20	40.42	31.66	51.15	66.26	46.21
R3 (% AD)	4.32	1.91	2.59	2.68	5.17	2.72
R4 (% AD)	2.18	1.46	0.51	0.44	2.72	1.32

SOLD Single oral low dose SOHD Single oral high dose MOLD Multiple oral low dose

ND Not detected

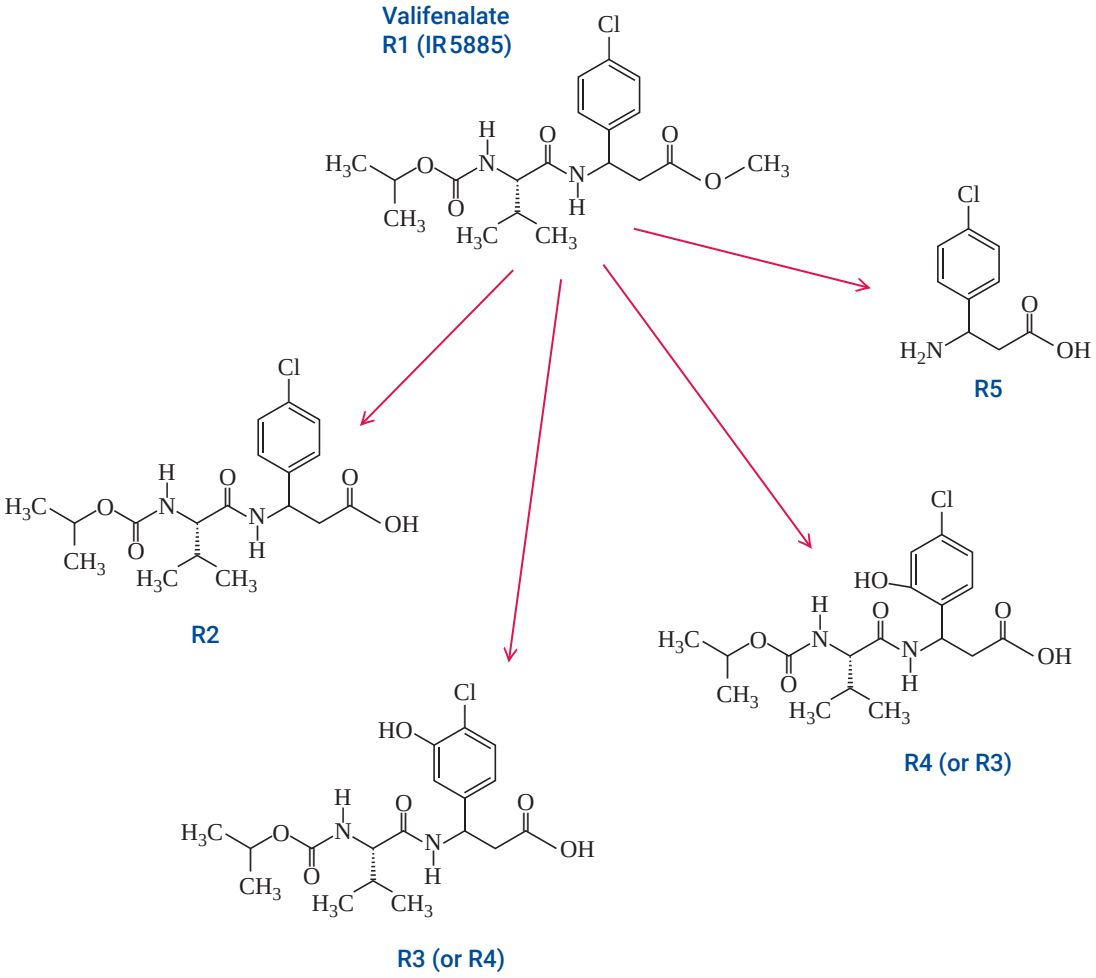
AD Administered dose

Source: Scacchi, Oriolo & Pizzingrilli, 2003

Unchanged valifenalate was eliminated primarily in the faeces and accounted for 5.3–7.8% of the administered radioactivity for the single and repeated low-dose groups. In high-dose groups, unchanged valifenalate was identified at a higher level (40.41%) in males, but accounted for only 9.5% of the dose in females. Pooled faecal extracts contained three metabolites of valifenalate (R2, R3, R4); R2 was the main faecal metabolite, R3 and R4 were identified as minor metabolites. R5, R6 and R7 were not detected in faecal extracts. Co-chromatography of representative samples proved that faecal metabolites from male rats corresponded to faecal metabolites from female rats. Furthermore, metabolites from rats administered a low dose corresponded to compounds from rats administered a high dose. Metabolites from rats administered with single low dose corresponded to compounds from rats administered with repeated low doses.

Following low, high and repeated oral administrations, the highest mean concentration of radioactivity was found in the GI tract in both sexes. Lower levels of radioactivity were found in the liver and kidneys, the only other organs showing significant radioactivity. Metabolic profiles from the liver showed only R2 and R3 and no traces of unchanged valifenalate (R1) The main metabolite was R2,with R3 accounting for only < 1% of the AD. R3 was not observed in liver extracts of female rats administered a single high dose or repeated low doses. Chromatographic analyses of extracts from kidneys of rats showed four metabolites of valifenalate (R2, R3, R4, R5); no traces of unchanged valifenalate were found in the kidneys and metabolite levels were < 1% of the AD. Compound R2 was identified as the main renal metabolite in both sexes; R4 was detected only in the kidneys of male rats treated with single oral doses. Metabolite R2, which was detected in large amounts in the faecal samples of noncannulated rats, was also prominent in the bile of cannulated rats, accounting for up to 59.58% and 46.35% of the AD in males and females respectively. R3 was detected in bile samples of both males and females, but in much smaller quantities (3.09% in males, 1.67% in females). R4 was observed in the bile of male rats only (1.59%). Unchanged valifenalate was not excreted in the bile. The main component of pooled urine samples from the bile duct-cannulated rats was R2, accounting for 3.96% and 24.90% of the AD in males and females, respectively. The amounts of R3, R4, R5 and R6 were comparable in the urine of both sexes. R7 was excreted in the urine of males only. Faecal extracts showed the presence of unchanged valifenalate, accounting for 8.08% and 6.65% of the AD in males and females respectively. R2 was also present in faecal samples at levels of 7.19% and 5.68% respectively. All biliary metabolites were also present in faecal samples from non-cannulated rats; faecal samples from non-cannulated rats also contained unchanged valifenalate, which was absent from the bile samples. The chemical structures of the metabolites isolated from faeces, urine, bile, liver and kidneys were identified on the basis of comparison of chromatographic and mass spectrometric data with those for reference standards. The metabolism of valifenalate (R1) was characterized by *O*-demethylation to produce valifenalate acid (R2), hydroxylation at the second or third carbons of the 4-chlorophenyl moiety to form R3 and R4, and side chain cleavage to form 3-amino-3-(4-chlorophenyl) propionic acid (R5, β-4-chlorophenylalanine) (Scacchi, Oriolo & Pizzingrilli, 2003).

Figure 2. Proposed metabolic pathway for valifenalate in rats



2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity, irritation and sensitization studies on valifenalate are summarized below in Tables 5 and 6.

Table 5. Studies of acute toxicity of valifenalate

Species	Strain	Sex	Route	Purity (%)	LD_{50} or LC_{50}	Reference
Rat	Sprague Dawley	M + F	Oral	98.9%	> 5000 mg/kg bw[a]	Yu, 2001a
Rat	Sprague Dawley	M + F	Dermal	98.9%	> 2000 mg/kg bw[b]	Yu, 2001b
Rat	Wistar	M + F	Inhalation	98.6%	> 3.118 mg/L[c]	Dotti, 2001

LC_{50} Median lethal concentration LD50 Median lethal dose

[a] Clinical signs consisted of transient piloerection in all animals on the day after treatment day

[b] No clinical signs were observed

[c] Nose-only exposure during 4 h. Animals were dosed at 3.118 mg/L, the highest technically achievable concentration. Mass median aerodynamic diameter: 2.42–2.45 µm. Clinical signs consisted of salivation during the last 2 h of the exposure period. Stagnation of body weight gain or even slight body weight loss was observed in all animals between days 1 and 4, thereafter body weight gain was normal.

Table 6. Studies of acute dermal and eye irritation, skin sensitization of valifenalate

Species	Strain	End-point	Purity (%)	Result	Reference
Rabbit	New Zealand White	Skin irritation	98.6%	Not irritating[a]	Renoldi, 2001a
Rabbit	New Zealand White	Eye irritation	98.6%	Not irritating[a]	Renoldi, 2001b
Guinea pigs	Dunkin-Hartley	Skin sensitization (Maximization)	98.6%	Not sensitizing[a]	Vigna, 2001

[a] No clinical signs were observed

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

Study 1

Valifenalate was tested in a 28-day study in mice. The study was not conducted according to GLP, but the study report did indicate that the work performed generally followed GLP principles. Valifenalate (purity 98.9%; batch number FCF/T/180-00 (ex ZI 068)) was administered in the diet for four weeks to groups of CD-1 mice (six/sex) at concentrations of 0, 110, 440, 1750, and 7000 ppm. The mean achieved doses were equal to 0, 18, 68, 266 and 1105 mg/kg bw per day for males, 0, 27, 96, 402 and 1536 mg/kg bw per day for females. Animals were observed at least twice daily and a more detailed weekly physical examination was performed. Body weight and food consumption were estimated at weekly intervals. Haematological and clinical chemistry investigations were performed on each animal at termination. All animals were subjected to a detailed necropsy and organ weights recorded for: adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, spleen, testes, thymus, thyroid (with parathyroids), and uterus (with cervix). Histopathology was limited to the brain, kidneys, spinal cord and spleen in control and high-dose mice. The liver and any grossly abnormal tissues were examined for all animals.

There was no mortality and no clinical signs of toxicity. Overall body weight gain by males at 1750 ppm and 7000 ppm was low compared to controls (81% and 63% of the control values respectively); these differences only attained statistical significance for weeks 1–2 with the 7000 ppm males. There was no clear evidence for body weight effects in females and no effect on food consumption in either sex. Haematology revealed reduced haematocrit (Ht) in males at 1750 and 7000 ppm and in females in all dose groups. Also, reduced haemoglobin (Hb) and erythrocyte count in males at 1750 and 7000 ppm, and in females in all dose groups without a dose-response. Clinical chemistry revealed high glucose concentrations in both sexes at 1750 and 7000 ppm, reduced triglycerides in females at 1750 and 7000 ppm (56% and 29% of control respectively), slightly higher cholesterol concentration in males at 7000 ppm (131% of control), elevated potassium concentrations in both sexes at 7000 ppm, slightly lower sodium concentrations in females at 1750 and 7000 ppm, slightly lower chloride concentrations 440, 1750, 7000 ppm. Total plasma protein and albumin concentrations were slightly reduced and albumin/globulin ratios were slightly increased for females at 1750 and 7000 ppm. No abnormalities were noted at necropsy. Elevated absolute and relative liver weights were observed for both sexes at 1750 and 7000 ppm and in 440 ppm females. Higher absolute (127% of control) and relative (145% of control, statistically significant) adrenal weights were apparent for males at 7000 ppm. Histopathology revealed centrilobular hepatocyte hypertrophy in both sexes at 1750 and 7000 ppm and in males at 440 ppm. Focal hepatocyte necrosis was observed in two males at 7000 ppm and one male at 110 ppm, this was of minimal or slight severity and is not considered to be treatment-related.

Table 7. Selected haematology, clinical chemistry and pathology findings in the 28-day study in mice receiving valifenalate in the diet

	Dose (ppm)				
	0	**110**	**440**	**1750**	**7000**
			Males		
Haematocrit (L/L)	0.446	0.446	0.441	0.429	0.403**
Haemoglobin (g/dL)	14.3	14.3	14.2	13.5*	12.7**
Erythrocytes ($\times 10^{12}$/L)	8.78	8.62	8.77	8.35	7.92**
Glucose (mmol/L)	12.33	12.24	11.45	17.00**	17.13**
Triglycerides (mmol/L)	1.96	1.91	1.78	1.81	1.86
Cholesterol (mmol/L)	2.85	3.46	3.29	3.16	3.74*
Potassium (mmol/L)	4.0	4.3	4.0	4.4	4.6*
Sodium (mmol/L)	147	147	148	146	147
Chloride (mmol/L)	107	108	105	105	108
Total protein (g/L)	48	49	48	48	46
Albumin/globulin ratio	1.34	1.33	1.39	1.47	1.39
Liver weight: absolute (g) [% diffence from controls]	2.033	2.371	2.216 [+9%]	2.350 [+16%]	2.749** [+35%]
Liver weight: relative (%) [% difference from controls]	4.864	5.591* [+15%)	5.584* [+15%]	6.345** [+30%]	7.414** [+52%]
Hepatocyte hypertrophy	0	0	6**	6**	6**
slight	0	0	6	6	4
moderate	0	0	0	0	2
			Females		
Haematocrit (L/L)	0.477	0.450**	0.447**	0.467	0.447**
Haemoglobin (g/dL)	15.3	14.5*	14.0**	14.8**	13.9**
Erythrocytes ($\times 10^{12}$/L)	9.22	8.54**	8.51**	8.86**	8.52**
Glucose (mmol/L)	8.8	9.29	7.49	11.60*	11.50*
Triglycerides (mmol/L)	2.18	2.87	1.94	1.22**	0.63**
Cholesterol (mmol/L)	2.33	1.97	2.30	2.31	2.45
Potassium (mmol/L)	3.1	3.1	3.3	3.4	3.7*
Sodium (mmol/L)	149	147	147	146*	146*
Chloride (mmol/L)	113	111	110*	109*	110*
Total protein (g/L)	50	48	47	48*	45**
Albumin/globulin ratio	1.59	1.61	1.58	1.62	1.65
Liver weight: absolute (g) [% diffence from controls]	1.414	1.397	1.523 [+8%]	1.705 [+21%]	2.043* [+44%]
Liver weight: relative (%) [% diffence from controls]	5.325	5.705	5.879* [+10%]	6.077 [+14%]	7.481** [+40%]
Hepatocyte hypertrophy	1	0	1	2	5
slight	1	0	1	2	5
moderate	0	0	0	0	0

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Webley, 2001a

A no-observed-adverse-effect level (NOAEL) of 110 ppm (equal to 18 mg/kg bw per day) in males was determined based on decreased body weight gain, increased liver weight and liver histopathology seen in males at doses above 110 ppm (Webley, 2001a).

