Pesticide residues in food — 2017

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2017

Part II – Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food – 2017

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, 12–21 September 2017

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* First full evaluation

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*** Follow-up evaluation.

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WHO Headquarters; Geneva, 12 to 21 September 2017

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Abbreviations used

AChE	acetylcholinesterase
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AhR	aryl hydrocarbon receptor
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
ALT	alanine aminotransaminase / alanine transaminase
ANOVA	analysis of variance
AR	androgen receptor
ARfD	acute reference dose
ASAT	aspartate aminotransferase
AST	aspartate transaminase (aspartate aminotransferase)
AUC	area under the concentration–time curve
AUC_0–last	area under the concentration–time curve from time 0 to end of study
AUC_τ	area under the plasma concentration-time curve during a dosage interval
B_{max}	concentration of receptor in tissue
BrdU	5-bromo-2′-deoxyuridine
BUN	blood urea nitrogen
bw	body weight
CAR	constitutive androstane receptor
CAS	Chemical Abstracts Service
CCPR	Codex Committee on Pesticide Residues
cDNA	complementary DNA
ChE	cholinesterase
CHO	Chinese hamster ovary
CI	confidence interval
C_{max}	maximum or peak concentration in blood or plasma
CMC	carboxymethylcellulose
CPS	cyclophosphamide
CYP	cytochrome P450
DMCF	N,N-dimethyl-1-cyanoformamide
DMO	methyl N′-methyl-N-[(methylcarbamoyl)oxy]-1-thiooxamimidate
DMOA	N,N-dimethyamino(oxo)acetic acid
DMSO	dimethyl sulfoxide
ECG	electrocardiograph
equiv.	equivalents
ER	estrogen receptor
7-ER	7-ethoxyresorufin-O-deethylase
F	female
F_0	parental generation
F_1	first filial generation
F_{1a} or F_{1A}	first filial generation, first litter
F_{1b} or F_{1B}	first filial generation second litter
F_2	second filial generation
F_{2a} or F_{2A}	second filial generation, first litter
F_{2b} or F_{2B}	second filial generation, second litter
F_{3a} or F_{3A}	third filial generation, first litter
F_{3b} or F_{3B}	third filial generation, second litter
FAO	Food and Agriculture Organization of the United Nations
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
FOB	functional observational battery
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GALT	gut-associated lymphoid tissue
GC-MS	gas chromatography–mass spectrometry
GGT	gamma-glutamyl transpeptidase or γ-glutamyltransferase
GIT	gastrointestinal tract
GLP	good laboratory practice
GPT	glutamic-pyruvic transaminase (alanine transaminase)
GSD	geometric standard deviation
GST	glutathione S-transferase
Hb	haemoglobin
hCG	human choriogonadotropin
HCT	haematocrit
HGPRT	hypoxanthine guanine phosphoribosyltransferase
HPAA	4-hydroxyphenylacetate
HPLA	4-hydroxyphenyllactate
HPLC	high-performance liquid chromatography
HPPA	4-hydroxyphenylpyruvate
HPPD	4-hydroxyphenylpyruvate dioxygenase
HPRT	hypoxanthine–guanine phosphoribosyltransferase
hPXR	human pregnane X receptor
i.p. or IP	intraperitoneal / intraperitoneal injection

IC_{50}	median inhibitory concentration
IgM	immunoglobulin M
IKI-3106	cyclaniliprole
IL	interleukin
IPCS	International Programme on Chemical Safety
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
IV	intravenous
IVF	in vitro fertilization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMAFF	Japanese Ministry of Agriculture, Forestry, and Fisheries
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
Kd	dissociation constant
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LLNA	local lymph node assay
LOAEC	lowest-observed-adverse-effect concentration
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOEC	lowest observed effect concentration
LOEL	lowest-observed-effect level
LOQ	limit of quantification
LSC	liquid scintillation counting
M	male
max.	maximum
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCT	medium chain triglyceride
MCTD	maximum clinically tolerated dose
MCV	mean cell volume / mean corpuscular volume
MIC	minimum inhibitory concentration
MIC_{50}	minimum concentration required to inhibit the growth of 50% of organisms
MIC_{90}	minimum concentration required to inhibit the growth of 90% of organisms
MIC_{avg}	average minimum inhibitory concentration
min.	minimum
miRNA	microribonucleic acid
MMAD	mass median aerodynamic diameter
MMC	mitomycin C
MNPCE	micronucleated polychromatic erythrocytes
MoA	mode of action
MODV	Modoc virus
mPXR	mouse pregnane X receptor
mRNA	messenger ribonucleic acid
MTD	maximum tolerated dose
MTO	methyl N-hydroxy-N′-methyl-1-thiooxamimidate
n.d. or ND	no data
n.d. or ND	not determined / not detected
n / *N*	sample size
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCE	normochromatic erythrocytes
ND	not detected / not determined
NMR	nuclear magnetic resonance
no. or No.	number
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NR	not reported
NSY-28	8-bromo-2-(3-bromo-1(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloroquinazolin-4(3H)-one
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OECD TG	Organisation for Economic Co-operation and Development Test Guideline
P	probability
P	parental (F_0) generation
PCE	polychromatic erythrocytes
PCR	polymerase chain reaction
PEG	polyethylene glycol
PICHV	Pichinde virus
PNA	p-nitroanisole-O-demethylase
PND	postnatal day
ppm	parts per million
PXR	pregnane X receptor
QA	quality assurance

(Q)SAR	quantitative structure–activity relationships
R243604	2-[2-chloro-3-ethoxy-4-(methylsulfonyl)benzoyl]-5-methylcyclohexane-1,3-dione
RBA	relative binding affinity
RBC	red blood cells
RNA	ribonucleic acid
RNAi	RNA interference
S9	9000 × *g* supernatant fraction from rat liver homogenate
SC	suspension concentrate
SER	smooth endoplasmic reticulum
SGOT	serum glutamic-oxaloacetic transaminase (or aspartate transaminase [AST])
SGPT	serum glutamic-pyruvic transaminase (or alanine transaminase, ALT)
siRNA	short-interfering ribonucleic acid
SRBC	sheep red blood cells
$t_{½}$	half-life
T_{max}	time to reach maximum concentration (C_{max})
T_3	triiodothyronine
T_4	tetraiodothyronine (thyroxine)
T_4-UGT	thyroxine-UDP-glucuronosyltransferase
TAT	tyrosine aminotransferase
TG	test guideline
TGAI	technical grade active ingredient
tk	thymidine kinase
TLC	thin-layer chromatography
TOCP	triorthocresyl phosphate
TOTP	tri-*o*-tolyl phosphate
TSH	thyroid-stimulating hormone
U	enzyme unit
UDPGT	uridine diphosphoglucuronosyltransferase / uridineglucuronosyltransferase
UDS	unscheduled DNA synthesis
UGT	uridine 5'-diphospho-glucuronosyltransferase
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
UV	ultraviolet
UVA	ultraviolet (radiation from about 320–400 nm in wavelength)
v/v	volume per volume
VEEV	Venezuelan equine encephalitis virus
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organization

Introduction

The toxicological monographs and monograph addenda contained in this volume were prepared by a WHO Core Assessment Group on Pesticide Residues that met with the FAO Panel of Experts on Pesticide Residues in Food and the Environment in a Joint Meeting on Pesticide Residues (JMPR) in Geneva, Switzerland, on 12–21 September 2017.

Seven of the substances evaluated by the WHO Core Assessment Group (bicyclopyrone, cyclaniliprole, fenazaquin, fenpyrazamine, fosetyl-aluminium, isoprothiolane, natamycin, propylene oxide, triflumezopyrim) were evaluated for the first time. Three compounds (chlormequat, fenpyroximate, oxamyl, thiophanate-methyl) were re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Reports and other documents resulting from previous Joint Meetings on Pesticide Residues are listed in Annex 1.

The report of the Joint Meeting has been published by the FAO as *FAO Plant Production and Protection Paper 232*. That report contains comments on the compounds considered, acceptable daily intakes and acute reference doses established by the WHO Core Assessment Group and maximum residue levels established by the FAO Panel of Experts. Monographs on residues prepared by the FAO Panel of Experts are published as a companion volume, as *Evaluations 2017, Part I, Residues*, in the FAO Plant Production and Protection Paper series.

The toxicological monographs and monograph addenda contained in this volume are based on working papers that were prepared by WHO experts before the 2017 Joint Meeting. A special acknowledgement is made to those experts and to the experts of the Joint Meeting who reviewed early drafts of these working papers.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological properties or toxicity of the compounds included in this volume should be addressed to:

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TOXICOLOGICAL MONOGRAPHS AND MONOGRAPH ADDENDA

BICYCLOPYRONE

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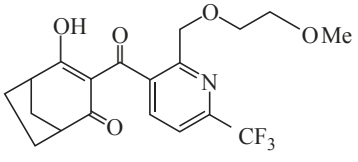
Explanation

Bicyclopyrone is the common name approved by the International Organization for Standardization (ISO) for 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1] oct-3-en-2-one (International Union of Pure and Applied Chemistry name [IUPAC]), with the Chemical Abstracts Service (CAS) number 352010-68-5. Bicyclopyrone is a herbicide that acts by inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD), leading to the destruction of chlorophyll in plants. This mode of action (MOA) is shared with several other herbicide active ingredients, for example, mesotrione, isoxaflutole, topramezone, tembotrione and pyrasulfatole.

The structure of bicyclopyrone is shown in Fig. 1.

Figure 1. Structure of bicyclopyrone



Bicyclopyrone 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), unless otherwise specified.

Evaluation for acceptable daily intake

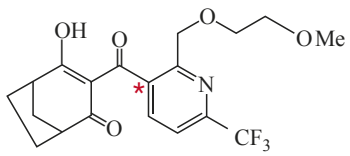
1. Biochemical aspects

1.1 Absorption, distribution and excretion

The metabolism of bicyclopyrone in mammals has been assessed in studies investigating the absorption, distribution and excretion of bicyclopyrone in rats following single and multiple gavage and single intravenous dosing. The quantitative and qualitative nature of metabolites formed was determined in a biotransformation study.

These studies used a single radiolabelled position because evidence shows that bicyclopyrone cleaves only minimally during the biotransformation process. The position of the radiolabel is shown in Fig. 2.

Figure 2. Radiolabelled form of bicyclopyrone used in absorption, distribution and excretion studies – structure and position of the radiolabel



* Denotes position of the radiolabel.

Because bicyclopyrone is soluble in water at pH 8, water with pH adjusted with 0.1 mol/L sodium carbonate was used as the dose vehicle for all the investigations.

(a) Oral route

Rats

In an excretion and tissue distribution study, rats (Crl:WI (Han); $n=4$/sex per group) received a single oral dose of [pyridinyl-3-^{14}C]bicyclopyrone (purity >99%; batch RDR-III) at a target dose level of either 2 or 200 mg/kg body weight (bw) or a single intravenous dose of [pyridinyl-3-^{14}C]bicyclopyrone at a target dose level of 2 mg/kg bw. At 7 days after exposure, the rats were killed and residual radioactivity was measured in blood and plasma, selected tissues and the remaining carcasses. Following analysis of radioactivity content, urine, cage wash and faeces samples collected from oral dose groups were transferred to a separate study for metabolite profiling (see section 1.2; Hardwick & Swalwell, 2012).