Study 2

In a 13-week study, valifenalate (purity 98.9%, batch number FCF/T/180-00 (ex ZI 068)) was administered in the diet to groups of CD-1 mice (ten/sex) at concentrations of 0, 110, 900, or 7000 ppm. Dietary concentrations were equal to achieved doses of 0, 15.3, 134 and 995 mg/kg bw per day for males, 0, 16.7, 148 and 1144 mg/kg bw per day for females. Animals were observed at least twice daily; a more detailed weekly physical examination was also performed. Body weight and food consumption measurements were at weekly intervals. Haematological and clinical chemistry investigations were performed on each animal at study termination. All animals were subjected to necropsy and the following weights recorded: brain, epididymides, heart, kidneys, liver, lungs, ovaries, spleen, testes, thymus and uterus. Histopathology was limited to the brain, kidneys, spinal cord and spleen in control and high-dose group mice. The liver and any grossly abnormal tissues were examined for all animals.

There were no deaths or signs of toxicity. Overall body weight gain by males at 7000 ppm was significantly lower (74% of the control value). Weight gain by males at 900 ppm was reduced (81% of the control value) but not significantly. Body weight gain by females at 900 ppm was slightly lower than controls, but was not considered treatment-related in the absence of a similar effect at 7000 ppm. Food consumption was lower for all groups of treated females, but this was not considered to be an effect of treatment in the absence of a dose–response relationship. Haematology revealed effects on erythrocyte parameters in both sexes: slightly lower Ht, Hb concentration, mean cell Hb and mean cell volume. Mean absolute and relative liver weights were higher in 900 ppm males and in both sexes at 7000 ppm. The livers of three males at 7000 ppm were visually enlarged. Histopathology showed an increased incidence and severity of centrilobular and periportal hepatocellular vacuolation in males but not in females. Specific staining of liver sections confirmed that vacuolation was due to fat accumulation.

Table 8. Selected findings of the 90-day study in mice receiving valifenalate in the diet

	Males				Females			
Dose (ppm)	**0**	**110**	**900**	**7000**	**0**	**110**	**900**	**7000**
Body weight gain weeks 0–13	19.0	17.8	15.3	14.1*	9.5	10.3	8.2	9.4
Haematocrit (L/L)	0.416	0.423	0.420	0.398	0.430	0.425	0.420	0.410*
Haemoglobin (g/dL)	14.3	14.6	14.3	13.7	14.8	14.6	14.6	14.3
MCH (pg)	16.1	15.9	15.7	15.3**	16.5	16.4	16.4	16.0
MCV (fL)	47.0	46.0	45.9	44.6**	48.1	47.8	47.0	46.1**
Liver weight (g)	2.27	2.19	2.44	3.14**	1.52	1.52	1.53	2.1**
[% diffence from controls]				[+38%]				[+38%]
Liver weight (%)	4.81	4.69	5.39**	7.26**	4.61	4.50	4.74	6.24**
[% diffence from controls]			[+12%]	[+51%]				[+35%]

	Males				Females			
Dose (ppm)	**0**	**110**	**900**	**7000**	**0**	**110**	**900**	**7000**
Hepatocyte vacuolation								
Centrilobular	2	2	4	8*	0	0	0	0
minimal	1	0	3	0	0	0	0	0
slight	1	1	0	4	0	0	0	0
moderate	0	1	1	4	0	0	0	0
Periportal	0	0	0	3	0	0	0	0
minimal	0	0	0	1	0	0	0	0
slight	0	0	0	1	0	0	0	0
moderate	0	0	0	1	0	0	0	0
Fat accumulation	8	7	9	10	-	-	-	-
minimal	3	3	4	2	-	-	-	-
slight	3	2	4	2	-	-	-	-
moderate	2	2	1	6	-	-	-	-

MCH Mean cell haemoglobin MCV Mean cell volume

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Webley, 2002a

Administration of valifenalate produced slight changes in erythrocyte parameters, fatty vacuolation in the liver of males at 7000 ppm, and non-specific toxicity in males at 900 and 7000 ppm. A NOAEL of 110 ppm (equal to 15.3 mg/kg bw per day) was determined for this study based on decreased body weight gain (Webley, 2002a)

Rat

Study 1

Valifenalate was tested in a four-week study in rats. The study was not conducted according to GLP, but the study report did indicate that the work performed generally followed GLP principles. Valifenalate (purity 98.9%; batch number FCF/T/180-00 (ex ZI 068)) was administered to groups of Han Wistar rats (five/sex) in the diet at concentrations of 0, 120, 600, 3000 and 15 000 ppm for up to 29 days. The mean achieved doses were 0, 13, 63, 311 and 1518 mg/kg bw per day for males, 0, 13, 64, 314 and 1537 mg/kg bw per day for females. Animals were observed at least twice daily and a weekly physical examination was also performed. Body weight and food consumption measurements were at weekly intervals. Terminal blood samples were taken for the assessment of haematological and clinical chemistry parameters. All animals were subjected to necropsy and the following weights recorded: adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, spleen, testes, thymus, thyroid and uterus. Histopathology was performed on the brain, kidneys, spinal cord and liver in the control and high-dose groups. In addition, the spleen, mandibular and mesenteric lymph nodes were examined in the control group and the groups dosed with 3000 and 15 000 ppm; moreover, the thymus and any grossly abnormal tissue were examined for all animals.

There were no deaths or signs of toxicity. Overall body weight gain for males at 15 000 ppm was reduced (75% of the control value). Haematology revealed minor effects on erythrocyte parameters not considered to be of toxicological significance. Lymphocyte counts were also slightly low in the 3000 and 15 000 ppm groups, which produced a concomitant reduction of total leucocyte count in these animals. Elevated serum aspartate transaminase (AST) activity was noted for 15 000 ppm females. Calcium concentrations were slightly reduced in all treated groups, without a dose–response relationship. Phosphorus concentration was also slightly lower for females at ≥ 600 ppm in a dose-related manner. Total plasma protein was slightly reduced in males at ≥ 600 ppm and in all treated groups of females. Albumin/globulin ratios were slightly higher in females at ≥ 600 ppm. No abnormalities were recorded at necropsy. Absolute and relative thymus weights were slightly lower in both sexes at 3000 and 15 000 ppm; histopathology revealed lymphocytolysis in males at ≥ 600 ppm and in females at 15 000 ppm, however without a clear dose-response.

Table 9. Selected haematology and pathology findings in the 28-day study in rats receiving valifenalate in the diet

	Males					Females				
Dose (ppm)	**0**	**120**	**600**	**3000**	**15 000**	**0**	**120**	**600**	**3000**	**15 000**
Number in group	5	5	5	5	5	5	5	5	5	5
Haematocrit (L/L)	0.474	0.462	0.452*	0.448*	0.451*	0.442	0.432	0.424	0.429	0.423*
Haemoglobin (g/dL)	16.3	16.1	15.7*	15.2**	15.5**	15.4	15.2	14.8	15.2	14.8*
Lymphocytes (10^9/L)	7.12	8.96	7.01	6.44	5.19*	6.15	6.10	5.92	4.01**	4.03**
APTT (s)	16.9	17.9	16.8	16.9	20.8*	19.3	17.3*	16.9*	17.9*	16.4**
AST (U/L)	74	65	79	78	79	59	69	63	64	73**
Calcium (mmol/L)	2.84	2.76**	2.72**	2.74**	2.75**	2.89	2.73**	2.76**	2.74**	2.75**
Phosphorus (mmol/L)	3.02	3.08	2.97	2.87	2.99	2.17	2.09	1.85*	1.75**	1.72**
Total protein (g/L)	63	63	61*	61*	61*	70	63*	66*	64*	65*
Albumin (g/L)	1.36	1.33	1.47	1.36	1.39	1.35	1.45	1.47*	1.52*	1.44*
Thymus weight (g)	0.180	0.208	0.183	0.178	0.142	0.244	0.238	0.245	0.214	0.215
Lymphocytolysis	0	0	3*	4*	2	2	1	3	3	4
slight	0	0	3	4	2	2	1	3	3	4
moderate	0	0	0	0	0	0	0	0	0	0

APTT Activated partial thromboplastin time AST Aspartate transaminase

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Webley, 2001b

Dietary concentrations of valifenalate up to 15 000 ppm produced non-specific toxicity at 15 000 ppm only. A NOAEL of 3000 ppm (equal to 311 and 314 mg/kg bw per day in males and females respectively) was determined for this study (Webley, 2001b).

Study 2

In a 13-week study, valifenalate (purity 98.9%; batch number FCF/T/180-00 (ex ZI 068)) was administered in the diet to groups of Han Wistar rats (ten/sex) at concentrations designed to achieve intakes of 0, 7, 150 and 1000 mg/kg bw per day. Further rats (five/sex) were assigned to the control and high-dose groups for a four-week recovery period following the 13-week treatment period. Animals were observed at least twice daily and a weekly physical examination with arena observations was also performed. Sensory reactivity, grip strength and motor activity were assessed for all animals during week 12. Terminal blood samples were taken for the assessment of haematological and clinical chemistry parameters. All animals were subject to necropsy and the following weights recorded: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid and uterus. The adrenals, brain, femur, heart, kidneys, liver, lungs, spinal cord, sternum, stomach, thyroid and uterus from all animals in the control and high-dose groups were examined microscopically; investigations in the intermediate dose groups were limited to the kidneys, liver, lung and any grossly abnormal tissues. There was no mortality and no clinical signs of toxicity. No treatment-related behavioural or functional performance nor sensory reactivity changes were noted. Body weight, body weight gain, food consumption and food conversion efficiency were unaffected by treatment. Haematology revealed slightly low Ht, Hb concentration and erythrocyte counts for males at 150 and 1000 mg/kg bw per day; differences were not always statistically significant. Lymphocyte and monocyte counts in males at 150 and 1000 mg/kg bw per day were also slightly lower than controls. Neutrophil counts were slightly reduced for females at 1000 mg/kg bw per day (0.47×10^9/L at 1000 mg/kg bw per day compared to 0.68×10^9/L in the control group; statistically significant at $p > 0.05$)). These differences, however, did not show any dose–response relationship in males and consequently are not considered to be of toxicological significance. Slightly higher platelet counts and slightly shorter prothrombin times were apparent for males at 150 and 1000 mg/kg bw per day. All haematological changes resolved by the end of the recovery period. Examination of bone marrow smears revealed an apparently high total myeloid count for males at 1000 mg/kg bw per day; however the differences compared to the control

group were small and females were unaffected. This finding was not considered to be toxicologically significant. Clinical chemistry revealed low plasma triglyceride and high chloride concentrations in males at 150 and 1000 mg/kg bw per day; a slightly elevated calcium concentration is reported for females at 1000 mg/kg bw per day. Triglyceride concentrations partially recovered but remained slightly (but not significantly) lower than controls after the four-week recovery period. Other findings showed a total recovery. Urinalysis revealed slightly higher volume and pH and slightly lower specific gravity for both sexes at 1000 mg/kg bw per day, without a dose–response relationship. A slightly higher urine pH was also recorded in both sexes at 150 mg/kg bw per day and in males receiving at 7 mg/kg bw per day. Findings had resolved by the end of the recovery period. Absolute and relative liver weights were elevated at 1000 mg/kg bw per day after 13 weeks treatment in both males and females; recovery was seen. Gross necropsy revealed a distended caecum in seven males and one female at 1000 mg/kg bw per day.

Table 10. Selected haematology, clinical chemistry, urinalysis and pathology findings in the 90-day study in rats receiving valifenalate in the diet

Dose	Main study (13-week exposure)				Recovery groups	
(mg/kg bw per day)	0	7	150	1000	0	1000
Males						
Haematocrit (L/L)	0.446	0.443	0.436	0.425**	0.443	0.439
Haemoglobin (g/dL)	15.8	15.6	15.3*	15.1**	15.8	15.9
Erythrocytes (10^{12}/L)	8.39	8.43	8.29	8.19	8.36	8.50
Lymphocytes (10^9/L)	6.91	5.91	4.99*	5.96*	6.04	6.43
Monocytes (10^9/L)	0.25	0.21	0.19*	0.18*	0.23	0.24
Neutrophils (10^9/L)	1.13	0.94	1.14	1.12	1.33	1.22
Platelets (10^9/L)	833	786	928**	895**	893	836
Prothrombin time (s)	15.7	15.0	14.5**	14.2**	15.1	15.6
Total myelocytes (%)	-	-	-	-	50.7	55.4*
Triglycerides (mmol/L)	1.36	1.20	0.90**	0.87**	1.63	1.11
Chloride (mmol/L)	106	106	107*	108**	104	103
Calcium (mmol/L)	2.93	2.91	2.87	2.98	–	–
Liver weight (g)	11.96	10.89	12.29	14.20	14.12	13.71
Liver weight (%)	3.336	3.201	3.380	3.846** (+ 15%)	3.627	3.553
Thymus weight (g)	0.320	0.329	0.355	0.344	0.361	0.285*
Thymus weight (%)	0.090	0.098	0.098	0.095	0.093	0.704*
Kidney weight (g)	1.97	1.85	1.95	2.15	1.96	2.18
Kidney weight (%)	0.550	0.549	0.540	0.585	0.505	0.567*
Distended caecum (*N*)	0	0	0	7**	0	0
Females						
Haematocrit (L/L)	0.414	0.407	0.405	0.411	-	-
Haemoglobin (g/dL)	14.6	14.4	14.3	14.5	-	-
Erythrocytes (10^{12}/L)	7.53	7.44	7.29	7.45	-	-
Lymphocytes (10^9/L)	3.38	3.15	2.86	3.18	4.22	4.44
Monocytes (10^9/L)	0.12	0.12	0.09	0.10	0.17	0.14
Neutrophils (10^9/L)	0.68	0.62	0.60	0.47*	1.05	0.85
Platelets (10^9/L)	876	908	915	890	-	-
Prothrombin time (s)	14.2	14.0	14.3	14.0	-	-
Total myelocytes (%)	-	-	-	-	41.9	43.8

Dose	Main study (13-week exposure)				Recovery groups	
(mg/kg bw per day)	0	7	150	1000	0	1000
Triglycerides (mmol/L)	0.52	0.58	0.49	0.49	-	-
Chloride (mmol/L)	107	107	108	108	-	-
Calcium (mmol/L)	2.93	2.96	2.93	3.02*	2.94	2.97
Liver weight (g)	7.66	7.00	7.46	8.53	7.42	7.55
Liver weight (%)	3.546	3.534	3.632	4.005** (+ 13%)	3.496	3.704
Thymus weight (g)	0.312	0.269	0.304	0.298	0.265	0.247
Thymus weight (%)	0.144	0.137	0.148	0.142	0.125	0.121
Kidney weight (g)	1.30	1.30	1.31	1.29	1.28	1.28
Kidney weight (%)	0.601	0.657**	0.637	0.607	0.600	0.626
Distended caecum	0	0	0	1	0	0

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Webley, 2002b

Treatment with valifenalate produced macroscopic changes in the caecum and adaptive changes in the liver, with no overt signs of toxicity. All treatment-related findings showed at least partial recovery within four weeks of cessation of treatment. A NOAEL of 150 mg/kg bw per day was determined for this study based on the finding of distended caecum at 1000 mg/kg bw per day (Webley, 2002b).

Dog

Study 1

In a range-finding study, valifenalate (purity 98.9%; batch number FCF/T/180-00, ex ZI 068) at dose levels of 0, 250, 500 or 1000 mg/kg bw per day was administered in gelatin capsules for 28 days to groups of Beagle dogs (three/sex). Control animals received empty capsules. Animals were observed at least twice daily. Body weights were recorded weekly and food consumption measured daily. Ophthalmoscopy was performed pretest and during week 4. Blood and urine were collected for analysis pretest and during week 4. All animals were subjected to necropsy and weights recorded of the adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid, and uterus. Tissues from all animals were investigated histopathologically. No deaths occurred; clinical signs were limited to white particles in the faeces of all treated animals, the incidence of which increased in a dose–dependent manner. Findings were attributed to unabsorbed test material. No effect due to treatment was observed on bodyweight or food consumption. No treatment-related ophthalmoscopic, haematological or urinalysis changes were noted. Clinical chemistry revealed reduced cholesterol and phospholipid levels in dogs administered 1000 mg/kg bw per day. Mild increases in serum alanine transaminase (ALT) activity were noted at 1000 mg/kg bw per day in males, without attaining statistical significance. Dose-dependent increases in alkaline phosphatase (ALP) and γ-glutamyl transferase (GGTP) activities were noted across all treated male groups and were statistically significant at 1000 mg/kg bw per day. In females, ALP activity increased without a dose–response relationship while GGT exhibited a dose–response relationship but without attaining statistical significance. Total protein levels were reduced in all treated groups in a dose-dependent manner and were accompanied by reductions in albumin, α_2 globulin and albumin : globulin ratios and increases in α_1 globulin. As a consequence of the reduction in albumin, calcium and magnesium levels were also reduced in all treated groups, while phosphorus levels were increased in males only. Enlarged livers were noted at necropsy in two 500 mg/kg bw per day males and in all dogs at 1000 mg/kg bw per day. Histopathology revealed minimal to marked hepatocellular hypertrophy in almost all treated dogs. Minimal to moderate numbers of eosinophilic cytoplasmic inclusions were also noted in most treated animals. Minimal to slight single cell necrosis was noted in all males and in one female at 1000 mg/kg bw per day and increased apoptosis was evident in one male at this dose level. A dose-dependent decrease in hepatocellular glycogen content was also noted.

Table 11. Selected haematology, organ weight and histopathological findings (at termination) in the four-week oral study in dogs

Dose	Males				Females			
(mg/kg bw per day)	0	250	500	1000	0	250	500	1000
Cholesterol (mmol/L)	3.19	1.86	1.87	1.28* (−60%)	3.41	2.75	1.65** (−52%)	1.14** (−67%)
Phospholipid (mmol/L)	3.47	2.10	2.14	1.64* (−53%)	3.77	3.17	2.12** (−44%)	1.47** (−60%)
ALT (µkat/L)	0.53	0.40	0.44	1.25	0.63	0.69	0.60	0.75
ALP (µkat/L)	6.92	10.10	12.48	20.95*	6.39	14.46*	13.54	13.73
GGTP(µkat/L)	50.5	66.9	69.2	90.9**	67.9	61.3	76.5	94.2
Total protein (g/L)	51.2	47.1*	46.8*	44.6**	54.3	50.0	46.7*	44.5**
Albumin (g/L)	26.56	20.52**	21.83**	21.27**	30.79	26.87	24.36*	23.70*
Calcium (mmol/L)	2.79	2.59*	2.61	2.57*	2.90	2.72	2.61*	2.58**
Magnesium (mmol/L)	0.81	0.75**	0.79	0.73**	0.87	0.81	0.79	0.81
Phosphorus (mmol/L)	2.62	2.26**	2.31**	2.14 **	2.25	2.05	2.09	2.22
Liver weight (g)	305.2	410.0* (+34%)	455.4** (+49%)	507.1** (+66%)	310.7	368.7 (+19%)	441.3* (+42%)	411.8 (+33%)
Liver weight (%)	3.3	4.5* (+36%)	5.2** (+56%)	5.3** (+61%)	3.4	4.1 (+21%)	5.2** (+53%)	4.8* (+41%)
Hepatocyte hypertrophy	1	3	3	3	0	3	3	3
Grade 1	1	2	1	0	0	1	0	0
Grade 2	0	1	0	0	0	1	1	0
Grade 3	0	0	0	0	0	1	2	2
Grade 4	0	0	2	3	0	0	0	1
[Mean severity]	[1.0]	[1.3]	[3.0]	[4.0]	[0]	[2.0]	[2.7]	[3.3]
Eosinophilic inclusions	0	2	3	3	0	1	2	2
Grade 1	0	1	1	0	0	1	1	0
Grade 2	0	1	1	2	0	0	1	0
Grade 3	0	0	1	1	0	0	0	2
[Mean severity]	[0]	[1.5]	[2]	[2.3]	[0]	[1]	[1.5]	[3]
Glycogen storage Total	2	3	3	0	3	3	3	1
Grade 1	0	0	1	0	0	2	0	1
Grade 2	1	1	1	0	1	1	3	0
Grade 3	1	2	1	0	1	0	0	0
Grade 4	0	0	0	0	1	0	0	0
[Mean severity]	[2.5]	[2.7]	[2.0]	[0]	[3.0]	[1.3]	[2.0]	[1.0]
Single cell necrosis	0	0	0	3	0	0	0	1
Grade 1	0	0	0	3	0	0	0	1
[Mean severity]	[0]	[0]	[0]	[1]	[0]	[0]	[0]	[1]
Increased apoptosis	0	0	0	1	0	0	0	0
Grade 2	0	0	0	1	0	0	0	0
[Mean severity]	[0]	[0]	[0]	[2]	[0]	[0]	[0]	[0]

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ kat katal (mol/second) Source: Brown, 2003

At ≥ 500 mg/kg bw per day, changes in clinical chemistry were observed with increased liver weight and liver histopathology. Therefore, the NOAEL in this study was 250 mg/kg bw per day (Brown, 2003).

Study 2

In a 13-week study, valifenalate (purity 99.56%, batch number T025/02) was administered to groups Beagle dogs (four/sex) in gelatin capsules at dose levels of 0, 50, 250 and 750 mg/kg bw per day. The control group received empty capsules. Treatment was suspended for one female at the highest dose level after seven weeks due to weight loss and evaluation of clinical findings; this animal remained untreated for the remainder of the study. Animals were observed at least twice daily. Detailed clinical observations were made daily during the pretest period and for the first week of treatment, then twice weekly during weeks 2–4, and weekly thereafter. Body weights were recorded weekly and food consumption was recorded daily. Ophthalmic examinations were carried out pretest and in week 13. Blood and urine samples were taken pretest and during weeks 6 and 13. All animals were subject to necropsy and weights of the folllowing organs recorded: adrenal gland, gall bladder, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid and uterus. Histopathology was performed on tissue from all animals.

There were no deaths. Occasional observations of discoloured faeces or white/yellow powder in faeces were noted throughout the study for the majority of dogs at 250 and 750 mg/kg bw per day. Significantly lower body weight gains were noted for both sexes at 750 mg/kg bw per day (−48% in males, −33% in females) and, to a lesser extent, males administered 250 mg/kg bw per day (−21%). Mean food consumption for both sexes at 750 mg/kg bw per day was lower from the beginning of the study, and showed a partial recovery from week 3 but remained consistently lower throughout the study. From week 8 onwards treatment was withdrawn from one female at 750 mg/kg bw per day. This dog showed bodyweight gain comparable to others of the same group, but a 10% loss in bodyweight was observed in weeks 5–7, and its week 8 bodyweight was lower than the initial value. Weight gain for this female was seen during the period off treatment and at termination weight was similar to others in the same dose group.

Haematological investigation of this dog in weeks 6 and 7 showed higher leucocyte, neutrophil and platelet counts and elevated prothrombin time compared to pretest values. Reversibility of haematological effects was seen after withdrawal of treatment. Clinical chemistry showed increases of serum ALP, ALT, AST and GGTP activities and marked decreases in cholesterol, total protein, albumin concentrations and albumin/globulin ratio compared to the pretest value. ALT, AST and GGTP activities were comparable to pretest values, however other effects did not indicate complete recovery. In the remaining dogs, haematology at weeks 6 and 13 showed higher mean platelet counts for both sexes at 750 mg/kg bw per day and for males at 250 mg/kg bw per day. A reduction in activated partial thromboplastin time (APTT) values was noted at week 6 for males at 250 and 750 mg/kg bw per day, but was not evident at week 13 or in females at either time point. Slightly lower erythrocyte counts were noted at 750 mg/kg bw per day in males at week 6 and both sexes at week 13; findings in males were associated with higher mean corpuscular Hb (MCH) and MCV values. Lower reticulocyte counts were noted at 750 mg/kg bw per day at week 6, but a dose–response relationship was not apparent and a similar effect was not seen at week 13. Clinical chemistry revealed elevated serum enzyme (most notably ALP) activities in dogs treated with 250 and 750 mg/kg bw per day.

Absolute liver weights were significantly higher in males at 250 and 750 mg/kg bw per day (+43% and +60%, respectively) and in females at ≥ 50 mg/kg bw per day (+33%, +34% and +70% with increasing dose). Gall bladder weights were increased for both sexes at 750 mg/kg bw per day (+36% in males, +39% in females) and males at 250 mg/kg bw per day (+26%); in the absence of any associated histopathology this is not considered be of toxicological significance. Thyroid weights were significantly higher in males at 250 and 750 mg/kg bw per day (+61% and +64%, respectively). Markedly lower prostate (−63%) and testis weights (−18%) and higher group mean epididymis weights (+14%) were noted for males at 750 mg/kg bw per day; in the absence of histopathological correlates for testis and epididymis, these findings are not considered to be of toxicological significance. Gross necropsy revealed enlarged liver in all dogs at 750 mg/kg bw per day and, to a lesser extent, at 250 and 50 mg/kg bw per day. Pale liver was also observed in the majority of dogs at 750 mg/kg bw per day. Histopathology revealed minimal to moderate hepatocyte hypertrophy, hepatocytes with pale cytoplasm and peripheral clumping and eosinophilic cytoplasmic inclusions. The incidence of thyroid follicular cell hypertrophy in thyroid glands was increased in males at 250 and 750 mg/kg bw per day and in all treated groups of females. Immature prostate was observed for all treated groups of males.