Following a single oral dose of [^{14}C]bicyclopyrone at 2 mg/kg bw, the major route of excretion was via urine. About 64% and 85% of the administered dose from male and female rat urine, respectively, was recovered within 7 days of dosing. In the same time period, about 27% and 5% of the dose was eliminated via faeces in male and female rats, respectively. Excretion was rapid, with the majority of the dose eliminated within 24 hours after dosing. No radioactivity was detected in expired air. Mean total recovery of radioactivity (including excreta, tissues and residual carcass) was 100% (males) and 99% (females). Seven days after this low dose was administered, radioactive residues in the majority of tissues were low and the tissue distribution of radioactivity was similar for males and females. The highest levels of radioactivity were found in the liver and kidney, accounting for about 4% and 0.3% of the administered dose in males and about 3% and 0.4% of the administered dose in females, respectively. Radioactivity in the thyroid equated to mean tissue concentrations of 0.074 and 0.089 µg equiv./g in the adrenals; this equated to mean tissue concentrations of 0.049 and 0.035 µg equiv./g in males and females, respectively, with each tissue accounting for <0.001% of the administered dose. In all other tissues, the mean concentrations were 0.015 µg equiv./g or less).

Following a single oral dose of [^{14}C]bicyclopyrone at 200 mg/kg bw, the major route of excretion was also via urine. About 68% and 88% of the administered dose was recovered within 7 days of dosing from male and female rat urine, respectively. In the same time period, about 23% and 6% of the dose was eliminated via faeces in male and female rats, respectively. Excretion was rapid, with the majority of the dose eliminated within 24 hours of dosing. No radioactivity was detected in expired air. Total recovery of radioactivity (including excreta, tissues and residual carcass) was about 97% for males and 98% for females. Seven days after dosing, radioactive residues in the majority of tissues were low and the tissue distribution of radioactivity was similar for both males and females. The highest levels of radioactivity found in the liver and kidney accounted for < 0.1% of the administered dose in both sexes. The highest concentration of radioactivity was detected in the thyroid (means of 5.625 and 7.513 µg equiv./g in males and females, respectively). Adrenals had mean tissue concentrations of 3.538 and 3.358 µg equiv./g in males and females, respectively, with each accounting for <0.001% of the administered dose. In all other tissues, the mean concentrations were 2.409 µg equiv./g or less.

Following a single intravenous dose of [^{14}C]bicyclopyrone at 2 mg/kg bw, the major route of excretion was also via urine. About 63% and 87% of the administered dose was recovered in urine within 7 days of dosing from male and female rats, respectively. In the same time period, about 29% and 5% of the dose was eliminated via faeces in male and female rats, respectively. Excretion was rapid, with the majority of the dose eliminated within 24 hours after dosing. No radioactivity was detected in expired air. Total recovery of radioactivity (including excreta, tissues and residual carcass) was about 100% for males and 102% for females. Seven days after administering the low dose intravenously to rats, radioactive residues in the majority of tissues were low and the tissue distribution of radioactivity was similar for both males and females. The highest levels of radioactivity were found in the liver and kidney, accounting for about 5% and 0.3% in males and 5% and 0.5% in females, respectively. Radioactivity in the thyroid equated to a tissue concentration of 0.076 and 0.078 µg equiv./g in the adrenals; this equated to mean tissue concentrations of 0.040 and 0.030 µg equiv./g in males and females, respectively, with each tissue accounting for <0.001% of the administered dose. In all other tissues, the mean concentrations were 0.03 µg equiv./g or less (Hurst, 2009).

In another excretion and tissue distribution study, groups of three male Han Wistar rats (Crl:WI (Han)) received a repeated oral dose of [pyridinyl-3-^{14}C]bicyclopyrone (purity 99.5%; batch RDR-VI-57) at a target dose level of 2 mg/kg per day and at a nominal radioactive dose of 1 mBq/kg bw per day for up to 28 days. At each time point, up to 840 hours (35 days) following dose 28, the rats were killed and residual radioactivity was measured in some tissues and the remaining carcasses. Following analysis of radioactivity content, samples of urine, cage wash and faeces collected from oral dose groups were transferred to a separate study for metabolite profiling (see section 1.2; Hardwick & Swalwell, 2012).

Mean concentrations of radioactivity in most tissues were at steady state by the tenth dose. With the exception of the thyroid, radioactivity was detected in all tissues investigated, but only at the first sampling time point for bone and renal fat. The recovery of radioactivity in the gastrointestinal tract and contents demonstrated that there was no accumulation of the dose through the digestive system following repeated doses. The greatest mean concentrations of radioactivity, which were present in the liver at all time points, contained between 3.6 and 4.3 µg equiv./g (accounting for up to 1% of the administered dose). The kidney contained the next highest concentration of radioactivity (between 0.9 and 1.0 µg equiv./g). The mean concentrations of radioactivity in all other tissues were less than 0.1 µg equiv./g (≤0.001% of the administered dose). Mean concentrations of total radioactivity were approximately equally distributed between whole blood and plasma at the time points investigated. Mean concentrations of radioactivity in the liver and kidney declined once dosing ceased, with elimination half-lives of 599 hours for the liver and 1300 hours for the kidney. Radioactivity concentrations in all other tissues apart from the kidney were at least 30-fold lower than in the liver and 24 hours after dosing ceased, accounting for ≤0.001% of the dose during the remainder of the study.

The excretion profiles were similar following both the single and repeated oral dosing regimens: of the administered dose, renal elimination accounted for more than 70%, faecal excretion for between 6% and 11%, and cage washes for less than 5% (Kendrick & Goodwin, 2010).

In another excretion study, bile duct-cannulated Han Wistar rats (Crl: WI (Han); $n = 4$/sex per group) received a single oral dose of [pyridinyl-3-^{14}C]bicyclopyrone (purity 99.1%; batch RDR-111-2) at a target dose level of either 2 or 200 mg/kg bw and a nominal radioactive dose of 5 mBq/kg. After 48 hours, the rats were killed and residual radioactivity was measured in the gastrointestinal tract and the carcasses. Following analysis of radioactivity content, samples of urine, cage wash and faeces underwent metabolite profiling (see section 1.2: Hardwick & Swalwell, 2012).

Following oral administration at 2 mg/kg bw, it was apparent that renal elimination was the predominant route of excretion; at this dose, about 42% and 55% were recovered in the urine of male and female rats, respectively. Males excreted about 14% in the faeces and about 16% in the bile, while females excreted about 5% in the faeces and about 2% in the bile. The high levels of radioactivity collected in cage washings, about 19% in males and 21% in females, were considered to be of urinary origin. Elimination was rapid, with the mean percentage of radioactivity about 79% for males and 70% for females 24 hours after dosing. A mean of about 83% and 87% of the administered dose was absorbed in males and females, respectively, as calculated from the radioactivity eliminated in urine and bile, together with that present in the residual carcass and cage washings (aqueous and organic). The mean total percentage recovery of administered radioactivity, including excreta, cage washings, cage debris, gastrointestinal tract and residual carcasses, was about 100% for males and about 96% for females.

Following oral administration at 200 mg/kg bw, urinary elimination accounted for about 46% (males) 69% (females) of the total recovered radioactivity. Biliary excretion amounted to about 19% and 7% in males and females, respectively, while faecal excretion accounted for about 8% of the administered dose in both sexes. Approximately 15% (males) and about 13% (females) of the administered dose was recovered in the cage washings (aqueous and organic), and was considered to be material of urinary origin. Elimination was rapid, with the mean percentage of radioactivity about 71% for males and about 91% for females 24 hours after the dosing period. A mean of about 86% and 90% of the administered dose was absorbed in males and females, respectively, as calculated from the radioactivity eliminated in urine, associated cage washings (aqueous and organic) and bile, together with that present in the residual carcass. The total mean percentage of administered radioactivity recovered from excreta, cage washings, cage debris, gastrointestinal tract and residual carcasses was about 98% for both male and female rats (Hurst, 2010).

In a pharmacokinetics study, Han Wistar rats ($n = 2$/sex per group) received either a single oral dose of [pyridinyl-3-^{14}C]bicyclopyrone (purity >99%; batch RDR-III-2) at a target dose level of 2 or 200 mg/kg bw, or a single intravenous dose of [pyridinyl-3-^{14}C]bicyclopyrone at a target dose level of 2 mg/kg bw. The study was designed to determine the pharmacokinetics of total radioactivity in blood and plasma by taking serial blood samples (from before dosing to 72 hours after dosing) and a terminal sample by cardiac puncture under anaesthesia at the final time point (96 hours). Three groups of rats ($n = 2$/sex per group) received the same oral and intravenous doses; at the approximate T_{max}, a single terminal blood sample was collected for plasma metabolite profiling in a separate study (section 1.2: Hardwick & Swalwell, 2012).

Following a single oral dose of [^{14}C]bicyclopyrone at a nominal dose level of 2 or 200 mg/kg bw, maximum mean plasma concentrations were reached within 1.3–2.3 hours, indicating a rapid absorption into the systemic circulation. Low-dose T_{max} values were 1.4 and 1.3 hours and high-dose T_{max} values were 2.3 and 1.3 hours for males and females, respectively. Peak mean plasma concentration values were 3.3 and 2.9 µg equiv./g following a low oral dose, and 425 and 441 µg equiv./g following a high oral dose, in males and females, respectively. Plasma concentrations generally remained close to the maximum plasma concentration (C_{max}) values up to 4 hours after dosing, after which levels declined rapidly, in a biphasic pattern. At 2 mg/kg bw, the mean plasma half-lives of the first phase (α phase) were 2.7 and 2.4 hours in males and females, respectively; at 200 mg/kg bw, these were 3.2 and 1.8 hours, respectively. At 2 mg/kg bw, it was not possible to calculate a half-life for the second phase (β phase) because the majority of samples were at or below the limit of detection. At 200 mg/kg bw, mean plasma β half-lives were 12.5 and 68.6 hours in males and females respectively.

Following a single intravenous dose of [^{14}C]bicyclopyrone at a nominal dose of 2 mg/kg bw, the concentration of radioactivity in plasma extrapolated to the zero time point was 6.1 and 6.6 µg equiv./g

in male and female rats, respectively. Plasma concentrations declined rapidly in a biphasic pattern with mean α half-lives of 1.4–2.0 hours.

At all dose levels and routes, systemic exposure as described by area under the plasma concentration–time curve (AUC) appeared to be slightly higher in males than in females. A comparison of the AUC values following the low oral dose and the intravenous dose indicates that oral bioavailability was quantitative. The mean values for C_{max} were generally proportional to dose, but AUC values for the high dose appeared to be supra-proportional to dose. However, this was attributed to differences in calculating the AUC for the two dose levels because of the definition of the β-phase in the high-dose level only. The concentration of radioactivity in blood followed the same pattern as plasma, with plasma concentrations generally slightly higher than those in blood, suggesting no preferential uptake into red blood cells in all dose groups analysed (Hurst & Stow, 2009).

In a tissue distribution study, Han Wistar rats ($n = 3$/sex per group) received a single oral dose of [pyridinyl-3-^{14}C]bicyclopyrone (purity 99.8%; batch RDR-V-2) at a target dose level of either 2 or 200 mg/kg bw and at a nominal radioactive dose of 4 mBq/kg. At each time point up to 144 hours after dosing, a group of rats was killed and residual radioactivity was measured in selected tissues and the remaining carcasses. Samples of liver were retained for metabolite profiling in a separate study (see section 1.2: Hardwick & Swalwell, 2012).

Following oral administration at a target dose of 2 mg/kg bw, the mean maximum levels of radioactivity (C_{max}) were observed at the first sampling time (two hours after dosing) in male and female rats. Levels of total radioactivity were detectable in all the tissues investigated at this time point, with the highest mean concentrations in liver (8.1 and 6.3 µg equiv./g in males and females, respectively) and kidney (4.4 and 3.5 µg equiv./g in males and females, respectively). Mean concentrations of total radioactivity in all tissues declined rapidly at the subsequent time points investigated, with $C_{max}/2$ achieved within six hours after dosing. Distribution of radioactivity was similar to that found at two hours after dosing. With the exception of the liver and kidney, radioactivity approached the limit of quantification (LOQ) for the majority of tissues 24 hours after dosing. At 144 hours after dosing, the liver and kidney contained the highest concentration of radioactivity (2.1 and 2.0 µg equiv./g in male and female liver, respectively, and 0.7 and 1.3 µg equiv./g in male and female kidney, respectively). Radioactivity in all other tissues was below or approaching the limit of detection. Residual radioactivity in the carcass accounted for less than 0.7% of the administered dose. Elimination half-lives were calculated for all tissues where concentrations were detected at more than three time points and where a terminal elimination phase could be unambiguously defined. In males, these tissues were adrenals, brain, renal fat, muscle and plasma; in females, blood, ovaries, pancreas and uterus. Half-lives ranged between 3.9 and 191.5 hours in males and between 1.4 and 15.3 hours in females.