Table 12. Haematology, clinical chemistry and pathology findings in the 90-day study in dogs receiving valifenalate by capsule

Dose (mg/kg bw per day)		Male				Female			
		0	50	250	750	0	50	250	750
Platelets (10^9/L)	Week 6	331	284	376	398	265	375	381	461*
	Week 13	299	280	363	398**	315	378	366	448*
APTT (s)	Week 6	23.4	24.0	20.7	19.8*	20.5	22.6	21.9	19.1
	Week 13	26.7	30.6	29.9	27.0	26.2	29.2	26.9	29.9
Erythrocytes (10^{12}/L)	Week 6	6.22	6.02	5.95	5.73	6.31	6.34	5.96	5.97
	Week 13	6.40	6.12	5.99	5.90	6.52	6.58	6.56	5.92
Mean cell Hb MCH (pg)	Week 6	21.2	22.3*	22.7**	23.2**	22.1	22.3	22.1	22.4
	Week 13	21.9	22.9*	23.3**	24.0**	22.5	22.6	23.0	23.1
Mean cell volume, MCV (fL)	Week 6	65.0	67.6*	67.9*	68.39**	66.7	66.6	66.9	68.0
	Week 13	67.5	69.4	70.4*	71.9**	68.4	68.8	69.6	70.6
ALP (U/L)	Week 6	103	208	366**	450**	122	211	233	574**
	Week 13	86	208**	456**	531**	110	257**	323**	601**
ALT (U/L)	Week 6	33	32	32	69	29	34	33	117
	Week 13	46	35	44	171	31	36	44	49*
AST (U/L)	Week 6	37	34	41	34	33	42	44	42
	Week 13	43	33	36	55	34	35	44	42
GGTP (U/L)	Week 6	3	4	4	5*	3	3	4	7**
	Week 13	3	3	5	7**	3	3	4*	6**
Cholesterol (mmol/L)	Week 6	4.10	3.92	2.17**	1.91**	3.47	3.22	2.21**	1.21**
	Week 13	3.91	3.81	2.36**	1.66**	3.50	3.46	2.43*	1.09**
Urea (mmol/L)	Week 6	2.79	3.49	4.24*	4.34*	3.38	3.70	3.65	3.93
	Week 13	3.09	3.56	4.45	4.63	3.76	3.98	3.97	3.93
Albumin:globulin ratio	Week 6	1.15	1.19	1.01	1.01	1.26	1.21	1.20	0.95**
	Week 13	1.15	1.27	1.00	0.96	1.28	1.16	1.16	0.94**
Liver									
Hepatocyte hypertrophy		0	4*	4*	4*	0	4*	4*	3*
	Minimal	0	3	0	0	0	2	1	0
	Slight	0	1	2	0	0	2	1	0
	Moderate	0	0	2	4	0	0	2	3
Eosinophilic inclusions		0	0	4*	4*	0	0	4*	3*
	Minimal	0	0	4	0	0	0	3	0
	Slight	0	0	0	2	0	0	1	1
	Moderate	0	0	0	2	0	0	0	2

Dose (mg/kg bw per day)		Male				Female			
		0	50	250	750	0	50	250	750
Pale cytoplasm		0	4*	4*	4*	0	4*	4*	3*
	Minimal	0	4	0	0	0	2	1	0
	Slight	0	0	4	2	0	2	1	0
	Moderate	0	0	0	2	0	0	2	3
Thyroid									
Follicular hypertrophy		0	0	1	2	0	1	2	2
	Minimal	0	0	1	1	0	0	0	2
	Slight	0	0	0	1	0	1	2	2
Prostate									
Immature		1	2	2	4	-	-	-	-
	Minimal	1	2	0	1	-	-	-	-
	Slight	0	0	1	0	-	-	-	-
	Moderate	0	0	1	3	-	-	-	-

APTT Activated partial thromboplastine time ALP Alkaline phosphatase ALT Alanine transaminase
AST Aspartate transaminase GGTP γ-glutamyl transpeptidase
* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Geary, 2003

The NAOEL in this study was 50 mg/kg bw per day based on changes in clinical chemistry, liver and thyroid histopathology at higher doses (Geary, 2003).

Study 3

Valifenalate (purity 99.56 or 99.63%; batch number T025/02) was administered to Beagle dogs by daily oral capsule for 52 weeks (main study) or for 13 weeks, followed by a recovery period of eight weeks. Four groups of dogs (four/sex) were dosed with valifenalate at dose levels of 0, 1, 7, 50 or 250 mg/kg bw per day for up to 52 weeks; the control group received empty capsules. Additional dogs (two/sex) were assigned to the control and high-dose groups and were treated for 13 weeks followed by an eight week recovery period. Animals were observed at least twice daily; more detailed clinical observations were performed daily during the first week of treatment, twice weekly during weeks 2–4, weekly during weeks 5–13 and fortnightly thereafter. Detailed observations were recorded immediately before dosing, up to 2 h after dosing and as late as possible in the working day. Blood samples were collected pretest and during weeks 13, 26, 39 and 52. Blood was collected from recovery animals pretest, during week 6 of treatment period and week 4 of the recovery period. Overnight urine was collected from all animals pretest and during weeks 6 (recovery animal only), 13, 26, 39 and 52. All animals were subject to gross necropsy; weights were recorded of adrenals, brain, epididymides, gall bladder, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thyroid and uterus. Histopathology was performed on tissues from all dogs.

One male dog from the 1 mg/kg bw per day group was sacrificed in week 34 due to poor clinical condition; the death was not considered to be treatment-related and was attributed to bacterial infection. There were no treatment-related deaths or signs of toxicity. Body weight gain and food consumption were unaffected by treatment. Ophthalmoscopy did not reveal any effects of treatment. From week 13, males at 250 mg/kg bw per day showed higher platelet values, attaining statistical significance from week 26. At weeks 13, 26 and 39, females at 50 and 250 mg/kg bw per day also showed higher platelet values, but with no dose–response relationship and without statistical significance. Individual values for these animals were within the concurrent control range, therefore the differences were not considered to be related to treatment. Platelet counts in male dogs assigned to the recovery group were similar to controls and/or their pretest value. In females there was an increase compared to controls in week 6 but by week 13 only one value was higher than control and both were lower than those in week 6. Following the recovery period platelet values were similar to controls. From week 13, higher serum ALP activities were noted in both sexes at 50 and 250 mg/kg bw per day. From week 13, dogs at 250 mg/

kg bw per day had elevated GGTP values; these were attributable to high values in three individuals. At week 52 all males from treated groups showed higher GGTP values, however there was no dose–response relationship and individual values were within the concurrent control range. At week 13, significantly lower cholesterol was noted in dogs at 250 mg/kg bw per day; cholesterol concentration remained lower at subsequent time points, but without statistical significance. There was no progression over time, and individual values tended to be within concurrent control values. Similar findings were noted in animals assigned to the recovery group; effects were reversible by week 4 of recovery. From week 13, males at 250 mg/kg bw per day showed higher mean triglyceride values compared to controls; higher values at week 39 were mainly due a single dog. At weeks 39 and 52, males at 50 mg/kg bw per day also showed higher triglyceride values due to higher values in individual animals. At week 39 females at 250 mg/kg bw per day showed higher mean triglyceride values; however values at week 52 were similar to controls. No effect on triglycerides was observed either at week 6 or 13 in animals assigned to the recovery group. From week 13, males at 250 mg/kg bw per day showed lower calcium concentrations; the difference from control was similar at all time points and similar effects were not apparent in females. From week 13, dogs at 250 mg/kg bw per day showed lower mean total albumin levels, resulting in lower total protein concentration in both sexes and lower A:G ratios in males and in females only at week 26. Lower albumin levels were also noted in females at 50 mg/kg bw per day at weeks 13 and 26. Albumin concentrations at weeks 6 and 13 in dogs assigned to the recovery groups were consistently lower and had recovered following withdrawal of treatment for four weeks. There were some differences in thyroid hormone levels between the control and treated groups; however there was no consistent correlation among parameters measured. Testosterone values recorded from week 13 of treatment onwards were highly variable in all treated and control animals, as normally seen at the performing laboratory in the case of single samples in dogs of similar age and strain. No differences between treated and control animals were apparent. There was no effect of treatment on urinalysis parameters in weeks 13, 26, 39 or 52.

Absolute liver weights in all treated groups were elevated compared to controls, however, a dose–response relationship was not apparent at 1 and 7 mg/kg bw per day. Relative liver weight was increased in both sexes at 250 mg/kg bw per day; liver weights following recovery were comparable to controls. After 52 weeks of treatment, dogs of both sexes at 50 and 250 mg/kg bw per day showed higher thyroid weights compared to controls. After 52 weeks of treatment lower mean prostate weights were noted in males at 250 mg/kg bw per day; values did not attain statistical significance. Recovery males also showed lower prostate weights compared to concurrent controls. Lower group mean absolute ovary and uterus/cervix weights were noted in females receiving 50 and 250 mg/kg bw per day; only mean weight attained statistical significance. After an eight week recovery period following 13 weeks of treatment, ovary weights for treated females were similar to controls. Significantly lower absolute and relative spleen weights were noted for all treated male groups compared to concurrent controls. In the absence of similar findings in females and no haematological or histopathological correlates, this finding is not considered to be of toxicological significance. There were no differences from controls in spleen weights in animals assigned to the recovery group.

Gross necropsy revealed enlargement of the livers in three males and two females at 250 mg/kg bw per day and in one female at 50 mg/kg bw per day. Necropsy of recovery group dogs did not show any treatment-related findings. Microscopically, generalized hepatocyte hypertrophy was seen in dogs of all treated groups. Hepatocytes with pale cytoplasm and peripheral clumping were seen in males at 250 mg/kg bw per day and females at 50 and 250 mg/kg bw per day. Eosinophilic concentric cytoplasmic inclusions in hepatocytes were seen in one male at 250 mg/kg bw per day. Minimal rarefaction of hepatocytes, compared to controls, was seen in recovery animals. Hypertrophy of the thyroid follicular epithelium was seen in both sexes at 250 mg/kg bw per day and in a single male and female at 50 mg/kg bw per day. No thyroid changes were observed in recovery animals. Minimal prostate immaturity was noted in the male recovery group. No corpora lutea were present in the ovaries of three females at 250 mg/kg bw per day.

Table 13. Findings in the one-year study in dogs receiving valifenalate by capsule

Dose (mg/kg	Males					Females				
bw per day)	0	1	7	50	250	0	1	7	50	250
Platelet (10^9/L)										
Week 13	272	307	268	307	344	296	277	306	384	357
Week 26	232	300	262	283	365*	291	268	329	388	344
Week 39	219	306	239	283	381**	322	280	295	377	377
Week 52	276	349	300	278	396**	369	259	316	408	349
Alkaline phosphatase, ALP (U/L)										
Week 13	77	88	98	178*	505**	86	100	158	240**	379**
Week 26	66	94	97	209*	915**	65	89	172**	324**	550**
Week 52	67	106	94	160	925**	78	167	170	220*	569**
Total cholesterol (mmol/L)										
Week 13	4.11	3.64	3.76	3.76	2.97**	4.32	4.03	4.23	3.94	3.25*
Week 26	3.57	2.73	3.16	3.63	3.21	4.00	4.46	5.04	4.48	3.39
Week 52	3.31	2.67	2.77	2.64	2.52	3.63	5.48	4.19	4.25	2.85
Triglycerides										
Week 13	0.32	0.28	0.29	0.27	0.36	0.48	0.41	0.45	0.40	0.36
Week 26	0.23	0.29	0.26	0.23	0.44**	0.31	0.46	0.33	0.38	0.35
Week 52	0.37	0.39	0.333	0.45	0.48	0.58	0.48	0.53	0.35	0.50
Liver										
Weight: abs. (g)	352	391 (+11%)	375 (+7%)	439* (+25%)	601** (+71%)	334	424 (+27%)	402 (+17%)	419 (+25%)	496** (+49%)
Weight: rel. (%)	2.8	3.4 (+21%)	2.9 (+4%)	3.2 (+14%)	4.5** (+61%)	2.8	3.3 (+18%)	3.3 (+18%)	3.5 (+25%)	3.8* (+36%)
Hepatocellular hypertrophy	0	2	1	4	4	0	2	2	4	4
Minimal	0	2	1	2	0	0	2	1	3	0
Slight	0	0	0	2	3	0	0	1	1	3
Moderate	0	0	0	0	1	0	0	0	0	1
Pale cytoplasm with peripheral clumping	0	0	0	0	4	0	0	0	1	3
Thyroid										
Weight: abs. (g)	0.866	0.776	0.761	1.044 (+21%)	1.245** (+44%)	0.792	0.810	0.794	0.971 (+23%)	1.199** (+51%)
Weight: rel. (%)	0.0071	0.0066	0.0059	0.0075	0.0093* (+31%)	0.0067	0.0063	0.0065	0.0080 (+19%)	0.0094 (+40%)
Follicular hypertrophy	0	0	0	1	3	0	0	0	1	2
Minimal	0	0	0	1	1	0	0	0	1	2
Slight	0	0	0	0	2	0	0	0	0	0

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Saunders, 2005

Microscopic changes seen in the liver were mainly adaptive hepatocyte hypertrophy. Recorded clinical chemistry changes were considered consistent with the microscopically evident liver changes. Due to the clinical chemistry changes, liver and thyroid alterations at 250 mg/kg bw per day, a NOAEL of 50 mg/kg bw per day was determined for this study (Saunders, 2005).

(b) Dermal application

Rats

In a 28-day dermal toxicity study, groups of ten male and ten female Wistar Hannover rats received occluded dermal applications of valifenalate (purity 99.63%; batch T025/02) at doses of 0 or 1000 mg/kg bw per day for six hours a day. All animals were dosed once a day, seven days a week for four consecutive weeks. Animals were checked twice a day for mortality. Observations were carried out daily before dosing and approximately 1, 2 and 6 h after dosing up to day 7. Detailed clinical examination was carried out before starting the treatment and once a week thereafter. Body weight and food consumption were measured weekly. Ophthalmoscopy was conducted just prior to treatment and during week 4 of treatment. At the end of the four-week treatment period urine samples were collected for urinalysis. On the same day, blood samples were drawn for haematological and clinical chemistry investigations. At terminal necropsy, selected organs were weighed and tissues were examined microscopically.