Following oral administration at a target dose of 200 mg/kg bw, the radioactivity C_{max} was observed at the first sampling time, two hours after dosing (except for the kidney in both male and female rats and, in males only, renal fat, where maxima were observed at six hours after dosing). Levels of radioactivity were detectable in all tissues investigated at this time point, with the highest mean concentrations in males and females, respectively, seen in plasma (301.2 and 206.6 µg equiv./g) and blood (210.6 and 147.4 µg equiv./g). Mean concentrations of total radioactivity in tissues declined rapidly at the subsequent time points investigated ($C_{max}/2$ between 6 and 12 hours after dosing), while distribution of radioactivity was similar to that found at two hours after dosing. By 48 hours after dosing, radioactivity was at or approaching the LOQ for the majority of tissues. At 144 hours after dosing, the liver had the highest concentration of radioactivity (5.1 and 3.9 µg equiv./g in males and females, respectively). Radioactivity in the kidney, spleen, thymus, heart, lung and muscle in both sexes and in the pancreas and uterus in females only was present at less than 2.2 µg equiv./g and was not detectable in any other tissues. Residual radioactivity in the carcass of male and female rats at 144 hours after dosing accounted for a mean of 0.3% of the administered dose. Elimination half-lives were calculated for all tissues, where concentrations were detected at more than three time points and where a terminal elimination phase could be unambiguously defined. In males, these tissues were adrenals, blood, bone, brain, renal fat, gastrointestinal tract plus contents, muscle, plasma and thyroid; in females, renal fat, kidney and thyroid. Elimination half-lives ranged between 2.3 and 51.2 hours in males and 1.7 and 481 hours in females (Hurst & Stow, 2010).

1.2 Biotransformation

One study was available on the biotransformation of bicyclopyrone in rats.

Samples of urine, faeces, bile, cage wash, plasma and liver obtained from rats following administration of [pyridinyl-3-^{14}C]bicyclopyrone via the oral and intravenous route were collected (described in section 1.1). Quantitative metabolic profiles and identification of metabolites in these samples were using radiochromatography and mass spectrometry.

Samples transferred from the following studies constituted the test system for this study:

- Han Wistar (Crl: WI (Han); $n = 4$/sex per dose) bile duct-cannulated rats received a single oral dose at nominal dose levels of either 2 or 200 mg/kg bw. Urine, faeces, bile and cage wash samples were collected up to 48 hours after dose (Hurst, 2010).
- Han Wistar (Crl: WI (Han); $n = 2$/sex) rats received a single oral dose at nominal dose levels of either 2 or 200 mg/kg bw or a single intravenous dose of 2 mg/kg bw. Plasma samples were collected up to 96 hours after dose administration (Hurst & Stow, 2009).
- Han Wistar (Crl: WI (Han); $n = 4$/sex) rats received a single oral or intravenous dose at nominal dose levels of either 2 or 200 mg/kg bw. Urine, faeces and cage wash samples were collected up to 168 hours after dose administration (Hurst, 2009).
- Han Wistar (Crl: WI (Han); $n = 3$/ sex) rats received a single oral dose at nominal dose levels of either 2 or 200 mg/kg bw. Liver samples were collected up to 144 hours after dose administration (Hurst & Stow, 2010).
- Han Wistar (Crl: WI (Han); $n = 3$/sex) rats received up to 28 oral doses at a nominal dose level of 2 mg/kg bw per day. Urine and faeces samples were collected up to 24 hours after the first, tenth and twenty-first dose administrations and 672 hours after the twenty-eighth dose administration (Kendrick & Goodwin, 2010).

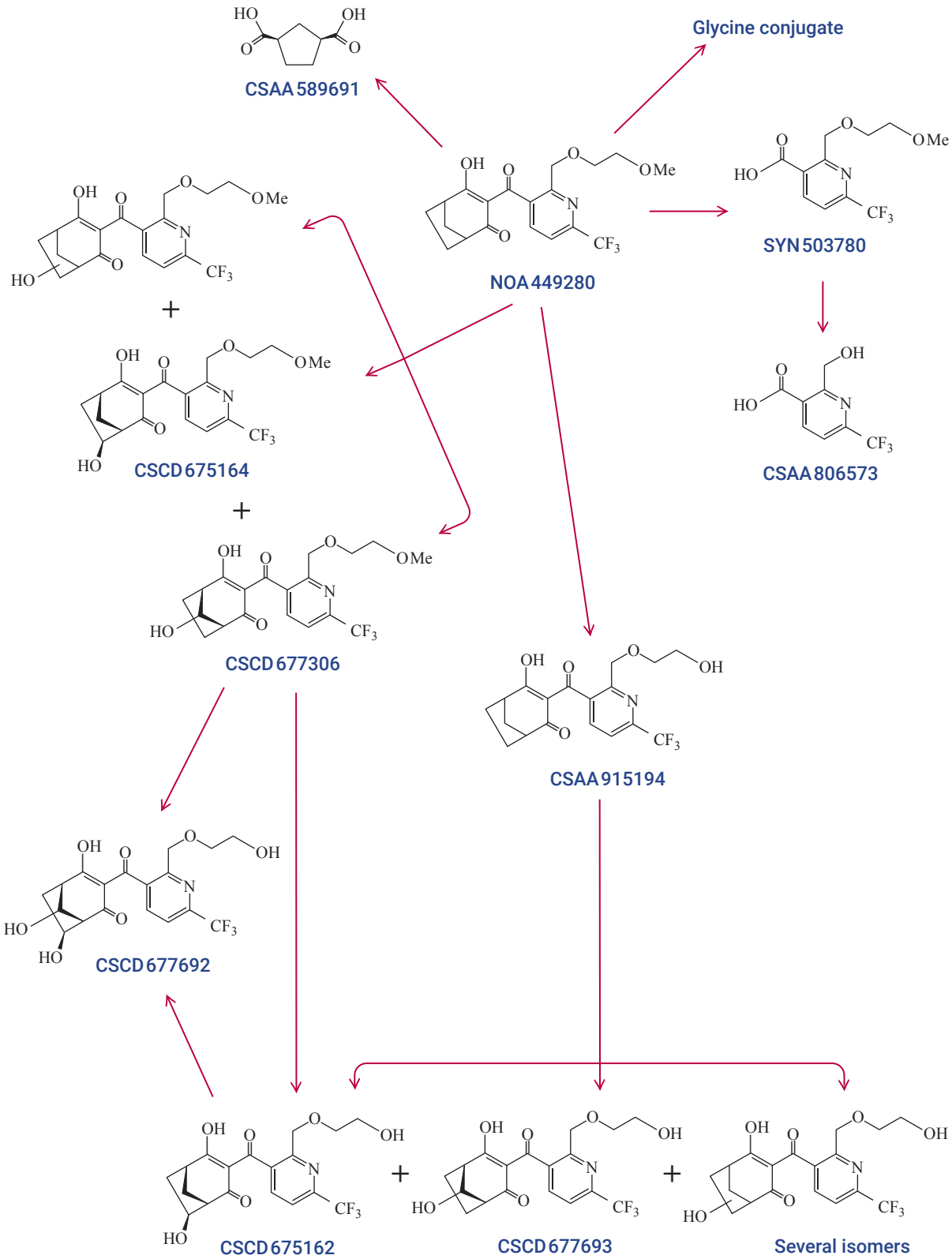
Bicyclopyrone was not extensively metabolized, with unchanged parent the principle radiolabelled component in all dose groups. The principal biotransformation routes were via oxidative phase 1 reactions. At both dose levels, the major radioactive component in the urine of non-cannulated rats was the parent compound, accounting for about 40% and 80% of the dose in males and females, respectively. The major urinary metabolite was CSAA915194, accounting for about 10% and 2% of the dose in males and females, respectively. Minor metabolites included CSCD6758164 and CSCD677306 and, detected in males only, CSCD675162 and CSCD677693. The major radioactive component in cage wash was parent compound, accounting for 2% and 5% of the administered dose in males and females, respectively. The parent compound was considered to originate from urinary contamination of the metabolic cage. The major faecal metabolite was CSCD675164, accounting for about 11% and 2% of the dose at 2 mg/kg bw and for about 8% and 0.4% at 200 mg/kg bw in males and females, respectively. Minor metabolites included CSCD675162 and CSCD677306. Parent compound accounted for less than 3% of the dose. The metabolite profile obtained from rats administered multiple low doses of bicyclopyrone was similar to that obtained from the equivalent single dose.

In bile duct-cannulated rats, the metabolite profile in urine was similar to that in intact rats. The major biliary metabolite was CSCD675164 accounting for about 7% and 1% of the low dose and for about 5% and 1% of the high dose in males and females, respectively. Minor metabolites included CSAA915194 and CSCD677306. Parent compound accounted for about 2% of the low dose for both males and females and for about 10% and 4% of the high dose for males and females, respectively. The major radioactive component in the faeces of cannulated rats was parent compound. The major circulating component in plasma was parent compound. Minor metabolites included CSAA915194, CSCD677692 and CSCD675164. The major component in liver was parent compound. Minor metabolites included CSAA915194 and CSCD677306.

Although the biotransformation of [^{14}C]bicyclopyrone was qualitatively independent of dose level and dose route, there was a quantitative sex difference in the metabolism, with males metabolizing a higher proportion of the parent compound. Repeated administration (performed in males only) had no significant effect on the metabolic profile, indicating no induction or inhibition of metabolic enzymes (Hardwick & Swalwell, 2012).

The proposed metabolic pathway of bicyclopyrone in rats is shown in Fig. 3.

Figure 3. Proposed metabolic pathway of bicyclopyrone in the rat



Source: Hardwick & Swalwell (2012)

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity, irritation and sensitization studies on bicyclopyrone are summarized in Table 1. The results of acute dermal, eye irritation and skin sensitization studies on bicyclopyrone are summarized in Table 2.

Table 1. Summary of acute toxicity studies with bicyclopyrone

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	HanRcc:WIST (SPF)	F	Oral	94.5[a]	LD_{50} > 5000 mg/kg bw	Arcelin (2007a)
Rat	HanRcc:WIST (SPF)	M + F	Dermal	94.5[a]	LD_{50} > 5000 mg/kg bw	Arcelin (2007b)
Rat	HanRcc:WIST (SPF)	M + F	Inhalation	94.5[a]	LC_{50} > 5.21 mg/L air	Decker (2008)

bw: body weight; F: females; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: males

[a] Batch SEZ3AP006/MILLED

Table 2. Summary of acute dermal and eye irritation and skin sensitization studies with bicyclopyrone

Species	Strain	End-point	Purity (%)	Result	Reference
Rabbit	New Zealand White	Skin irritation	94.5[a]	Not irritating	Arcelin (2007c)
Rabbit	New Zealand White	Eye irritation	94.5[a]	Slightly irritating	Arcelin (2007d)
Mouse	CBA/Ca (CBA/Ca CruBR)	Skin sensitization (LLNA)	94.5[a]	Not sensitizing	Pooles (2008)

LLNA: local lymph node assay

[a] Batch SEZ3AP006/MILLED

2.2 Short-term studies of toxicity

Bicyclopyrone belongs to a class of herbicides that inhibit the liver enzyme HPPD. HPPD is involved in the catabolism of tyrosine (an amino acid derived from proteins in the diet). Inhibition of HPPD can result in elevated tyrosine levels in the blood, a condition called tyrosinaemia. HPPD-inhibiting herbicides have been found to cause ocular, developmental, liver and kidney effects in laboratory animal studies. Of these, the ocular effect (corneal opacity) was found to be highly correlated with the elevated blood tyrosine levels. In fact, rats dosed with tyrosine alone show ocular opacities similar to those seen with HPPD inhibitors. Although the other toxicity effects may be associated with chemically induced tyrosinaemia, other mechanisms may also be involved.

There are marked differences between species in the ocular toxicity associated with inhibition of HPPD. Ocular effects following treatment with HPPD-inhibiting herbicides are seen in the rat, but not the mouse; monkeys also seem to be recalcitrant to the ocular toxicity induced by HPPD inhibition. The reason for this species-specific response in ocular opacity is related to species differences in tyrosine clearance. A metabolic pathway to remove tyrosine from blood involves a liver enzyme called tyrosine aminotransferase (TAT). In contrast to rats, in which ocular toxicity is observed following exposure to HPPD-inhibiting herbicides, mice and humans are unlikely to achieve the levels of plasma tyrosine necessary to produce ocular opacities because TAT activity is much greater than in rats. Thus, humans and mice have a highly effective metabolic process for handling excess tyrosine.

HPPD inhibitors such as nitisinone (NTBC) are effective therapeutic agents used to treat patients with the rare genetic diseases of tyrosine catabolism. Treatment starts in childhood and is often sustained throughout a patient's life. Nitisinone at therapeutic doses (1 mg/kg bw per day) has an excellent safety record in infants, children and adults. Serious adverse health outcomes have not been observed in the population followed for approximately a decade. Rarely, ocular effects are seen in patients with high plasma tyrosine levels, but these effects are transient and can be readily reversed upon adherence to a restricted protein diet. This indicates that an HPPD inhibitor in and of itself cannot easily overwhelm the tyrosine clearance mechanism in humans.

Therefore, due to an efficient metabolic process for handling excess tyrosine, exposure to environmental residues of HPPD-inhibiting herbicides are unlikely to result in high blood levels of tyrosine and ocular toxicity in humans. Single chemical risk assessments for which the hazard end-point is from a rat toxicity study and the effect observed is ocular opacity due to tyrosinaemia should be considered a worst case scenario.

For more details, see mode of action studies and relevance to human risk assessment in Appendix 1.

(a) Oral administration

Mouse

In a dose range-finding study for an 80-week carcinogenicity study, bicyclopyrone (purity 94.5%: batch SEZ3AP006/MILLED) was administered in the diet to CD-1 (Crl:CD-1(ICR)) mice ($n = 10$/sex per group) for 90 days at dose levels of 100, 3500 or 7000 ppm; equal to 15.4, 543 and 1127 mg/kg bw per day for males and 20.8, 809 and 1344 mg/kg bw per day for females, respectively). Clinical signs were checked daily, and body weight and feed consumption were measured weekly. Opthalmoscopic examination was performed prior to study initiation and in week 13, and haematology samples were obtained at terminal kill. All animals underwent a detailed necropsy, and selected organs were weighed. Tissues from all animals in the control and high-dose groups underwent a comprehensive histological evaluation. In addition, liver from all low- and mid-dose animals was examined. Additional samples of the liver were taken for possible genomics and immunohistochemistry analysis.

Administration of 7000 ppm did not adversely affect body weight, feed consumption or haematological and ophthalmological parameters. Dose levels of 3500 and 7000 ppm resulted in increased liver weight accompanied by minimal hepatocyte hypertrophy. A dose-related increase in absolute and adjusted liver weight was noted in males at 3500 and 7000 ppm. At 3500 and 7000 ppm, adjusted weights were 23% and 36% higher than the control, respectively. Adjusted liver weights were increased in all female treatment groups. Adjusted weights at 100, 3500 and 7000 ppm were 14%, 19% and 19%, respectively, higher than the control. Minimal centrilobular hepatocyte hypertrophy was seen in the livers of 6/10 males and 1/10 females at 3500 ppm and 9/10 males and 4/10 females at 7000 ppm. There was no evidence of centrilobular hepatocyte hypertrophy in mice at 100 ppm. At 3500 ppm, the liver weight was increased over 15% without further histopathological changes. Hepatocellular hypertrophy would normally be considered an adaptive response, but since no clinical chemistry was performed, concomitant liver effects in blood could not be assessed and, as a precaution, the liver effects at 3500 ppm are considered adverse. At 100 ppm, minimal increase (<15%) in liver weight in females only was not accompanied by any change in liver histopathology.

The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 15.4 mg/kg bw per day) based on the increased liver weights at 3500 ppm (equal to 543 mg/kg bw per day). Plasma tyrosine levels were not measured (Shearer & Wood, 2009).

Rat

In a 90-day dietary toxicity study, Alpk:APfSD (Wistar-derived) rats ($n = 8$/sex per group) were fed diets containing bicyclopyrone (analytical grade, purity 96%, batch K16353/13) at 0, 500, 2000 or 5000 ppm (equal to 0, 51.2, 208 and 503 mg/kg bw per day for males and 0, 50.5, 202.2 and 494.6 mg/kg bw per day for females, respectively) and bicyclopyrone (technical grade: purity 90%, batch K16353/14) at 5000 ppm (equal to 518 mg/kg bw per day for males and 499.8 mg/kg bw per day for females) for at least 90 consecutive days. Clinical observations, body weights and feed consumption were measured throughout the study. Ophthalmoscopy was performed pretest and in the week prior to scheduled kill. At the scheduled necropsy, cardiac blood samples were taken for haematology, clinical pathology and plasma tyrosine and bicyclopyrone analyses; selected organs were weighed; specified tissues were taken for subsequent histopathological examination; and liver was taken for TAT, HPPD and cytochrome P450 (CYP) isoenzyme analysis.

There were no unscheduled deaths or compound-related systemic clinical changes. Ocular effects, observed in both sexes at all dose levels, included hazy corneal opacity, corneal opacity,

persistent pupillary membrane, iris congested and iris partially fixed. Body weight and feed consumption were lower than in controls and feed utilization was less efficient in males at 5000 ppm. There were no compound-related effects on blood clinical chemistry parameters. Plasma tyrosine levels were uniformly higher than controls in all treatment groups.

Kidney weights were higher than control values in all treated males; there was no dose–response relationship. Liver weights were higher than control values in all treated males and in females at 2000 or 5000 ppm; there was no dose–response relationship. After adjustment for terminal body weight, thymus weights were lower than control values in both sexes at 5000 ppm (analytical grade). Since thymus weight were not reduced in females at 5000 ppm (technical grade) and relative thymus weights were not affected, this effect on thymus weight was considered probably not treatment related.

The effect on liver and kidney weights are in line with the known MOA of bicyclopyrone, that is, inhibition of HPPD in the liver, resulting in an increase in tyrosine concentrations, with increased elimination via the kidney. Since the tyrosine concentrations were also not dose related (see Table 3), the lack of a dose response in the liver and kidney effects does not mean these effects are not treatment related. Macroscopic changes, confined to the eyes, were observed in a number of animals in all the treatment groups; there was no dose–response relationship. Microscopic changes were also confined to the eyes and were present in at least 50% of the animals in all treatment groups.

Table 3. Plasma concentrations of bicyclopyrone and tyrosine in a 90-day dietary toxicity study in rats

Plasma concentrations per dose level of bicyclopyrone									
0 ppm		**500 ppm**		**2 000 ppm**		**5 000 ppm (analytical grade)**		**5 000 ppm (technical grade)**	
M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Bicyclopyrone (µg/mL)									
0.01	0.01	10.90	1.50	43.20	5.88	106.05	24.66	101.59	32.16
Tyrosine (nmol/mL)									
133.1	107.7	2480.0**	2023.8**	2176.33**	2101.3**	2430.0**	1798.9**	2636.3**	1892.5**

F: females; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student *t*-test, 2-sided)

Source: Rattray (2003)

There were no compound-related differences between the technical and analytical grades.

No NOAEL could be determined as ocular toxicity effects (opacity and keratitis) were seen in males and females in the low-dose group. The lowest-observed-adverse-effect level (LOAEL) was 500 ppm (50.5 mg/kg bw per day; Rattray, 2003).

In another three-month dietary toxicity study, Han Wistar rats ($n = 10$/sex per group) were fed diets containing bicyclopyrone (purity 94.5%; lot/batch SEZ3AP006) at 0, 2.5, 10, 2500 or 5000 ppm (equal to 0, 0.18, 0.72, 183 and 363 mg/kg bw per day for males and 0, 0.22, 0.88, 229 and 442 mg/kg bw per day for females, respectively) for at least 90 days. The animals were monitored regularly for mortality and clinical signs of toxicity or reaction to treatment. Detailed functional observational battery (FOB) tests were performed once over a two-week period. Body weights and feed consumption were measured and recorded at predetermined intervals from prior to treatment initiation until scheduled necropsy. All animals underwent ophthalmoscopic examination three times throughout the study. Blood and urine samples for laboratory investigations were collected during the last week of treatment. All animals underwent a detailed necropsy after completion of treatment. Tissues from all control and high-dose animals and the eyes from all animals underwent a comprehensive histological evaluation. Additional liver tissue from all animals was taken and stored for possible investigative studies.

Treatment-related effects on body weight and feed consumption were observed in males at 2500 and 5000 ppm and in females at 5000 ppm. In animals at 2500 and 5000 ppm, opaque eyes and the absence of pupillary reflex were related to histopathological findings of keratitis. Increases in kidney weight in males at 2500 and 5000 ppm were considered treatment related, but due to the lack of corroborating histological evidence, the changes were considered not toxicologically significant.

The NOAEL was 10 ppm (equal to 0.72 mg/kg bw per day) based on ocular toxicity (opaque eyes, opacities, an absent pupillary reflex in the cornea and keratitis) at 2500 ppm (equal to 183 mg/kg bw per day). Plasma tyrosine levels were not measured (Shearer & Robertson, 2009).

Dog

In a 28-day oral dose range-finding study, bicyclopyrone (purity 96%; batch KI6353/13 on day 1 and KI6353/19 thereafter) was administered daily by capsule to beagle dogs ($n = 1$/sex per group) at concentrations of 0, 10, 100 or 250 mg/kg bw per day. Clinical observations and veterinary examinations (including ophthalmoscopy) were undertaken, and body weight, feed consumption, clinical pathology and toxicokinetic parameters were measured periodically throughout the study. Plasma samples for toxicokinetic and tyrosine analyses were taken after dosing on day 1; before dosing on days 4, 6, 8, 16; and at several time points after dosing on day 26. Following completion of the scheduled treatment, the animals were necropsied, selected organs were weighed and specified tissues were histopathologically examined.

The animals at 250 mg/kg bw per day were euthanized ahead of schedule because of the adverse clinical signs and ocular effects, body weight loss and reduced feed consumption observed in the male on study day 7. Various clinical pathology parameters were affected, and urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were elevated. Macroscopic examination showed treatment-related ocular effects (retinopathy) and reduced cortical lymphocytes in the thymus. This dose level was considered to exceed the maximum clinically tolerated dose (MCTD), which is why the female at this dose was also killed, although she did not show any clinical signs.

There were no bicyclopyrone-attributable effects on clinical signs, body weights, feed consumption or clinical pathology parameters at either 10 or 100 mg/kg bw per day. However, as expected based on the MOA of this class of chemical, urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were elevated.