No mortalities occurred during the study and no abnormalities were noted at daily observations or weekly detailed clinical observations. No effects on body weight or food consumption were observed. Ophthalmoscopy did not reveal any treatment-related findings. No treatment-related changes in haematology, clinical chemistry or urinalysis were noted. No effects on organ weights were found. The histopathological findings observed in all remaining tissues/organs examined were recognised as those commonly observed in this species and were considered to be spontaneous or incidental in origin. The dermal toxicity of valifenalate when given by daily topic administration (6 h exposure) to rats at a dose level of 1000 mg/kg per day, has been investigated over a period of four consecutive weeks. On the basis of the results, no signs of an effect due to the test item were seen at the dose level investigated. Therefore, the dose level of 1000 mg/kg per day was considered the NOAEL (Longobardi, 2005).

(c) Exposure by inhalation

No study available.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study, valifenalate (purity 99.56%; batch number T025/02) was administered in the diet for 78 weeks to groups of Crl:CD-1 (ICR) BR mice (50/sex) at concentrations of 0, 150, 850 or 5000 ppm. Overall achieved dose levels were equal to 0, 16.8, 97.2 and 657 mg/kg bw per day in males, 0, 21.6, 124 and 756 mg/kg bw per day in females. Animals were observed at least twice daily; a more detailed physical examination (including palpation) was performed pretest and weekly throughout the study. Bodyweights and food consumption were recorded at weekly intervals for the first 14 weeks and once every four weeks thereafter. Blood smears were prepared during weeks 52 and 78 from tail vein samples of all surviving animals; smears from the control and high-dose groups were examined for differential leukocyte count, abnormal morphology and unusual cell types. All animals were subject to gross necropsy and weights recorded of the adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes and uterus. Histopathology was performed on tissues from all control and high-dose mice and for all mice killed or found dead. Investigations in the intermediate dose groups were limited to the kidneys, liver and lungs and all grossly abnormal tissues.

Mortality was unaffected by treatment. Survival with increasing dose levels was 70, 60, 74 and 64% in males. For females survival was 78, 80, 72 and 60% with increasing dose levels. There were no clinical signs of toxicity. Overall body weight gain was lower in males at 5000 ppm (78% of the control value); findings were largely due to reduced weight gain from week 14. Similar effects were not seen in females or in males at lower dose levels. Food consumption in males at 5000 ppm was higher than controls (110% of the control value), resulting in a slightly lower food conversion efficiency. Examination of blood smears taken during weeks 52 and 78 revealed no treatment-related changes. Absolute and relative liver weights were higher in both sexes at 850 and 5000 ppm and in males at 150 ppm; males were affected to a greater extent than females. Absolute and relative kidney weights were also marginally higher in 5000 ppm females (+11% and +12% of control, respectively). Gross necropsy revealed a higher incidence of masses, pale areas and accentuated lobular patterning

in the livers of males at 850 and 5000 ppm. A slightly higher incidence of dark areas on the liver was observed for females at 5000 ppm. Non-neoplastic findings were seen in the liver and gall bladder. An apparent reduction in the incidence of centrilobular hepatocyte hypertrophy in males at 850 and 5000 ppm is related to the increased incidence of generalized hepatocyte hypertrophy. Gall bladder findings that attained statistical significance were limited to an increased incidence of choleliths in females at 5000 ppm. A higher incidence of hepatocellular tumours was observed in mice of both sexes at 850 and 5000 ppm. The incidences of adenoma in both groups exceed the historical control range (males, 7.8–21.2%; females, 0–1.9%). Considering liver carcinoma, historical control data indicate a range of 1.9–8.0% in males. The value found in males at 850 ppm is at the upper limit of the historical control range and the value found at 5000 ppm is outside the historical control range. It should be noted that the historical control data (HCD) are outside the two-year time frame, as the underlying studies for these HCD were conducted between January 1994 and November 1998.

Table 14. Selected findings in the 78-week study in mice receiving valifenalate in the diet

	Males				Females			
Dose (ppm)	**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Liver								
Weight: absolute (g)	2.772	3.258	3.568** (+29%)	4.851** (+75%)	1.974	2.100	2.434	2.407** (+22%)
Weight: relative (%)	4.807	5.522	6.183** (+29%)	9.469** (+97%)	4.685	4.482	5.415	5.768** (+23%)
Masses	3	2	1	5	0	1	2	2
Pale areas	1	0	3	2	0	1	5*	3
Accentuated lobular pattern	0	1	1	3	1	0	1	1
Centrilobular hepatocyte hypertrophy	21	34*	26	8**	8	9	12	25**
Slight	19	21	18	5	8	8	12	22
Moderate	2	13	8	2	0	1	0	3
Marked	0	0	0	1	0	0	0	0
Generalized hepatocyte hypertrophy	3	6	13*	29**	2	2	7	5
Slight	3	5	10	18	2	2	6	5
Moderate	0	1	3	11	0	0	1	0
Centrilobular hepatocyte vacuolation	11	14	33**	32**	2	8	8	2
Minimal	3	4	2	0	2	1	4	1
Slight	7	7	20	11	0	7	4	0
Moderate	1	3	11	20	0	0	0	1
Marked	0	0	0	1	0	0	0	0
Cytoplasmic eosinophilia	0	1	1	29**	1	0	0	6
Pigmented macrophages	1	2	4	12**	12	20	13	31**
Pigmented hepatocytes	1	0	0	18**	0	0	3	13**
Eosinophilic foci	1	0	1	1	0	1	1	4

	Males				Females			
Dose (ppm)	**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Gall bladder								
Choleliths	2	0	1	0	1	0	1	7*
Neoplastic findings								
Liver adenoma	7 (14%)	2 (4%)	14 (28%)	16* (32%)	0	0	2 (4%)	5* (10%)
Liver carcinoma	2 (4%)	4 (8%)	4 (8%)	10* (20%)	0	1 (2%)	0	0

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Webley, 2004b

Dietary concentrations of up to 5000 ppm were generally well tolerated. Histopathological examination identified the liver as the target organ with significantly increased neoplastic changes at 5000 ppm and an increase in liver adenomas outside the historical control range at 850 ppm. Reduced body weight gain and food conversion efficiency in males at 5000 ppm is indicative of non-specific toxicity and indicates that the maximum tolerated dosage (MTD) had been reached. There were no findings considered of toxicological significance in mice at 150 ppm and this dietary dose level (equal to 16.8 mg/kg bw per day) is considered to be the chronic NOAEL. Based on the increase in liver adenomas in males and females at the mid dose and high dose and liver carcinomas in males at the high dose, the NOAEL for carcinogenicity is set at 150 ppm (equal to 16.8 mg/kg bw per day) (Webley, 2004b).

Rat

A combined carcinogenicity and chronic toxicity study was conducted in Han Wistar rats. Valifenalate (purity 99.56 or 99.63%; batch number T025/02) was administered in the diet to groups of 50 rats/sex at concentrations designed to achieve constant dose levels of 0, 15, 150 or 1000 mg/kg bw per day; this lasted for 104 weeks, the carcinogenicity phase. A further 20 rat/sex were assigned to each group and sacrificed after 52 weeks (the toxicity phase). Animals were observed at least twice daily; more detailed physical examinations including palpation were performed pretest and weekly throughout the study. Terminal blood smears were taken from the carcinogenicity phase animals. For the toxicity phase animals, arena observations were performed weekly, in addition to the physical examination. During week 50, sensory reactivity and grip strength assessments were performed on ten rats/sex per group in the toxicity phase. Body weights and food consumption were recorded weekly for the first 16 weeks and once every four weeks thereafter. Water consumption was recorded by weight over a three-day period during week 24. Blood and overnight urine samples were collected at various time points from animals in both phases. All animals were subject to gross necropsy and weights recorded for the following organs: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid and uterus. Tissues from control and 1000 mg/kg bw per day rats were assessed histopathologically; the kidneys, liver and lungs were examined for all rats and the thyroid was examined for all toxicity phase males.

Mortality was unaffected by treatment. A slightly increased incidence of yellow perineal area was observed in females at 1000 mg/kg bw per day; brown staining on the cage paper was observed for males (and transiently for females) in this group. Arena observations were unaffected by treatment. Animals at 1000 mg/kg bw per day (and to a lesser extent at 150 mg/kg bw per day) showed slightly increased motor activity scores during week 50. Sensory reactivity and grip strength were unaffected by treatment. Overall body weight gain was reduced in the carcinogenicity phase males at 150 and 1000 mg/kg bw per day (92% and 91% of controls respectively) No bodyweight effects were observed in females or in toxicity phase rats of either sex. Initial food consumption by males taking 1000 mg/kg bw per day was slightly reduced; however overall food consumption was unaffected by treatment. Water consumption was comparable in all groups. Haematology indicated lower Hb concentration in males at 1000 mg/kg bw per day (by around 3%), associated at weeks 13 and 26 with reduced erythrocytes counts (by around 3%) and mean cell Hb concentration (by around 2%). Erythrocyte counts, Ht and Hb concentration were also slightly low at weeks 13, 26 and 52 (respectively for the three parameters) in females at 1000 mg/kg bw per day. Differences in females were less consistent than those seen in males.

Examination of the week 52, 78 and 104 blood smears from the carcinogenicity phase animals did not reveal any changes considered to be related to treatment. There were no toxicologically significant changes in clinical chemistry profile. Urinalysis revealed a tendency to slightly higher urine volume, lower specific gravity and lower protein concentration for females at 1000 and (to a lesser extent) 150 mg/kg bw per day at weeks 12, 25 and 51. Conversely, specific gravity and protein concentrations were slightly higher for males at 1000 mg/kg bw per day at some time points. A slight increase in urinary ketones was also observed in males at 1000 mg/kg bw per day. At the 52-week sacrifice, slightly higher absolute and relative liver weights were observed for both sexes at 1000 mg/kg bw per day. Relative kidney weights were also marginally higher in 1000 mg/kg bw per day males. At terminal sacrifice, slightly higher absolute and relative liver weights were observed in both sexes at 1000 mg/kg bw per day; differences were minimal and only the relative values attained statistical significance. Gross necropsy at 52 and 104 weeks did not reveal any treatment-related findings.

Treatment-related histopathological changes at the interim sacrifice were limited to an increased incidence of thyroid follicular cell hypertrophy in males at 1000 mg/kg bw per day. Follicular carcinoma was seen in a single male rat at 150 mg/kg bw per day; follicular adenoma was seen in one male rat at 150 mg/kg bw per day. Findings were not considered to be related to treatment in the absence of any increase in the incidence of these tumours in the carcinogenicity phase of this study. In the carcinogenicity phase there were no treatment-related neoplastic findings and no statistically significant differences in tumour incidences. An increased incidence of pelvic/papillary epithelial hyperplasia in the kidney was seen in females at 1000 mg/kg bw per day.

Table 15. Selected findings in the two-year study in rats receiving valifenalate in the diet

Dose	**Males**				**Females**			
(mg/kg bw per day)	**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Survival (%)								
Toxicity phase	95	95	100	100	100	100	95	85
Carcinogenicity phase	66	86	82	78	74	68	78	70
Liver								
Weight: absolute (g)								
Week 52	14.04	14.37	14.50	16.05* (+14%)	8.54	8.66	9.00	9.53* (+12%)
Week 104	16.27	15.46	15.55	16.71	11.08	10.90	10.76	11.90
Weight:relative (%)								
Week 52	2.890	3.093	3.095	3.441** (+19%)	3.196	3.178	3.301	3.587** (+12%)
Week 104	2.876	2.805	2.906	3.161** (+10%)	3.203	3.071	3.083	3.446** (+8%)
Kidney								
Weight: absolute (g)								
Week 52	2.28	2.28	2.28	2.35	1.64	1.62	1.63	1.58
Week 104	2.78	2.76	2.70	2.72	1.98	2.03	1.96	2.03
Weight:relative (%)								
Week 52	0.472	0.490	0.490	0.508* (+8%)	0.617	0.599	0.598	0.595
Week 104	0.491	0.490	0.517	0.519	0.577	0.574	0.563	0.592
Pelvic hyperplasia	4	1	3	2	9	10	5	25**
Slight	4	1	2	1	7	7	4	17
Moderate	0	0	1	1	2	3	1	7
Marked	0	0	0	0	0	0	0	1

Dose	Males				Females			
(mg/kg bw per day)	0	15	150	1000	0	15	150	1000
Thyroid								
Follicular cell hypertrophy	3	2	5	11*	1	0	0	1
Slight	3	2	5	10	1	0	0	1
Moderate	0	0	0	1	0	0	0	0

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Webley, 2004a

Oral administration of valifenalate to Han Wistar rats for up to 104 weeks identified the thyroids and kidneys as target organs. A slightly low overall body weight gain for males at 150 and 1000 mg/kg bw per day suggests slight nonspecific toxicity. In males at 1000 mg/kg bw per day there was a persistent slight anaemia and, at week 52, thyroid changes. Changes to renal function were attributed to the excretion of valifenalate. A chronic NOAEL of 150 mg/kg bw per day was determined for this study, based on the effects on thyroid (follicular cell hypertrophy) and kidney (pelvic hyperplasia). There were no treatment-related neoplastic findings, therefore, the NOAEL for carcinogenicity was 1000 mg/kg bw per day, the highest dose tested (Webley, 2004a).

2.4 Genotoxicity

Valifenalate is not mutagenic to bacterial cells in vitro, did not induce chromosome aberrations in vitro and is not clastogenic in an in vitro mammalian mutation assay at the thymidine kinase locus in L5178Y cells. Valifenalate was not genotoxic in an in vivo micronucleus assay in NMRI mice. Overall, valifenalate shows no genotoxic potential either in vitro or in vivo.