Peak blood concentrations of bicyclopyrone and the AUC increased approximately in proportion to the administered dose. Compared to females, males tended to reach maximum concentrations earlier. Plasma profiles for bicyclopyrone after the twenty-sixth dose were similar to those after the first dose, and the AUC was similar at both time points, suggesting no steady-state plasma concentrations. The plasma concentration in the male dog at 250 mg/kg bw per day that was euthanized on day 7 was approximately five-fold higher than the concentration at the same time point after dosing on day 1. In contrast, shortly before termination on day 8 the female at 250 mg/kg bw per day had plasma concentrations of bicyclopyrone similar to day 1 levels.

Compared to pre-exposure levels, tyrosine concentrations were markedly elevated following administration of bicyclopyrone. Plasma tyrosine concentrations 24 hours after the first dose and after subsequent doses were similar for all dose levels, indicating that even the lowest dose led to a maximal elevation.

In control dogs, plasma tyrosine concentrations were markedly elevated at day 26, and there was evidence of a progressive elevation at earlier time points. This was more marked in females than in males. In addition, both bicyclopyrone and its putative metabolite were detected in plasma from the control dogs. The AUC at day 26 indicates that the exposure in the female was equivalent to about one-tenth of the lowest dose, and somewhat less in the male. This is believed to be due to the control animals being co-housed with the low-dose animals.

As only one animal/sex per dose was treated in this dose range-finding study, no NOAEL could be determined (Twomey, 2003).

In a 13-week oral toxicity study, beagle dogs ($n = 4$/sex per group) were dosed orally by capsule with bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) at 0, 5, 25 or 125 mg/kg bw per day. Clinical signs, body weight and feed consumption were recorded throughout the study. Ophthalmoscopy and veterinary examinations were performed pretest and at week 13, and blood and urine samples were collected for clinical laboratory investigations at intervals throughout the study. Following completion of the scheduled treatment, a detailed necropsy was performed on all animals and various organs were weighed. A full set of tissues and organs was prepared and examined histopathologically.

All animals survived the scheduled treatment period. No treatment-related clinical signs were observed, and there were no treatment-related effects on feed consumption, body weight or ophthalmological and haematological parameters.

The clinical biochemistry investigation found decreased cholesterol values in animals at 125 mg/kg bw per day. The group mean liver weight in females at 125 mg/kg bw per day was statistically significantly higher than in the concurrent control group. However, the difference was not accompanied by any histopathological changes in the liver and a similar increase was not seen at the same dose level in the one-year dog study. This difference was considered to be an adaptive effect in the liver and not toxicologically significant.

There were no treatment-related macroscopic findings. An increased incidence and severity of inflammation/fibrosis/apoptosis was noted in pancreas of 2/4 male dogs at all dose levels. As this pathological finding was not noted in the one-year study in dogs using the same dose levels, it was considered incidental to treatment. Minimal degeneration of the sciatic nerve was observed in one male at each dose level and in one control and one high-dose female; this too was considered to be incidental.

The NOAEL was 125 mg/kg bw per day, the highest dose tested. Tyrosine concentration in plasma were not measured (Jackson, 2009).

In a one-year oral toxicity study, beagle dogs ($n = 4$/sex per group) were dosed orally by capsule with bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) at 2.5, 25 or 125 mg/kg bw per day. Clinical signs, body weight and feed consumption were recorded throughout the study. Ophthalmoscopy was performed pretest and in weeks 13, 26 and 52. Veterinary examinations were also performed, and blood and urine samples were collected at intervals during the study for clinical laboratory investigations. Blood samples were collected for analysis of plasma bicyclopyrone, metabolite CSAA915194 and tyrosine levels. Following the scheduled treatment, all the animals were necropsied and selected organs were weighed. A full set of tissues and organs was prepared and examined histopathologically.

One male at 125 mg/kg bw per day was found dead on day 336, after one day of low feed consumption. All other animals survived the scheduled treatment period. No adverse treatment-related clinical signs were observed at any dose level. There were no treatment-related differences in veterinary examination results, haematology or urine analysis parameters, organ weights or macroscopic findings.

Corneal opacities were seen in the eyes of control and treated dogs (Table 4). According to the study report, incidental transient opacity can be distinguished from treatment-related opacity, the latter being a typical star-shaped opacity. Persistent corneal opacities that developed over the course of treatment were observed in two males (nos. 10 and 12) and one female (no. 25) at 25 mg/kg bw per day and in one male (no. 14) and one female (no. 29) at 125 mg/kg bw per day. The opacity seen pretest in male no. 14 was similar to opacities in untreated animals, but developed the typical star-shaped treatment-related opacity during the course of the study. The treatment-related opacities were observed as early as week 13. Of the affected animals, one male (no. 12) at 25 mg/kg bw per day and one female (no. 29) at 125 mg/kg bw per day showed sensitivity to light during the final (week 52) examination.

Decreased cholesterol levels were noted in weeks 13, 26 and 52 in animals at 125 mg/kg bw per day. In the absence of any effects on other biochemical parameters or any histopathological changes in the liver, this finding was considered not toxicologically significant.

Minimal to moderate chromatolysis and swelling of some neurons occurred in the dorsal root ganglia in two males and three females at 2.5 mg/kg bw per day, all males and females at 25 mg/kg bw per day, and three males and two females at 125 mg/kg per day. The toxicological relevance of this finding is questionable: it occurred in animals treated with the test item only and is not known to occur as a background finding. However, there were no clinical signs of neurotoxicity.

Table 4. Corneal opacity and selected neuropathy findings in a 1-year dog (N = 4) study with bicyclopyrone

Finding	Animal ID no. or number of findings per dose level of bicyclopyrone							
	0 mg/kg bw per day		2.5 mg/kg bw per day		25 mg/kg bw per day		125 mg/kg bw per day	
	M	F	M	F	M	F	M	F
Corneal opacity (animal ID no., eye)								
Pretest	0	18R, 19R	0	0	0	0	14R	0
Week 13	0	18R, 19R	0	0	11L, 12L[a] R[a]	25R[a], 27L[a] R[a]	14R[a]	29L[a] R[a], 31L[a]
Week 26	1L	0	0	22R, 23LR	10R, 12L[a] R[a]	25L[a] R[a], 27L	14R[a]	29L[a] R[a], 31L[a]
Week 52	0	19R	0	0	10R, 12L[a,b], R[a,b]	25L[a] R[a]	14R[a]	29L[a,b] R[a,b], 32L[a]
Ganglion, dorsal root, chromatolysis/swelling (*n*)								
Grade 1	0	0	1	1	1	2	1	0
Grade 2	0	0	0	2	2	1	2	2
Grade 3	0	0	1	0	1	1	0	0
Total no. affected	0	0	2	3	4	4	3	2
Mean grade/tissue affected	0	0	2.0	1.7	2.0	1.8	1.7	2.0
Degeneration of sciatic nerve fibres (*n*)								
Grade 1	1	0	2	1	2	1	2	2
Degeneration of spinal nerve roots (*n*)								
Grade 1	1	0	1	0	0	0	0	1
Grade 2	0	0	1	0	0	0	1	0

bw: body weight; ID no.: identification number; F: females; L: left eye; M: males; no.: number; R: right eye

[a] Star-shaped characteristic of treatment-related opacity.

[b] Light sensitive.

Source: Braun (2010)

In addition, minimal degeneration of nerve fibres of the sciatic nerve was observed in one control male, two males and one female at 2.5 mg/kg bw per day, two males and one female at 25 mg/kg bw per day, and two males and two females at 125 mg/kg bw per day. Minimal to slight degeneration of nerve fibres occurred in the spinal nerve roots of one control male, two males at 2.5 mg/kg bw per day, and one male and one female at 125 mg/kg bw per day.

Exposure was primarily to the parent compound. Metabolite CSAA915194 reached only 0.8–5.4% of the AUC_{τ} (AUC during a dosage interval) of bicyclopyrone. When a 50-fold dose range was investigated, exposure to bicyclopyrone increased with AUC_{τ} ratios of 15 to 42. For CSAA915194, AUC_{τ} increased in males with ratios of 23 to 52 and in females with ratios of 41 to 55. Following treatment with bicyclopyrone at 2.5 mg/kg bw per day, tyrosine AUCτ increased by a factor of 6.2–22 compared to controls. No further increase in tyrosine AUC_{τ} (ratios: 0.9 to 1.3) was found in dogs at 25 and 125 mg/kg bw per day.

There was no consistent sex differences through all treatment groups and when sampling for parent compound, metabolite and tyrosine, except at 2.5 mg/kg bw per day, where exposure to CSAA915194 was lower in females than in males. In all bicyclopyrone-treated groups, exposure to parent compound, metabolite and tyrosine remained similar after 4, 13, 26 and 52 weeks of repeated dosing.

Ketones were apparently detected in the urine of all animals after treatment with bicyclopyrone during weeks 26 and 52. Further laboratory investigations demonstrated that the tyrosine-derived metabolite 4-hydroxyphenylpyruvate (HPPA) can cause a red–brown change in the urine dipsticks used to measure ketones. When analysed using an automated method, this was incorrectly identified as the presence of ketones in the urine; visual examination can clearly distinguish between the red-brown change caused by HPPA and the purple-violet change caused by acetoacetate. As an HPPD inhibitor, bicyclopyrone blocks the conversion of HPPA to homogentisate following HPPA production from tyrosine, leading to elevated HPPA levels. The apparent detection of ketones in bicyclopyrone-treated animals in this study is due to cross-reactivity of HPPA with the dipsticks used to measure ketones.

As bicyclopyrone treatment cannot be excluded as a cause for the histopathological findings, no NOAEL could be determined. The LOAEL was 2.5 mg/kg bw per day, based on minimal histopathological effects of chromatolysis, swelling and/or degeneration of ganglia and/or neurons, which might indicate a possible neurological effect (Braun, 2010).

(b) Dermal application

Wistar rats ($n = 10$/sex per group) were dermally exposed (using a semi-occlusive bandage) to bicyclopyrone (purity 94.5%; SEZ3AP006/MILLED) at 0 (control), 50, 250 or 1000 mg/kg bw per day for six hours a day, five days a week over 28 days. Clinical signs, feed consumption and body weights were recorded periodically during acclimatization and treatment periods. In the acclimatization period, all animals underwent ophthalmoscopic examinations. In the final week of treatment, all animals underwent FOB evaluations, including measurement of grip strength and quantitative measurement of locomotor activity. Control and high-dose animals also underwent ophthalmoscopic examination. At the end of the treatment period, blood samples were drawn for haematology and plasma chemistry analyses and urine samples were collected for urine analyses. All animals were subsequently necropsied, and a selection of organs/tissues were weighed. Histological examinations were performed on all animals from the control and high-dose group. The eyes with optic nerve and Harderian glands of low- and mid-dose animals were also examined.

All animals survived the scheduled treatment. No treatment-related effects or toxicologically relevant changes in any of the haematology parameters were noted. Alkaline phosphatase activity was lower in all male treatment groups compared to their controls. Higher liver weights were observed in all treated males, and higher kidney weights in mid- and high-dose males. In the absence of any histopathological changes or correlative clinical chemistry changes, the lower alkaline phosphatase activity and the weight differences were considered not toxicologically significant. Of the animals at 250 mg/kg bw per day, histological examination of two males and three females found minimal degeneration of the corneal epithelium. In two males at 1000 mg/kg bw per day, minimal to moderate degrees of keratitis were recorded.

Ketone levels in the urine appeared to increase in a dosage-related manner in all treatment groups. This apparent increase in ketones is attributable to metabolic products of tyrosine. Bicyclopyrone inhibits HPPD, the second enzyme in the tyrosine catabolic pathway. This leads to an increase in plasma tyrosine and its metabolite, HPPA. HPPA has been shown to cross-react with the ketone-detecting dipstick (Combur 10 Test M®) in a dose-dependent manner. This result does not represent an adverse change in ketone levels, but of an increase in the tyrosine metabolites caused by inhibition of HPPD.

The NOAEL was 50 mg/kg bw per day, based on eye lesions (keratitis or degeneration of corneal epithelium) at 250 mg/kg bw per day. Tyrosine concentration in plasma were not measured (Sommer, 2009).

(c) Exposure by inhalation

No studies were available.

2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of bicyclopyrone was evaluated in a 104-week study in the rat, which included a 52-week long-term toxicity phase, and in an 80-week carcinogenicity study in the mouse.

Mouse

In an 80-week carcinogenicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was administered to groups of CD-1 mice ($n = 50$/sex per group) in the diet at dose levels of 0, 70, 1700 and 7000 ppm (equal to 0, 8.7, 233 and 940 mg/kg bw per day for males and 0, 9.2, 242 and 1027 mg/kg bw per day for females, respectively). Animals were monitored regularly for mortality and clinical signs of toxicity or reaction to treatment. Body weights and feed consumption were measured and recorded at predetermined intervals from pretest until the completion of treatment. At week 80, prior to terminal kill, blood samples were collected from all surviving animals for haematological analysis. Blood films from all surviving animals were made during week 53/54 and week 80; however, blood cell morphology was not performed as no treatment-related effects were seen on white cell haematological parameters at scheduled kill. All surviving animals underwent a detailed necropsy after completion of treatment. Tissues from all animals underwent a comprehensive histological evaluation.

There were no treatment-related clinical signs and no effects on mortality. Slight but statistically significant decreases in body weight and body weight gain compared to controls were seen in both sexes at 7000 ppm (Table 5). Initially, body weight in males at 1700 ppm was statistically significantly lower than concurrent control, but this group had a lower starting weight and initial differences were not considered treatment related. Males treated at 1700 and 7000 ppm had statistically significantly lower feed consumption than controls pretest; thereafter, there was no evidence of a treatment effect. Less efficient feed utilization was noted for males and females treated at 7000 ppm; the differences from control were statistically significant.

SC-0774, a known liver carcinogen in the mouse, is associated with moderate centrilobular hypertrophy in short-term toxicity studies, a fact that was borne out in this study. The other compounds at top dose caused only minimal centrilobular hypertrophy, which could reflect a possible weak carcinogenic effect in the liver in longer-term studies but only at high doses. Some associated increases in liver weights were observed in males at 10 ppm and above and in females at 7000 ppm, which identifies bicyclopyrone as a slight to moderate inducer of CYP2B isoenzymes, and as a minimal inducer of CYP1A and CYP4A isoenzymes in both sexes. In males, microsomal uridine diphosphoglucuronosyltransferase (UDPGT) was slightly induced at 100 ppm and above. The liver enzyme induction profile of bicyclopyrone at 7000 ppm was similar to that observed with SC-0774 at 3500 ppm (Noakes, 2007).

Table 5. Comparison of body weight gain and feed utilization in an 80-week mouse study with bicyclopyrone

Weeks	Weight per dose level of bicyclopyrone							
	0 ppm		70 ppm		1700 ppm		7000 ppm	
	M	F	M	F	M	F	M	F
Body weight gain (g)								
0–1	2.1	1.2	2.3	0.6	1.5*	1.4	1.5*	1.1
0–2	2.9	2.0	3.2	1.6	3.6**	2.8*	3.4	3.1**
0–4	5.9	4.1	6.4	4.2	5.9	4.3	4.9*	3.8
0–8	10.7	7.7	10.9	8.2	10.1	7.6	8.7**	7.2
0–26	22.5	19.6	22.2	21.5	19.8	18.8	17.9**	14.0**
0–52	26.4	27.9	27.0	29.7	25.3	27.3	23.0*	21.6**
0–80	26.5	29.4	27.9	31.4	24.7	27.3	23.3	24.9

(*Continued on next page*)

Weeks	Weight per dose level of bicyclopyrone							
	0 ppm		70 ppm		1700 ppm		7000 ppm	
	M	F	M	F	M	F	M	F
Feed utilization (g)								
1–4	3.5	3.5	3.8	3.3	3.5	3.2	2.9**	2.9
5–8	2.7	2.6	2.4	2.8	2.2**	2.1	2.1**	2.2
9–13	1.6	2.5	2.0	1.4**	1.5	2.2	1.7	0.9**
1–13	2.5	2.8	2.7	2.4	2.3	2.4	2.2*	1.9**

F: females; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test)

Source: Robertson & Perry (2012a)

Compared to concurrent controls, liver weights (adjusted for terminal body weight) were statistically significantly increased in all male treatment groups and in females at 1700 and 7000 ppm, although no dose–response relationship was apparent. These increased liver weights were accompanied by a statistically significant increase in the incidence of mild centrilobular hypertrophy in males at 7000 ppm only. These changes are indicative of an adaptive response in the liver and are considered not adverse.

Incidence of subcapsular cell hyperplasia in the adrenals of both sexes at 7000 ppm (generally where the grading was mild) was statistically significantly lower than in controls. These adrenal changes are common spontaneous finding in mice of this strain and age, and thus a lower incidence of this change is considered not to be adverse.

There were no treatment-related tumours. There was no difference in the overall number of tumours, the number of tumour-bearing animals or the time to onset of any tumour type. The incidence of bronchial-alveolar adenoma was higher in males at 7000 ppm than in concurrent controls; there were no differences in female mice. The incidence of these benign tumours in the male lung at 7000 ppm was not accompanied by any other histopathological changes in the lungs and was in the range of the incidence seen in control groups from concurrent studies. Therefore, the bronchial-alveolar adenoma in males at 7000 ppm was considered not treatment-related.

The NOAEL was 1700 ppm (equal to 233 mg/kg bw per day for males and 242 mg/kg bw per day for females, respectively) based on decreases in body weight and body weight gain and less efficient feed utilization in males and females at 7000 ppm (equal to 940 mg/kg bw per day). There were no treatment-related tumours (Robertson & Perry, 2012a).

Rat

In a 104-week combined chronic toxicity and carcinogenicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was administered in the diet to Han Wistar (CrL:WI(Han)) rats. Rats in the carcinogenicity study ($n = 52$/sex per group) were fed diets containing 0, 5, 500, 2500 or 5000 ppm (equal to 0, 0.28, 28.4, 141 and 280 mg/kg bw per day in males and 0, 0.35, 35.8, 178 and 368 mg/kg bw per day in females, respectively) for at least 104 weeks. Rats in the chronic toxicity part of the study ($n = 12$/sex per group) were dosed in an identical fashion for 52 weeks. As a result of severe eye lesions and corneal opacity and damage, males at 5000 ppm were fed a control diet over nine days during weeks 4 and 5. Dosing recommenced after this period and continued uninterrupted to the end of the study.

The following were assessed at pre-determined intervals from before the trial until study completion: clinical observation including ophthalmology, body weight change, feed consumption, haematology, coagulation and clinical chemistry. The carcinogenicity study animals underwent ophthalmoscopy prior to dosing initiation and at weeks 50 and 102, and urine analysis occurred at predetermined intervals. The chronic toxicity study animals were given a detailed FOB assessment once during treatment (week 51/52). All surviving carcinogenicity and chronic toxicity study animals were necropsied and underwent a comprehensive histological evaluation after 104 or 52 weeks of treatment, respectively.

There were no statistically significant differences in mortality between the controls and treatment groups. Dose levels of 2500 and 5000 ppm were associated with a significantly lower body weight and

a corresponding decrease in body weight gain in both sexes, generally from study start. Lower feed consumption was seen at these dose levels over the first 13 weeks of treatment.

Clinical observations of severe ocular findings were noted throughout treatment from approximately week 4: animals at 500 ppm and above were seen to have opaque eyes and/or corneal damage (neovascularization) (Table 6). An absent pupillary reflex was noted in 2/12, 4/12 and 2/12 males and in 4/12, 4/12 and 5/11 females treated at 500, 2500 and 5000 ppm, respectively. Histological examination showed that the corneal damage was associated with the occurrence of squamous cell carcinoma and squamous cell papilloma in the cornea in treated males only (total incidence 0, 3, 3, 5) and keratitis with regenerative hyperplasia of the corneal epithelium of the eye in treated animals of both sexes. The corneal tumours seen in male rats were associated with and likely attributable to significant damage to and regenerative hyperplasia of the cornea seen during the course of the carcinogenicity study with bicyclopyrone at concentrations of 500 ppm and above. The identified MOA of HPPD-inhibiting herbicides results in significantly elevated plasma tyrosine in rats, particularly males. The occurrence of corneal tumours is also seen for some other HPPD-inhibiting herbicides, like fenquinotrione and tembotrione. However, the human relevance of this finding cannot be excluded (see mode of action studies and relevance to human risk assessment in Appendix 1).

Statistical increases in urinary ketones at 500 ppm and above were noted in both sexes. Phenylketones are excreted as a consequence of the inhibition of HPPD by bicyclopyrone, and the increase in urinary ketones in this study is considered to reflect an increase in phenylketones, metabolic products of tyrosine catabolism. Blood pigments were also increased at 2500 and 5000 ppm.

Table 6. Selected findings in a 2-year rat study with bicyclopyrone

Finding	Measures per dietary concentration of bicyclopyrone									
	0 ppm		5 ppm		500 ppm		2500 ppm		5000 ppm	
	M	F	M	F	M	F	M	F	M	F
Non-neoplastic findings										
Eyes opaque										
No. of animals [a]	1	0	3	1	51	52	51	52	51	50
Days [b]	408–737	–	387–737	640–710	24–737	24–738	23–737	24–738	23–737	24–738
Corneal damage										
No. of animals [a]	0	0	1	0	51	51	51	52	51	50
Days [b]	–	–	352–359	–	24–737	24–738	24–737	24–738	24–737	24–738
Thyroid after 1 year (n = 11 or 12)										
Focal cell hypertrophy [a]	0	0	0	0	9***	0	10***	5*	8***	6*
Focal follicular cell hyperplasia [c]	4 (33%)	1	2 (18%)	1	2 (17%)	0	3 (25%)	4	4 (33%)	0
Thyroid after 2 years										
Focal cell hypertrophy [a]	0	0	1	0	2	1	1	2	1	2
Focal follicular cell hyperplasia [c]	2 (4%)	3	10* (19%)	3	12** (23%)	10*	12** (23%)	6	17*** (33%)	2
Neoplastic microscopic findings										
Squamous cell										
Carcinoma [a]	0	0	0	0	2	0	2	0	2	0
Papilloma [a]	0	0	0	0	1	0	1	0	3	0

F: females; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Dunnett test)

[a] Number of animals with the finding.

[b] Time point when the finding was noted.

[c] Presented as the number of animals with the finding, and in parentheses, as a % of the number of animals examined.

Source: Robertson & Perry (2012b)

Kidney and/or liver weights in males at 500, 2500 or 5000 ppm at 52 weeks (relative kidney weights only) and at 104 weeks (absolute and relative kidney and liver weights) were increased compared to concurrent controls. Mean heart weights (absolute and relative) were statistically significantly reduced in males at 2500 ppm and above (week 104) and in females at 5000 ppm (weeks 52 and 104).

An increased incidence of focal follicular hypertrophy of the thyroid was noted in males at 500 ppm and above and in females at 2500 ppm and above after one year of treatment. After two years of treatment, the incidence of focal thyroid follicular cell hyperplasia was statistically significantly increased in males at all dose levels; at dose levels of 500 ppm and above, this was consistent with a continued mild perturbation of thyroid function. Some thyroid hypertrophy and hyperplasia was seen in females, but to a lesser extent and at higher dose levels only, and in many cases without a clear dose response.