Table 16. Overview of genotoxicity with valifenalate [a, b]

End-point	Test object	Concentration	Batch and Purity	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, T1537, TA102	Up to 5000 µg/plate with and without S9 mix	FCF/T/180-00 (ex ZI068) 98.9%	Negative	Wollny, (2002a)
Chromosomal aberrations	Chinese-hamster ovary (CHO) cells	Up to 200 µg/mL without S9 mix and up to 400 µg/mL with S9 mix	FCF/T/180-00 (ex ZI068) 98.9%	Negative	Schultz, (2002)
Gene mutation	Mouse lymphoma L5178Y ($tk^{+/-}$) cells	Up to 800 µg/mL without S9 mix and up to 400 µg/ml with S9 mix	FCF/T/180-00 (ex ZI068) 98.9%	Negative	Wollny, (2002b)
In vivo					
Micronucleus test	Male and female NMRI mice (6/sex per dose)	0, 500, 1000 and 2000 mg/kg bw	T 025/02 99.56%	Negative	Honarvar, (2003)

[a] Positive and negative (solvent) controls were included in all studies

[b] Statements of adherence to quality assurance and GLP were included unless indicated otherwise

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

A preliminary study was carried out in HanBrl:WIST rats to determine suitable dose levels for the two-generation study described below. Valifenalate (purity 99.56%; batch number T025/02) was administered in the diet to groups of rats (eight/sex) during a 21-day premating period, then during mating, and gestation/lactation of the resulting litters. Selected offspring were maintained on the treated diets for a seven-day treatment period after the 21-day lactation period. Dietary concentrations of 0, 1400, 7000 and 14 000 ppm were reduced to 900, 4500 and 9000 ppm during lactation and following weaning. No mortalities or clinical signs were observed; food consumption and weight gain were unaffected by treatment. Reproductive parameters were comparable in all groups. Slightly lower food consumption was noted in F1 pups at 9000 ppm (Marburger, Knuppe & Weber, 2002).

A two-generation reproductive toxicity study was conducted in HanBrl:WIST (SPF) rats. Valifenalate (purity 99.56%; batch number T025/02) was administered to groups of 24 rats/sex in the diet at concentrations of 0, 1250→850, 4300→2900, 15 000→10 000 ppm over two successive generations. Dietary concentrations were reduced during lactation to maintain a more constant intake throughout the study. Achieved doses were 81, 277 and 986 mg/kg bw per day in males, 93, 319 and 1146 mg/kg bw per day in females. Parental (F0) rats received valifenalate for 70 days prior to mating, during mating, gestation and lactation. At weaning, administration of the test substance was continued for F1 offspring during premating, mating, gestation and lactation until weaning of the F2 generation. Parental animals were examined for their reproductive performance, mating and parturition. Sperm parameters were evaluated for male rats. All parental animals were subject to gross necropsy and organ weights were recorded. F2 pups were subject to gross necropsy; weight of the brain, spleen and thymus were recorded for one randomly selected male and female pup from each litter. Histopathology was performed for all control and high-dose group parental animals and on one male and one female pup of each F2 litter (control and high-dose groups).

Ruffled fur was noted in a small number of dams at 4300 and 15 000 ppm during early lactation in the F1 generation. There were no clear effects of treatment on parental body weight. During lactation, reduced food consumption was noted in dams at 4300 and 15 000 ppm. Gross necropsy revealed enlarged livers in F0 males at the highest dose level and in F1 males in the mid- and high-dose levels. Enlarged liver was noted in one high-dose F1 female. Increased liver weights were noted for both sexes in the mid- and high-dose groups of the F0 and F1 generations. Histopathology revealed centrilobular hepatocellular hypertrophy in many rats of these groups. Reduced kidney weight was noted for high dose F1 females.

Reproductive performance was unaffected by treatment. Estrus cyclicity and spermatogenic end-points (sperm motility, morphology and sperm head count) were comparable in all groups. Neonatal mortality in F1 pups was increased in the highest two dose groups; the resulting viability indices were 85.2% and 84.8% respectively, compared to 92.6% in the control group. Pup mortality was also increased in these groups. F1 pup weights were unaffected by treatment at any dose. F2 pup weights at day 1 were unaffected by treatment, however a reduction in pup weight gain during lactation was seen at the two highest dose levels. In F2 pups during lactation, the incidence of the finding 'no milk in the stomach' was increased in the mid- and high-dose groups between days 2 and 5 of lactation. Most of the affected pups were found dead or were missing (i.e. cannibalized) on one of the following days. This increased incidence was considered to be treatment-related. No treatment-related effect on sex ratio was noted in either generation. Attainment of sexual maturation (balanopreputial separation and vaginal patency) in the F1 generation was not adversely affected. There were no treatment-related effects at any dose level on the parameters of the behaviour tests, observed in the modified Irwin Screen. A lower mean overall locomotor activity was seen in high-dose females compared to the control, however, the difference was considered to be too small to be of toxicological relevance. Gross necropsy of F1 and F2 pups did not reveal any effects of treatment. Brain and thymus weights in F2 pups were unaffected by treatment. Spleen weights of male and female F2 pups at the two highest dose levels were significantly lower, however histopathological examination did not reveal any correlating findings. Therefore, it was concluded that the lower spleen weights were attributable to the lower pup weights noted in these two

dose groups. F2 pups of both sexes showed some decrease in the incidence and/or degree of glycogen deposition in the liver at 4300 and 15 000 ppm; these findings were not considered to be adverse but reflecting the nutritional state of the animals.

Table 17. Parental findings in the reproductive toxicity study with valifenalate

Dose (ppm)	0	1250→850	4300→2900	15 000→10 000
		F0 generation males		
Brain weight (g)	2.03	2.02	2.00	1.97**
Liver weight (g)	12.45	12.32	12.52	14.45**
		F0 generation females		
Relative food consumption (g/kg bw per day)				
Lactation days 1–7	123.1	119.3	115.2	114.2
Lactation days 7–14	178.7	172.2	166.4*	162.6**
Brain weight (g)	1.88	1.87	1.87	1.85
Liver weight (g)	12.09	12.07	12.84*	13.95**
		F1 generation males		
Brain weight (g)	2.06	2.04	2.02	2.00*
Liver weight (g)	14.49	14.65	15.35	16.18**
		F1 generation females		
Relative food consumption (g/kg bw per day)				
Lactation days 1–7	127.4	116.9	114.7	106.5*
Lactation days 7–14	177.1	187.0	181.4	170.1
Brain weight (g)	1.87	1.88	1.89	1.87
Liver weight (g)	12.37	12.24	12.27	13.30

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

Source: Marburger, Knuppe & Weber, 2004

Table 18. Reproductive and pup findings in the reproductive toxicity study with valifenalate

Dose (ppm)	0	1250→850	4300→2900	15 000→10 000
		F0 generation		
Fertility index (%)	100	100	95.8	100
Gestation index (%)	100	100	100	100
Duration of gestation (days)	21.6	21.5	21.7	21.5
Postimplantation loss (%)	1.6	2.4	1.8	1.3
Birth index (%)	98.4	97.6	98.2	98.7
Viability index (%)	99.0	96.5*	96.4*	99.3
Weaning index (%)	100	97.2*	100	96.4**
Postnatal loss days 0–4 PP (%)	1.0	3.5	3.6	0.7
Total number of pups lost	3	10*	10*	2
		F1 generation		
Fertility index (%)	87.5	95.8	95.8	95.8
Gestation index (%)	100	100	95.7	100
Duration of gestation (days)	21.8	21.6	21.8	21.6

Dose (ppm)	0	1250→850	4300→2900	15 000→10 000
Postimplantation loss (%)	8.6	11.4	14.2	11.4
Birth index (%)	91.4	88.6	85.8*	88.6
Viability index (%)	92.6	96.8*	85.2**	94.8**
Weaning index (%)	97.5	99.4	94.2	93.9
Postnatal loss days 0–4 PP (%)	7.4	3.2	14.8	15.2
Total number of pups lost day 4 PP	18	8	35**	39**
Breeding loss days 5–12 PP (%)	2.5	0.6	5.8	6.1
		F1 Pups		
Pup body weight (g)				
Day 1 PP	5.6	5.7	5.7	5.8
Day 4 PP	8.5	8.5	8.3	8.2
Day 7 PP	14.0	13.9	13.5	13.2
Day 14 PP	29.2	29.7	28.9	28.0
Day 21 PP	45.8	45.5	44.3	42.7
Body weight gain PP days 1–21 (g)	40.2	39.8	38.6	36.9
Preputial separation				
Day PP	26.7	26.1*	26.2	26.1*
Weight (g)	75.18	71.65	69.96*	71.40
Vaginal patency				
Day PP	35.1	34.9	34.8	35.4
Weight (g)	111.06	108.84	105.93	106.17
		F2 Pups		
Pup body weight (g)				
Day 1 PP	5.7	5.4	5.8	6.0
Day 4 PP	8.6	7.9	8.1	8.3
Day 7 PP	14.3	13.3	13.3	13.2
Day 14 PP	30.5	28.8	28.1	28.2
Day 21 PP	49.0	46.9	45.1	45.8
Body weight gain PP days 1–21 (g)	43.3	41.5	39.3	39.8
No milk in stomach (Number of pups/number of litters)	4 / 2	5 / 1	46 / 7	31 / 4
Spleen weight: male/female (g)	0.277/0.282	0.242/0.246	0.206**/0.210**	0.227**/0.217**

PP Postpartum Source: Marburger, Knuppe & Weber, 2004

* significantly different to controls, $p < 0.05$) ** $p < 0.01$

In the absence of effects the parental NOAEL was set at the highest dose tested of 15 000 ppm (equal to 986 mg/kg bw per day).

In the absence of effects, the reproductive NOAEL was set at 15 000 ppm (equal to 986 mg/kg bw per day), the highest dose tested.

The offspring NOAEL was set at 1250 ppm (equal to 81 mg/kg bw per day) based on decreased F2 pup body weight gain during lactation (Marburger, Knuppe & Weber, 2004).

(b) Developmental toxicity

Rat

A preliminary study was carried out in groups of eight pregnant Crl:CD(SD)BR rats. Valifenalate (purity 98.9%; batch number FCF/T/180-00, ex ZI068) was administered by gavage in 0.5% aqueous methylcellulose at dose levels of 0 (vehicle), 250, 500 or 1000 mg/kg bw per day on days 6–15 of gestation. Effects of treatment on maternal animals were limited to a slightly higher body weight gain in all treated groups, which displayed a dose relationship. Lower fetal weight observed at 250 mg/kg bw per day was attributable to a significantly lower value for male fetuses and is considered to be incidental in the absence of a dose–response relationship and because values were within the historical control range. One fetus with umbilical hernia was observed at 1000 mg/kg bw per day group (Comotto, 2001a).

The main rat developmental toxicity study was conducted in groups of 25 pregnant Crl:CD(SD) BR rats. Valifenalate (purity 98.9%; batch number FCF/T/180-00; ex ZI068) was administered by gavage (in 0.5% aqueous methylcellulose) at dose levels of 0 (vehicle), 100, 300 or 1000 mg/kg bw per day on days 6–19 of gestation.

There was no mortality or clinical signs of toxicity. As in the preliminary study, body weight gain in the treated groups was slightly higher than controls, attaining statistical significance only at 1000 mg/kg bw per day during the early treatment phase, gestation days (GDs) 6–12. This finding is not considered to be of toxicological significance. Food consumption was unaffected by treatment. No effects were noted on fetal weight, placental weight, gravid uterus weight or litter weight. A single incidence of agnathia was observed at 300 mg/kg bw per day; there were no further external, visceral or skeletal malformations. A significantly higher frequency of skeletal variants observed at 100 mg/kg bw per day was considered to be incidental in the absence of similar findings at higher dose levels.

Table 19. Summary of findings in the rat developmental toxicity study with valifenalate

Dose (mg/kg bw day)		0	100	300	1000
Maternal body weight (g)	Day 0	247.2	246.0	250.3	245.4
	Day 6	277.7	279.4	280.4	275.1
	Day 8	281.4	284.7	285.1	282.0
	Day 10	286.0	291.3	292.2	289.3
	Day 19	361.0	369.8	369.7	367.9
	Day 20	376.7	387.6	388.1	385.1
Maternal body weight gain (g)	Day 6–8	3.74	5.36	4.68	6.95
	Day 6–10	8.26	11.95	11.86	14.24
	Day 6–12	18.32	22.09	21.95	25.48**
	Day 6–20	99.05	108.23	107.77	110.05
Mated (*N*)		25	25	25	25
Pregnant (*N*)		19	22	22	21
Corpora lutea (*N*)		17.32	17.95	17.77	17.43
Implantations (*N*)		14.63	15.27	14.45	14.67
Preimplantation loss (%)		14.48	14.95	17.48	16.04
Postimplantation loss (%)		6.81	5.08	4.16	4.64
Live fetuses (*N*)		13.63	14.55	13.86	13.95
Fetal weight (g)		3.97	3.97	4.00	3.96
Percent males (sex ratio, %)		49.42	50.31	47.21	47.44

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Comotto, 2002

There was no evidence of teratogenicity in this study. In the absence of any findings of toxicological significance, maternal and developmental NOAELs of 1000 mg/kg bw per day were determined for this study (Comotto, 2002).

Rabbit

A preliminary study was conducted in groups of eight pregnant New Zealand White rabbits. Valifenalate (purity 98.9%; batch number FCF/T/180-00, ex ZI068) was administered by gavage (in 0.5% aqueous methylcellulose) on GDs 6–18 at dose levels of 0 (vehicle), 250, 500 or 1000 mg/kg bw per day. During the study one female in the 250 mg/kg bw per day group aborted on GD 17 and another in the 500 mg/kg bw per day group aborted on GD 28. Three deaths occurred during the study: one control female died on GD 23, one female in the 250 mg/kg bw per day group died on GD 16 and one female in the 500 mg/kg bw per day group died on GD 20. All three deaths were caused by pneumonia and were considered incidental. Slightly higher body weight gain was observed at 500 and 1000 mg/kg bw per day during the treatment period, but without a dose–response relationship. Slightly fewer live fetuses were observed in the 250 and 500 mg/kg bw per day groups but a similar finding was not observed at the highest dose level (Comotto, 2001b).