While the thyroid effects may be due to elevated tyrosine levels in mainly male rats, the relevance for humans cannot be excluded, although it is recognized that humans are unlikely to reach the plasma tyrosine levels at which these effects were seen. (See also mode of action studies and relevance to human risk assessment in Appendix 1.)

There was a statistically significant increase in the incidence of chronic progressive nephropathy in the kidney of males at all dose levels. At 500 ppm and above, this finding was associated with an increase in kidney weight and increases in urinary specific gravity and urinary proteins. The incidence of chronic progressive nephropathy at 5 ppm in males was within the control range for the laboratory and was not associated with any other changes in the kidney. Chronic progressive nephropathy has no strict human counterpart. Consequently, chemically induced exacerbation of chronic progressive nephropathy in rats should not be considered an indicator of human toxic hazard (WHO, 2015).

Other treatment-associated findings were a decrease in heart weight at 2500 ppm and above in both sexes; in males, this was accompanied only by a decreased incidence of cardiomyopathy. Incidence of acinar cell atrophy of the exocrine and Harderian gland alteration (and inflammation) of the exorbital lacrimal glands was also increased in males at 2500 ppm and above.

No NOAEL for chronic toxicity could be determined as focal follicular cell hyperplasia of the thyroid gland was observed in males at all doses. The LOAEL was 5 ppm (equal to 0.28 mg/kg bw per day) based on focal follicular cell hyperplasia of the thyroid gland in males (Robertson & Perry, 2012b).

2.4 Genotoxicity

Bicyclopyrone is not mutagenic to bacterial or mammalian cells in vitro (Booth, 2006; Callander, 2007; Sokolowski, 2010) and is not clastogenic in human lymphocytes in vitro (Fox, 2006). It is not clastogenic and does not induce DNA repair in vivo (Clay, 2007; Honarvar, 2008). Bicyclopyrone shows no genotoxic potential either in vitro or in vivo (Table 7).

Table 7. Summary of genotoxicity studies with bicyclopyrone [a, b]

End-point	Test object	Concentration	Batch and purity	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, T1537; *Escherichia coli* WP2 and WP2*uvr*A	Up to 5000 µg/plate with and without S9-mix	SEZ3AP006 94.5%	Not mutagenic	Callander (2007)
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, T1537; *E. coli* WP2 and WP2*uvr*A	Up to 5000 µg/plate with and without S9 mix	SMU0BP0028 95.9%	Not mutagenic	Sokolowski (2010)
Chromosomal aberrations	Human lymphocytes	Up to 1500 µg/mL with S9 mix and up to 1250 µg/mL without S9 mix	SEZ3AP006 94.5%	Not clastogenic	Fox (2006)
Gene mutation	Mouse lymphoma L5178Y $Tk^{+/-}$	Up to 3994 µg/mL (10 mmol/L) with and without S9 mix	SEZ3AP006 94.5%	Not mutagenic	Booth (2006)
In vivo					
Chromosomal aberrations	Rat bone marrow	2000 mg/kg bw	SEZ3AP006/ MILLED 94.5%	Not clastogenic	Honarvar (2008)
Unscheduled DNA synthesis	Han Wistar rat hepatocytes	2000 mg/kg bw	SEZ3AP006 94.5%	Not mutagenic	Clay (2007)

bw: body weight; DNA: deoxyribonucleic acid; GLP: good laboratory practice; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *Tk*: thymidine kinase

[a] Positive and negative (solvent) controls were included in all studies.

[b] Statements of adherence to quality assurance and GLP were included.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

In a GLP-compliant but not guideline-compliant multigeneration dose range-finding study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was administered to young adult Crl:WI (Han) rats (four groups; n = 12/sex per group) in the diet at 0, 25, 500, 2500 or 5000 ppm (dose levels in mg/kg bw per day not provided in the study report) for 10 weeks. They were then paired (one male with one female) for mating. Formulated diet was available for the males throughout the mating period until necropsy and for females during the mating period, gestation and lactation and up to scheduled kill when the F_1 generation rats were 22 days old.

The time of onset and completion of parturition were recorded. The females were allowed to rear their offspring to weaning on lactation day 22, and nursing and nesting behaviour of the maternal

animals was observed. All animals were examined for effects on general condition, body weight and feed consumption. All F_0 animals underwent ophthalmoscopy during week 10; before pairing (day 64); and 1 week prior to necropsy (day 92 for males and day 117 for females). The males were killed once littering was completed, and the females were killed on lactation day 22; all animals were subsequently macroscopically examined. For all F_0 animals, terminal body weight, liver and kidney weights were recorded; liver and kidneys were retained for possible subsequent examination. The implantation scars in each uterine horn of all mated F_0 females were counted. For any apparently non-pregnant F_0 female, the uteri were stained with ammonium sulfide to confirm pregnancy status. The total litter size was recorded after completion of littering, and litter size was recorded daily thereafter. Pups were sexed on lactation days 1, 4, 7, 14, 21 and 22. After the completion of littering, all pups were examined for malformations. They were examined daily for clinical signs of toxicity and weighed on days 1, 4, 7, 14 and 21. Where possible, one pup/sex per group underwent ophthalmoscopy between days 19 and 22. Any pups that died or were euthanized prematurely were necropsied. Surviving F_1 pups were killed on lactation day 22 and, where possible,. three pups/sex per group were examined. Terminal body weight and liver and kidney weights from one pup/sex per litter were recorded, and these pups were macroscopically examined. Liver and kidneys together with any grossly abnormal tissue were retained for possible subsequent examination.

Statistically significantly lower body weight gains were apparent for males at 5000 ppm for the first week of the pre-mating period only. Lower body weights and body weight gains were evident in females at 5000 ppm during the pre-mating and gestation periods. In addition, there was a statistically significant decrease in body weight gain on lactation days 1–14 and 1–17 for females at 2500 or 5000 ppm. Feed consumption of F_0 females at 5000 ppm was lower than controls throughout lactation.

Unilateral or bilateral opacity was observed in the eyes of most males and females at 500, 2500 and 5000 ppm from treatment day 36 onwards; corneal opacity was even evident in some males at 25 ppm. A high incidence of ocular findings was observed in treated animals on day 64 and at study end. Although uni- or bilateral corneal opacity was apparent for both males and females, a dose–response relationship was not observed; for males at 5000 ppm and for females in all treatment groups, the incidence of opacities increased over time. Vascularization was seen in all treated animals except in females at 25 ppm.

There was no effect of treatment on time taken to mate, fertility, length of gestation or proportion of pups born live. Females at 2500 or 5000 ppm had lower mean numbers of pups on day 1 (9.6 and 9.8, respectively) compared with controls (11.9). The number of uterine implantation scars in females at 2500 or 5000 ppm was correspondingly lower. There was no effect of treatment on the pup survival indices or sex ratio. The percentage of pups surviving to day 22 was lower than expected (70–76%) in all groups.

Corneal opacities were observed in F_1 pups receiving 500, 2500 and 5000 ppm (only one pup/sex per litter was investigated). Group mean pup body weights during the first week of lactation were similar to controls for all groups. On lactation day 14, group mean male pup body weights in all treatment groups were slightly lower than in controls; mean values were below the background data range (25.5–26.9 g) for all groups, including controls. Female pup weights were similar to controls, although all values were below the background data range (26.0–26.1 g). On lactation day 21, mean body weight for both males and females was slightly lower than controls at all dose levels. Body weight gains for pups on lactation days 1–21 were lower than those of controls; these values were below the background data range for all treatment groups and achieved statistical significance for male pups at 5000 ppm on days 14–21.

Liver and kidney weights were increased in F_0 males, with no evidence of a dose response. There was no effect on liver or kidney weights in F_0 females or in pups.

At necropsy, the incidence of abnormally coloured eyes in treated parental animals increased in a dose-related manner. When compared with controls, treated animals had fewer implantation scars in the uterine horns; mean values showed a dose-related trend and, for females at 2500 or 5000 ppm (11.3 and 10.5, respectively), were below the background data range (11.7–12.2). At necropsy, early decedent pups from groups at 500, 2500 or 5000 ppm were found to have an increased incidence of no milk in the stomach.

As this was a dose range-finding study, no NOAELs or LOAELs were determined (Davies & Penn, 2009).

In a two-generation reproductive toxicity study, bicyclopyrone (batch SEZ3AP006/MILLED, purity 94.5%) was fed to young adult Crl:WI (Han) rats (n = 25/sex per group) in the diet at dose levels of 0, 25, 500 or 5000 ppm (equal to 0, 1.9, 38.4 and 377 mg/kg bw per day for males and 0, 2.1, 42.2 and 410 mg/kg bw per day for females, respectively, based on F_0 pre-mating feed consumption data) for 10 weeks. These F_0 rats were then paired (one male with one female) for mating. The formulated diet was available for the males throughout the mating period until necropsy and for females during the mating period, gestation and lactation and up to scheduled kill when the F_1 animals were weaned (at age 21 days).

Weaned F_1 animals (n = 25/sex per group) had formulated diet available from birth through maturation and pairing (at approximately 14 weeks old) until necropsy (for males) and until weaning (21 days of age) of the F_2 generation (for females). The F_2 litters received the formulated diet from birth until necropsy at 21 days of age. The F_0 and F_1 parental animals and F_1 and F_2 pups (n = 3/sex per litter, where possible) were necropsied. Any remaining pups were killed and discarded without undergoing necropsy. Clinical signs, ophthalmoscopy (week 10 prior to mating), body weights, feed consumption, fertility and mating performance, organ weights and macroscopic abnormalities at necropsy were recorded for all parental animals. Microscopic examination of selected organs was conducted. Litter size, clinical condition, ophthalmoscopy (n = 1 pup/sex per group between days 19 and 22), growth and development to weaning, organ weights and macroscopic abnormalities at necropsy were recorded for the offspring.

There were no treatment-related deaths or effects on reproductive performance.

In parental animals, lower body weight gains and feed consumption were noted in F_0 and F_1 animals at 5000 ppm and F_1 animals at 500 ppm. Treatment-related ocular findings, known to be associated with the class of chemistry of bicyclopyrone, were evident in all animals at 500 and 5000 ppm. Increased kidney weights in F_0 and F_1 males at 500 and 5000 ppm and in females at 5000 ppm were accompanied in F_1 animals by an increased incidence of pelvic dilatation. Increased thyroid weights were observed in F_0 and F_1 males at 500 and 5000 ppm, and histopathological changes in the thyroid reflecting minor perturbations of thyroid homeostasis were observed in F_0 and F_1 males and females at 500 and 5000 ppm. Liver weight increases were seen in F_0 and F_1 animals at 500 and 5000 ppm, and were accompanied in males only by minimal centrilobular hypertrophy, reflecting an adaptive effect of treatment in the liver.

At 25 ppm, treatment-related ocular effects were evident in F_0 males and F_1 males and females. Minor histopathological changes and/or weight increases were observed in the liver, kidney and thyroid at this dose level (Table 8).