The main developmental toxicity study was conducted in groups of 22 pregnant New Zealand White rabbits. Valifenalate (purity 98.9%; batch number FCF/T/180-00, ex ZI068) was administered by gavage (in 0.5% aqueous methylcellulose) at dose levels of 0 (vehicle), 100, 300 or 1000 mg/kg bw per day on GDs 6–28. One female in the 100 mg/kg bw per day group aborted on day 20. There was no treatment-related mortality; however deaths due to dosing accidents or incidental pneumonia occurred in all groups (four each in the control and low-dose groups, one in the mid-dose group and three in the high-dose group). A slightly higher body weight gain was observed at 300 and 1000 mg/kg bw per day during the treatment period, but without a dose–response relationship. Food consumption was unaffected by treatment. No treatment-related effects were noted on any of the litter parameters. One fetus with arthrogryposis was observed at 1000 mg/kg bw per day; one external malformation (absent testis) was observed at 100 mg/kg bw per day. Skeletal examination showed two malformed fetuses in separate litters at 1000 mg/kg bw per day group, one with misshapen sternum and one with scoliosis. A significantly higher frequency of skeletal anomalies and variants at 300 and 1000 mg/kg bw per day was not considered to be related to treatment in the absence of a dose–response relationship. There were no visceral malformations, anomalies or variants in any group.

Table 20. Summary of findings in the rabbit developmental toxicity study with valifenalate

Dose (mg/kg bw day)		**0**	**100**	**300**	**1000**
Maternal body weight (kg)	Day 0	3.96	4.02	3.94	4.07
	Day 6	4.13	4.19	4.13	4.24
	Day 28	4.37	4.37	4.42	4.52
	Day 29	4.39	4.39	4.44	4.54
Maternal body weight gain (g)	Day 6–10	60.7	32.5	37.7	46.7
	Day 6–28	241.3	186.9	294.1	281.3
	Day 6–29	260.0	201.9	313.5	292.7
Pregnant (*N*)		18	20	18	18
Deaths (*N*)		3	4	1	3
Litters (*N*)		15	16	17	15
Corpora lutea (*N*)		8.27	9.69	9.82	9.33
Implantations (*N*)		6.67	7.94	7.94	7.07
Preimplantation loss (%)		20.40	17.87	19.00	25.33
Postimplantation loss (%)		6.45	21.28	9.75	8.43
Live fetuses (*N*)		6.13	7.00	7.62	6.47
Fetal bodyweight (g)		48.04	46.38	46.02	46.43
Percent males (sex ratio, %)		54.35	56.12	53.28	56.70
Fetuses with skeletal variants (%)		53.35	58.16	74.59**	56.70
Fetuses with skeletal anomalies (%)		4.35	11.22	19.67**	17.53**

* significantly different to controls, $p < 0.05$) ** $p < 0.01$ Source: Comotto, 2003

There was no evidence of teratogenicity in this study. In the absence of any findings of toxicological significance, maternal and developmental NOAELs of 1000 mg/kg bw per day were determined for this study (Comotto, 2003).

2.6 Special studies

(a) Neurotoxicity

No specific studies regarding neurotoxicity are available for valifenalate.

(b) Immunotoxicity

No specific studies regarding immunotoxicity are available for valifenalate.

(c) Mechanistic studies

No mechanistic studies are available for valifenalate.

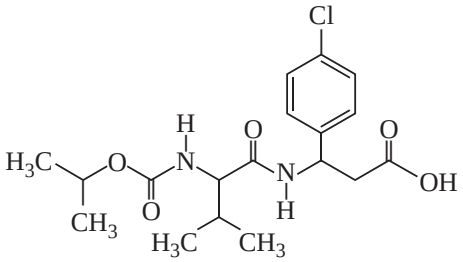
(d) Studies on metabolites or impurities

No studies on impurities are available. Studies have been performed on two metabolites of valifenalate: IR5839 (valifenalate acid, R2) and PCBA (4-chlorobenzoic acid).

IR5839 (valifenalate acid, R2)

For metabolite IR5839 an acute oral toxicity study in rats showed a median lethal dose (LD_{50}) > 2000 mg/kg bw (Freulon, 2004). Metabolite IR5839 did not induce gene mutations in an Ames test (Sokolowsky, 2004) and did not induce mutations at the *tk* $^{+/-}$ locus of mouse lymphoma L5178Y cells (Poth, 2004). In an in vitro chromosome aberration assay, metabolite IR5839 induced structural chromosome aberrations; in this assay no increase in polyploidy was observed (Schultz, 2004). As a follow-up, metabolite IR5839 was tested in a bone marrow micronucleus assay in mice (Honarvar, 2004a). In this study IR5839 did not induce micronuclei. Overall this metabolite is considered not genotoxic. Furthermore, this metabolite was detected in the rat metabolism study as one of the main metabolites (see Table 4; metabolite coded R2 was found up to 36% in urine). It can therefore be assumed that the toxicity of this metabolite is covered by the studies conducted with the parent compound valifenalate.

Figure 3. Chemical structure of IR5839 (valifenalate acid, R2)

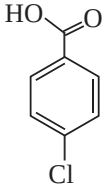


PCBA (4-chlorobenzoic acid)

Metabolite PCBA (4-chlorobenzoic acid) did not induce mutations at the *tk* $^{+/-}$ locus of mouse lymphoma L5178Y cells (Poth, 2003); a non-significant increase was seen at the two high doses in experiment I after 4 h exposure without S9; this observation was not reproduced in experiment II. Therefore, according to the criteria in OECD 476, PCBA is considered non-mutagenic in this mouse lymphoma assay. In addition, PCBA was tested in an in vitro chromosome aberration assay using human lymphocytes

(Schultz, 2003). In this assay, PCBA induced structural chromosome aberrations and a slight increase in polyploidy was observed compared to the negative control. As a follow-up, metabolite PCBA was tested in an in vivo micronucleus study in NMRI mice (Honarvar, 2004b). In this assay, PCBA did not induce the formation of micronuclei. Overall, this metabolite is considered not genotoxic. The threshold of toxological concern (TTC) approach could be used for PCBA (Cramer class III) at 1.5 μg/kg bw per day.

Figure 4. Chemical structure of PCBA (4-chlorobenzoic acid)

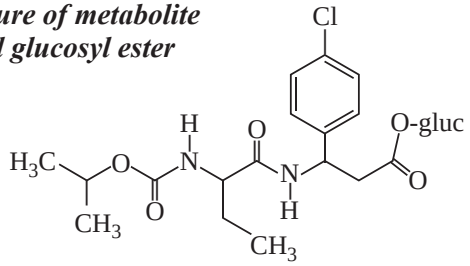


Other metabolites

Information was requested for two additional metabolites: valifenalate acid glucosyl ester and β-4-chlorophenylalanine-*N*-glucoside.

Valifenalate acid glucosyl ester was not detected in the rat metabolism study and no specific data regarding this metabolite was provided. However, it is the glucosyl ester of the R2 metabolite (valifenalate acid, IR5839) which was one of the main rat metabolites (found up to 36% in rat urine). It is proposed to read-across to the metabolite valifenalate acid, which is considered covered by the studies conducted with the parent valifenalate. Thus, metabolite valifenalate acid glucosyl ester is also considered to be covered by the studies conducted with valifenalate.

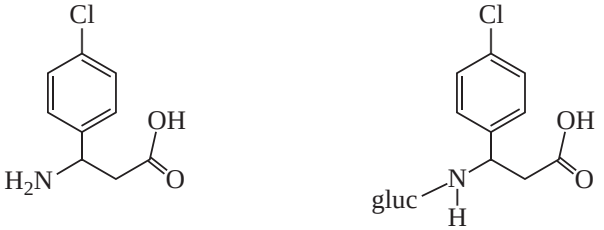
Figure 5. Chemical structure of metabolite valifenalate acid glucosyl ester



No specific data was provided for metabolite β-4-chlorophenylalanine-*N*-glucoside (conjugate of R5 in the rat metabolism study). The sponsor, however, indicated the following regarding metabolite R5:

> this metabolite is indeed appearing in the rat metabolism study. Observed levels are in the kidney and the urine respectively at < 0.10% (single low dose) and 3.6% of the administered dose. This correlates to around 11 mg/kg in kidneys. Although the metabolite appears present lower than 10% of the AD, we consider that the metabolite itself is covered by the parent because the dietary exposure is low, the toxicological studies were dosed at high levels, and the toxicological profile of the parent substance is considered well characterized. The dietary exposure is considered low because the metabolite itself is only found in the potato metabolism where generally speaking the residue levels of parent are very low (100% < LOQ of 0.01 mg/kg). In other crops, the metabolite is not characterized/found back. Consequently, human exposure through plants can be considered very low. Furthermore potatoes are not subjected to this CODEX request as EU MRL is 0.01 mg/kg for this crop.

Figure 6. Chemical structure of metabolites β-4-chlorophenylalanine (R5, left) and its conjugate β-4-chlorophenylalanine-N-glucoside (right)



As there is no specific data provided for β-4-chlorophenylalanine (R5) or its conjugate β-4-chlorophenylalanine-*N*-glucoside, no conclusions can be made regarding its (geno)toxicity. The TTC approach could be used in case a reference value is needed. Since no information on genotoxicity was provided and genotoxicity can thus not be excluded, the TTC value of 0.0025 μg/kg bw per day applies.

3. Observations in humans

No clinical cases or poisoning incidents have been recorded at pilot plant production level. About ten operators (chemists), one shift incharge [sic] and one production line manager are involved on every shift in day-to-day valifenalate production. No clinical incidents have happened to date.

Medical surveillance on manufacturing plant personnel and monitoring studies

Every year examinations will be conducted on all persons, including managers.

Reports of adverse effects from product end users

There are no reported incidents from product end users.

4. Microbial aspects

No information for valifenalate was available concerning mechanism and type of antimicrobial action, effects on the microbiome of the human GIT, antimicrobial spectrum of activity or antimicrobial resistance mechanisms and genetics.

Comments

Biochemical aspects

The toxicokinetics and metabolism of ^{14}C-radiolabelled valifenalate have been investigated in the rat, following oral dosing. Following a single oral dose of valifenalate, concentrations of radioactivity in whole blood increased rapidly to reach C_{max} at 1–2 h post dose. The maximum concentration following a single high dose of 1000 mg/kg bw was markedly lower than 10 times that seen following a low dose of 100 mg/kg bw, indicating saturation of absorption. Radioactivity was rapidly excreted, mainly in the faeces, with a lower proportion in urine. The majority of the radioactivity in tissues was found in the GI tract. The liver and kidneys also contained concentrations higher than those in the blood. Residual radioactivity declined rapidly with time and was below the limit of quantification at 72 hours post dose (Kidd & Gedik, 2003).

Repeated administration of the low dose (100 mg/kg bw per day) given for 14 days with unlabelled valifenalate followed by a single radiolabelled dose, did not result in any increase in tissue residues. Bioaccumulation is not predicted based on such rapid excretion. A sex difference in the excretion of radioactivity was apparent: excretion in the faeces was higher in male (83%) than in female rats (58%), with a lower proportion eliminated in the urine in males (9%) compared to females (34%). The majority of the administered radioactivity was excreted within 48 h of dosing. In bile-cannulated rats, the majority of the administered dose was excreted in bile (65% and 49% of the dose in male and female rats, respectively). Based on these results, approximately 80% of the administered dose was absorbed. Valifenalate or its metabolites were not eliminated via expired air (Kidd & Gedik, 2003).

Valifenalate was found to be extensively metabolized in rats; six metabolites were identified. At the low dose level of 100 mg/kg bw only a small proportion of the administered radioactivity was eliminated as unchanged valifenalate (5–8% in faeces; not detected in urine). In high-dose groups (1000 mg/kg bw), more unchanged valifenalate was excreted compared to the low-dose groups, with females showing more extensive metabolism at this high dose compared to males (unchanged valifenalate excreted was 40% in males and 10% in females). The metabolites were the products of

primary metabolism, (Scacchi, Oriolo & Pizzingrilli, 2003), mainly:

- *O*-demethylation – forming R2, identified as RS-β-alanine; *N*-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl; valifenalate acid); found up to 36% in urine;
- hydroxylation at both carbons 2 and 3 of the chlorophenyl moieties of the parent molecule –forming R3 (only found in faeces up to 5%) and R4 (found in urine up to 0.8%);
- side-chain cleavage – forming R5, found up to 3.6% in urine.

The diastereoisomeric ratio (S,R:S,S) of the unchanged parent compound and of R2 did not alter notably as measured in rat urine and faeces.

Toxicological data

The acute oral LD_{50} of valifenalate was > 5000 mg/kg bw (Yu, 2001a) and the dermal LD_{50} was > 2000 mg/kg bw (Yu, 2001b). The inhalation median lethal concentration (LC_{50}) of valifenalate was > 3.118 mg/L (Dotti, 2001). Valifenalate was not irritating to skin or eyes in rabbits (Renoldi, 2001a, b) and was not considered to be a skin sensitizer in the guinea pig maximization test (Vigna, 2001).

In repeated-dose toxicity studies on mice (28-day and 90-day), rats (28-day and 90-day) and dogs (28-day, 90-day and one-year), the main effects were reduced body weight gain, increased liver weight and hepatocellular hypertrophy, along with changes in clinical chemistry parameters.

In a 90-day toxicity study in mice, valifenalate was administered at dietary concentrations of 0, 110, 900 and 7000 ppm (equal to 0, 15.3, 134 and 995 mg/kg bw per day for males, 0, 16.7, 148 and 1144 mg/kg per day for females). The NOAEL was 110 ppm (equal to 15.3 mg/kg bw per day), based on decreased body weight gain and liver histopathology (vacuolation in males due to fat accumulation) at 900 ppm (equal to 134 mg/kg bw per day) (Webley, 2002a).

In a 90-day toxicity study in rats valifenalate was administered in the diet at varying concentrations to obtain dietary doses of 0, 7, 150 or 1000 mg/kg bw per day (ten rats/sex per dose). The NOAEL for this study was 150 mg/kg bw per day based on the macroscopic change of distended caecum at 1000 mg/kg bw per day (Webley, 2002a).

In a 28-day study in dogs valifenalate was administered in gelatin capsules at doses of 0, 250, 500 or 1000 mg/kg bw per day (three dogs/sex per dose). The main target organ was the liver. The NOAEL was 250 mg/kg bw per day based on increased liver weight and liver histopathology (hepatocellular hypertrophy), and clinical chemistry changes at 500 mg/kg bw per day (Brown, 2003).

In a 13-week study in dogs valifenalate was administered in gelatin capsules at dose levels of 0, 50, 250 and 750 mg/kg bw per day (four dogs/sex per dose). Similarly to the 28-day dog study, the liver was a target organ, however, effects on the thyroid were also observed after 90 days (follicular cell hypertrophy). The NOAEL was 50 mg/kg bw per day based on changes in clinical chemistry (increased alkaline phosphatase), liver (hepatocellular hypertrophy) and thyroid (follicular cell hypertrophy) at 250 mg/kg bw per day (Geary, 2003).

In a one-year dog study valifenalate was administered in gelatin capsules at dose levels of 0, 1, 7, 50 or 250 mg/kg bw per day (four dogs/sex per dose). The NOAEL was 50 mg/kg bw per day based on changes in clinical chemistry, liver effects (increased weight and hepatocellular hypertrophy) and thyroid alterations (follicular cell hypertrophy) at 250 mg/kg bw per day (Saunders, 2005).

In a 78-week dietary toxicity and carcinogenicity study, mice received valifenalate at dietary levels of 0, 150, 850 or 5000 ppm (equal to 0, 16.8, 97.2 and 657 mg/kg bw per day for males, 0, 21.6, 124 and 657 mg/kg bw per day for females). The NOAEL for carcinogenicity was 150 ppm (equal to 16.8 mg/kg bw per day) with a lowest-observed-adverse-effect level (LOAEL) of 850 ppm (equal to 97.2 mg/kg bw per day) based on liver adenomas exceeding the historical control range at the mid and high dose, and at the high dose also an increase in liver carcinoma outside the historical control range that was seen in males. The NOAEL for chronic toxicity was 150 ppm (equal to 16.8 mg/kg bw per day) based on increased liver weight accompanied by

histopathological changes in the liver (hepatocellular hypertrophy, centrilobular hepatocellular vacuolation) at 850 ppm (equal to 97.2 mg/kg bw per day) (Webley, 2004b).

In a two-year toxicity and carcinogenicity study, rats received valifenalate in the diet at varying concentrations to obtain dietary doses of 0, 15, 150 or 1000 mg/kg bw per day. No neoplastic lesions related to treatment were observed. The NOAEL for chronic toxicity was 150 mg/kg bw per day based on thyroid (follicular cell hypertrophy) and kidney (pelvic hyperplasia) changes at 1000 mg/kg bw per day (Webley, 2004a).

The meeting concluded that valifenalate is carcinogenic in mice, but not in rats.

Valifenalate was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The meeting concluded that valifenalate is unlikely to be genotoxic.

In view of the lack of genotoxicity, the finding of malignant liver tumours in male mice only at the highest dose, which is expected to show a threshold, and the absence of carcinogenicity in rats, the Meeting concluded that valifenalate is unlikely to pose a carcinogenic risk to humans via the diet.

In a two-generation study rats were fed diets containing valifenalate at concentrations of 0, 1250, 4300 and 15 000 ppm (equal to 0, 81, 277 and 986 mg/kg bw per day in males, 0, 93, 319 and 1146 mg/kg bw per day in females). In the absence of adverse effects, the parental NOAEL was 15 000 ppm (equal to 986 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 1250 ppm (equal to 81 mg/kg bw per day) based on decreased F2 pup body weight gain during lactation, at 4300 ppm (equal to 277 mg/kg bw per day). The reproductive NOAEL was set at 15 000 ppm (equal to 986 mg/kg bw per day), the highest dose tested (Marburger, Knuppe & Weber, 2004).

In a developmental toxicity study in rats, valifenalate was administered via oral gavage at dose levels of 0, 100, 300 or 1000 mg/kg bw per day from GD 6–19. No signs of maternal or developmental toxicity were observed in this study, therefore the maternal and embryo/fetal NOAELs were 1000 mg/kg bw per day, the highest dose tested (Comotto, 2002).

In a rabbit developmental toxicity study valifenalate was administered by oral gavage from GD 6 to GD 28 at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. No signs of maternal or developmental toxicity were observed in this study, therefore the maternal and embryo/fetal NOAELs were 1000 mg/kg bw per day, the highest dose tested (Comotto, 2003).

The Meeting concluded that valifenalate is not teratogenic.

No evidence of neurotoxicity was reported in routine toxicological studies with valifenalate.

The Meeting concluded that valifenalate is unlikely to be neurotoxic.

No evidence of immunotoxicity was reported in routine toxicological studies with valifenalate.

The Meeting concluded that valifenalate is unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

Metabolite IR5839 (valifenalate acid, R2), found in plants, rats and other animals, was not acutely toxic via oral exposure (LD_{50} > 2000 mg/kg bw). Metabolite IR5839 did not induce gene mutations in an Ames test (Sokolowsky, 2004), nor did it induce mutations at the $tk^{+/-}$ locus of mouse lymphoma L5178Y cells (Poth, 2004). Metabolite IR5839 induced structural chromosome aberrations in an in vitro assay, without any increase in polyploidy (Schultz, 2004). In a follow-up in vivo micronucleus assay in mice, metabolite IR5839 did not induce micronuclei (Honarvar, 2004b). IR5839 (R2) was one of the main metabolic products of valifenalate in rats (up to 36% in urine) and can be considered covered by studies conducted with the parent compound.

Metabolite PCBA (4-chlorobenzoic acid), found in soil, did not induce mutations at the *tk*$^{+/-}$ locus of mouse lymphoma L5178Y cells (Poth, 2003). PCBA induced structural chromosome aberrations in an in vitro assay with human lymphocytes and a slight increase in polyploidy was observed (Schultz, 2003). In a follow-up in vivo micronucleus assay in mice, PCBA did not induce micronuclei (Honarvar, 2004b). It was concluded that TTC Cramer class III can be applied (value 1.5 µg/kg bw per day).

Valifenalate acid glucosyl ester is the glucosyl ester of the metabolite IR5839 (valifenalate acid, R2) and will therefore not be more toxic than R2. As R2 is covered by studies conducted with the parent compound, the Meeting concluded that this will also hold for this glucosyl ester of R2.

Metabolite β-4-chlorophenylalanine (coded R5) was found in the rat metabolism study, its level at most 3.6% of administered dose. No further data is available for this metabolite or on its conjugate β-4-chlorophenylalanine-*N*-glucoside. As no genotoxicity data has been submitted, the TTC approach could be used for both metabolites in which case TTC value is 0.0025 µg/kg bw per day.

The Meeting concluded that metabolites valifenalate acid (IR5839, R2) and valifenalate acid glucosyl ester are toxicologically relevant and of equal potency to the parent. The Meeting concluded that a TTC approach could be used for metabolites PCBA (4-chlorobenzoic acid), β-4-chlorophenylalanine and its conjugate β-4-chlorophenylalanine-*N*-glucoside.

Microbiological data

No information is available.

Human data

No clinical cases or poisoning incidents have been recorded at pilot plant production level.

The Meeting concluded that the existing database on valifenalate was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

An acceptable daily intake (ADI) of 0–0.2 mg/kg bw was established on the basis of the NOAEL of 16.8 mg/kg bw per day in the 78-week study in mice and supported by the NOAEL of 15.3 mg/kg bw per day set by the 90-day study in mice, and employing a safety factor of 100. This provides a margin of 600 with respect to the LOAEL for benign liver tumours found in mice.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for valifenalate in view of its low acute oral toxicity, the absence of developmental toxicity or any other toxicological effects likely to be elicited by a single dose.

Levels relevant to risk assessment of valifenalate

Species	Study	Effect	NOAEL	LOAEL
Mouse	90-day study of toxicity[a]	Toxicity	110 ppm, equal to 15.3 mg/kg bw per day	900 ppm, equal to 134 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	150 ppm, equal to 16.8 mg/kg bw per day	850 ppm, equal to 97.2 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 16.8 mg/kg bw per day	850 ppm, equal to 97.2 mg/kg bw per day
Rat	90-day study of toxicity[a]	Toxicity	150 mg/kg bw per day	1000 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	150 mg/kg bw per day	1000 mg/kg bw per day
		Carcinogenicity	1000 mg/kg bw per day[b]	-
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	15 000 ppm, equal to 986 mg/kg bw per day[b]	-
		Parental toxicity	15 000 ppm, equal to 986 mg/kg bw per day[b]	-
		Offspring toxicity	1250 ppm, equal to 81 mg/kg bw per day	4300 ppm, equal to 277 mg/kg bw per day
	Developmental toxicity[c]	Maternal toxicity	1000 mg/kg bw per day[b]	-
		Embryo and fetal toxicity	1000 mg/kg bw per day[b]	-
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1000 mg/kg bw per day[b]	-
		Embryo and fetal toxicity	1000 mg/kg bw per day[b]	-
Dog	90-day study of toxicity[d]	Toxicity	50 mg/kg bw per day	250 mg/kg bw per day
	One-year study of toxicity[d]	Toxicity	50 mg/kg bw per day	250 mg/kg bw per day

[a] Dietary administration [b] Highest dose tested [c] Gavage administration [d] Capsule administration

Acceptable daily intake (ADI), applies to valifenalate, valifenalate acid and valifenalate acid glucosyl ester,expressed as valifenalate

0–0.2 mg/kg bw

Acute reference dose (ARfD), applies to valifenalate, valifenalate acid and valifenalate acid glucosyl ester, expressed as valifenalate

Unnecessary

Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

Critical end-points for setting guidance values for exposure to valifenalate

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid absorption (T_{max} 1–2 h) and approximately 80% absorbed at 100 mg/kg bw (based on urine, bile, cage wash and tissue/carcass)
Dermal absorption	No data
Distribution	Highest tissue levels found in GI tract, liver and kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid (ca 99% within 48 h); in bile-cannulated rats predominantly in bile (65–49%), urine (13–31%) and faeces (17–16%)
Metabolism in animals	Extensively metabolized; main metabolite R2 (valifenalate acid); oxidation and cleavage reactions
Toxicologically significant compounds in animals and plants	Valifenalate, valifenalate acid and its glucosyl ester, PCBA (4-chlorobenzoic acid), β-4-chlorophenylalanine and β-4-chlorophenylalanine-*N*-glucoside
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.118 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight gain (mice), liver weight and histopathology (mice, dog), caecum histopathology (rat), thyroid histopathology and clinical chemistry (dog)
Lowest relevant oral NOAEL	15.3 mg/kg bw per day (mouse)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rat; highest dose tested)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver (mouse); Thyroid and kidneys (rat)
Lowest relevant NOAEL	16.8 mg/kg bw per day (mouse)
Carcinogenicity	Carcinogenic in mice, not carcinogenic in rats [a]
Genotoxicity	No evidence of genotoxicity [a]
Reproductive toxicity	
Target/critical effect	Decreased pup body weight gain
Lowest relevant parental NOAEL	986 mg/kg bw per day, highest dose tested
Lowest relevant offspring NOAEL	81 mg/kg bw per day
Lowest relevant reproductive NOAEL	986 mg/kg bw per day, highest dose tested

Developmental toxicity	
Target/critical effect	None
Lowest relevant maternal NOAEL	1000 mg/kg bw per day (rat, rabbit; highest dose tested)
Lowest relevant embryo/fetal NOAEL	1000 mg/kg bw per day (rat, rabbit; highest dose tested)
Neurotoxicity	
Acute neurotoxicity NOAEL	No specific data; unlikely to be neurotoxic
Subchronic neurotoxicity NOAEL	No specific data; unlikely to be neurotoxic
Developmental neurotoxicity NOAEL	No specific data; unlikely to be neurotoxic
Immunotoxicity	No specific data; unlikely to be immunotoxic [a]
Human data	No clinical cases or poisoning incidents have been recorded.

Studies on toxicologically relevant metabolites

Acute toxicity

IR5839 (valifenalate acid, R2), rat, oral	LD_{50} > 2000 mg/kg bw (rat)

Genotoxicity

IR5839 (valifenalate acid, R2)	Ames: negative In vitro mammalian cell gene mutation: negative In vitro chromosome aberration: positive In vivo micronucleus: negative
PCBA (4-chlorobenzoic acid)	In vitro mammalian cell gene mutation: negative In vitro chromosome aberration: positive In vivo micronucleus: negative

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

Summary

	Value	Study	Safety factor
ADI	0–0.2 mg/kg bw [a]	78-week mouse study, supported by the 90-day mouse study	100
ARfD	Unnecessary		

[a] applies to valifenalate, valifenalate acid and valifenalate acid glucosyl ester, expressed as valifenalate

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