Table 8. Incidence of opacity and roughness and vascular keratitis in reproductive toxicity study in rats

Generation / ocular effect	No. of animals with the finding per dose level of bicyclopyrone			
	0 ppm	25 ppm	500 ppm	5000 ppm
F_0 parental animals (n = 25/sex per group)				
Corneal opacity & roughness – males	0	3	5	5
Corneal opacity & roughness – females	0	0	1	9
Vascular keratitis – males	0	15	20	21
Vascular keratitis – females	0	0	25	18
F_1 parental animals (n = 25/sex per group)				
Corneal opacity & roughness – males	0	2	0	0
Corneal opacity & roughness – females	0	4	0	0
Vascular keratitis – males	0	22	25	25
Vascular keratitis – females	0	0	25	25

(*Continued on the next page*)

Generation / ocular effect	No. of animals with the finding per dose level of bicyclopyrone			
	0 ppm	25 ppm	500 ppm	5000 ppm
F_1 pups[a]				
Number of litters	24	25	23	22
Corneal opacity & roughness – males	0	0	8	8
Corneal opacity & roughness – females	0	0	10	8
Vascular keratitis – males	0	0	2	4
Vascular keratitis – females	0	0	4	3
F_2 pups[a]				
Number of litters	22	22	21	22
Corneal opacity & roughness – males	0	0	11	14
Corneal opacity & roughness – females	0	0	8	14
Vascular keratitis – males	0	0	2	11
Vascular keratitis – females	0	0	3	8

[a] Only 1 pup/sex per litter was investigated. *Source*: Davies & Penn (2012)

In pups, body weights and body weight gain of treated animals were similar to controls to day 14. Thereafter, body weight gains were reduced for F_1 and F_2 pups at 5000 ppm and for F_1 pups at 500 ppm. Treatment-related ocular effects were recorded in F_1 and F_2 pups at 500 and 5000 ppm. Increased kidney and liver weights were evident in both generations at 500 and 5000 ppm. There was no effect on vaginal opening. Although there was no difference in the body weight of males when preputial separation occurred, there was a slight but statistically significant delay in the time of preputial separation at all dose levels. There was no effect on anogenital distance in the F_2 generation. The lack of any effect on vaginal opening in F_1 females or on anogenital distance in F_2 pups, together with the lack of effects on subsequent reproductive performance, reproductive organ weights or histology in F_1 adults, led to the conclusion that the slight delay in preputial separation was not toxicologically significant. At 25 ppm, there were no adverse effects on body weight and no evidence of systemic toxicity in F_1 or F_2 pups.

No NOAEL for systemic toxicity in parental animals could be determined as ocular effects and effects on liver, thyroid and kidney were observed at all doses. The LOAEL for systemic toxicity in parental animals was 25 ppm (equal to 1.9 mg/kg bw), the lowest dose tested.

The NOAEL for systemic toxicity and sexual development in pups was 25 ppm (equal to 1.9 mg/kg bw per day for males and 2.1 mg/kg bw per day for females, respectively) based on ocular effects and effects on liver, thyroid and kidney at 500 ppm (equal to 38.4 mg/kg bw per day).

The NOAEL for reproductive toxicity was 5000 ppm (equal to 377 mg/kg bw per day), the highest dose tested (Davies & Penn, 2012).

(b) Developmental toxicity

Rat

In a dose range-finding developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% carboxymethylcellulose (CMC) in purified water, was administered by gavage to mated female rats (RccHan:WIST(SPF); $n = 10$/group) at dose levels of 0, 100, 500 or 1000 mg/kg bw per day on gestation days 5–20. Animals were examined/observed twice daily for signs of toxicity and mortality. Clinical signs were assessed at least twice daily for signs of reaction to the treatment and/or symptoms of ill health. Ophthalmoscopy was carried out once during acclimatization, and once towards the end of the gestation period. Body weights were recorded daily from gestation days 0 to 21. Feed consumption was recorded over 5-day intervals as follows: gestation days 0–5, 5–8, 8–11, 11–14, 14–17 and 17–21. All females were killed on gestation day 21, and the fetuses were removed by caesarean section. Dams and fetuses were examined in accordance with international recommendations.

All female animals survived until scheduled necropsy. At 500 and 1000 mg/kg bw per day, individual dams were noted moving their heads through the bedding material, considered a nonspecific

sign of discomfort. Mean feed consumption was statistically significantly reduced between days 5 and 17 in animals at 500 mg/kg bw per day and between days 5 and 14 in animals at 1000 mg/kg bw per day. Mean feed consumption at 100 mg/kg bw per day was slightly, but statistically significantly, lower than control on gestation days 5–8. Maternal body weights and/or maternal body weight gains were statistically significantly lower in all dose groups compared to the controls; this paralleled the lower feed consumption in these groups (Table 9). At 500 and 1000 mg/kg bw per day, maternal body weights were beginning to diverge from control values on days 9 and 7, respectively. Mean weight gain was affected as early as gestation day 6 in all dose groups. However, these effects were transient and, by gestation day 21, both body weight and weight gain were comparable across all dose groups.

There was no effect on preimplantation loss, implantation rate, postimplantation loss or number of live fetuses. No treatment-related effects were observed at ophthalmological examination or scheduled necropsy. There were no effects on fetal sex ratios. At 1000 mg/kg bw per day, the mean body weight of fetuses was statistically significantly reduced. At scheduled caesarean section, no treatment-related macroscopic findings were noted in the pups. Numerous visceral variations were scattered across all dose groups, including controls. Though the overall incidence of visceral variations appeared to be higher at 500 and 1000 mg/kg bw per day than in controls, the vast majority of these were common variations in the rat and were within historical control ranges. No skeletal abnormalities were observed in any dose group. The incidences of pelvic girdle displaced (caudal), also known as 27 pre-pelvic (presacral) vertebrae, were statistically significantly increased in all dose groups compared to controls. In addition, the incidences of costal cartilages asymmetrically aligned at the sternum were increased at 500 and 1000 mg/kg bw per day. All other skeletal variations were considered normal variability in this species.

Table 9. Maternal body weight gain and feed consumption in a range-finding developmental toxicity study in rats

Maternal parameter / Day	Measure per dose level of bicyclopyrone			
	0 mg/kg bw per day	**100 mg/kg bw per day**	**500 mg/kg bw per day**	**1000 mg/kg bw per day**
Body weight (g)				
0	212	208	210	209
3	225	220	223	222
4	229	224	225	225
5	230	227	228	227
6	232	227	227	225
7	235	231	230	227*
8	240	235	233	231*
9	244	239	235*	235*
Body weight gain (g) from gestation day 5 (treatment initiation)				
6	2.5	−0.7*	−1.4*	−1.2*
7	5.1	3.5	1.2**	0.5**
8	10.0	7.2	4.4**	4.1**
9	14.4	11.4*	6.7**	8.2**
14	38.0	33.5	26.1**	30.2*
21	111.0	119.2	110.4	113.1
Feed consumption (g/rat per day)				
0–5	21.9	21.4	21.7	21.1
8–11	24.8	23.5	21.3**	20.5**
11–14	24.4	24.2	21.1**	20.8**
17–21	26.5	27.7	25.9	25.9

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test) *Source*: Gerspach (2011a)

The litter incidence of unossified cervical vertebrae, the presence of supernumerary and/or rudimentary ribs and the litter incidence of long costal cartilage 11 were increased in all dose groups compared to controls (Table 10). In addition, the litter incidence of supernumerary costal cartilage was increased at 1000 mg/kg bw per day. All other cartilage variations were considered to reflect the normal variability in this species.

No NOAEL for maternal toxicity could be determined as transiently reduced feed consumption accompanied by lower maternal body weights and body weight gains were seen at the lowest dose level. The LOAEL was 100 mg/kg bw per day, the lowest dose tested.

No NOAEL for embryo/fetal toxicity could be determined as skeletal effects consisting of increases in caudal displacement of the pelvic girdle (27 pre-pelvic vertebrae), supernumerary ribs ossification delays and cartilage changes were observed in all treatment groups. The LOAEL was 100 mg/kg bw per day, the lowest dose tested (Gerspach, 2011a).

Table 10. Fetal skeletal findings in a developmental toxicity study in rats

Skeletal findings	**No. of findings per dose level of bicyclopyrone**			
	0 mg/kg bw per day	**100 mg/kg bw per day**	**500 mg/kg bw per day**	**1000 mg/kg bw per day**
Number of fetuses examined	52	55	63	64
Pelvic girdle displaced (caudal)	3	10	14*	13*
Costal cartilages asymmetrically aligned at sternum	0	0	4	3
Unossified cervical vertebral body 1	5	1	3	8
Unossified cervical vertebral body 2	3	13*	19**	34**
Unossified cervical vertebral body 3	2	17**	27**	31**
Unossified cervical vertebral body 4	0	14**	20**	31**
Unossified cervical vertebral body 5	0	8**	9**	22**
Unossified cervical vertebral body 6	0	4	5	12**
Unossified cervical vertebral body 7	0	2	2	0
Supernumerary, one rib, left	0	6*	5	9**
Supernumerary, one rudimentary rib, left	13	27*	38**	36**
Supernumerary, one rudimentary rib, right	17	30*	31	35*
Long costal cartilage 11, left	0	11**	20**	7*
Long costal cartilage 11, right	1	10**	18**	7
Supernumerary costal cartilage, left	0	5	5	9**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test) *Source*: Gerspach (2011a)

In a developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% CMC in purified water was administered by gavage to mated female rats (RccHan:WIST(SPF); $n = 24$/group) at dose levels of 0, 100, 500 or 1000 mg/kg bw per day on gestation days 6–20. Animals were examined twice daily for signs of toxicity and mortality. Clinical signs were assessed at least twice daily for signs of reaction to the treatment and / or symptoms of ill health. Body weights were recorded daily from gestation day 0 to 21. Feed consumption was recorded at three-day intervals as follows: gestation days 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–21. All females were killed on gestation day 21, and the fetuses were removed by caesarean section. Dams and fetuses were examined in accordance with international recommendations.

All animals survived until scheduled necropsy. No treatment-related clinical signs were noted. At 500 and 1000 mg/kg bw per day, statistically significantly decreased feed consumption was observed. Dose-related decreases in maternal body weight gain were observed at all dose levels beginning immediately after treatment initiation (Table 11). These differences, though transient at 100 mg/kg bw per day, persisted throughout treatment at 500 and 1000 mg/kg bw per day. Mean maternal body weight gain (corrected for gravid uterus weight) was also decreased by more than 10% at 500 and 1000 mg/kg bw per day. There was no effect on any of the evaluated reproduction parameters.

Table 11. Maternal body weight gain and feed consumption in a developmental toxicity study in rats

	Measure per dose level of bicyclopyrone			
Gestation day	0 mg/kg bw per day	100 mg/kg bw per day	500 mg/kg bw per day	1000 mg/kg bw per day
Body weight (g)				
0	200	203	201	201
3	213	218*	215	214
4	216	222*	218	218
5	218	226*	221	222
6	221	229*	225	225
7	226	230	225	225
8	230	235	228	227
9	235	239	233	231
Body weight gain (g) from gestation day 6 (treatment initiation)				
7	4.23	0.86**	−0.01**	0.12**
14	36.9	34.1	29.8*	27.9**
21	115.5	110.8	105.9	102.0*
Feed consumption (g/rat per day)				
3–6	18.9	20.3	20.1	19.5
6–9	22.5	22.2	20.9*	19.4**
12–15	23.4	24.3	22.7	21.7*
18–21	24.8	25.2	25.1	23.9

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test)

Source: Gerspach (2011b)

Effects on fetuses were found at all tested dose levels. Fetal weights were decreased at 500 and 1000 mg/kg bw per day. Although there was no increase in the incidence of external, visceral or skeletal abnormalities (malformations) in any dose group, at all dose levels, skeletal evaluation revealed increased incidences of pelvic girdle malpositioned caudal; of costal cartilages asymmetrically aligned at the sternum; of unossified cervical vertebrae; of full or rudimentary supernumerary ribs; and of costal cartilage (11 long and interrupted). In addition, statistically significant increased incidences of a number of variations of bone or cartilage structures and of delays in ossification occurred at 1000 mg/kg bw per day (Table 12).

No NOAEL for maternal toxicity could be determined as maternal body weights and body weight gains were transiently reduced in all dose groups. The LOAEL was 100 mg/kg bw per day, the lowest dose tested.

No NOAEL for embryo/fetal toxicity could be determined as skeletal effects consisting of increases in caudal displacement of the pelvic girdle (27 pre-pelvic vertebrae), supernumerary ribs, ossification delays and cartilage changes were observed in all treatment groups. The LOAEL was 100 mg/kg bw per day, the lowest dose tested (Gerspach, 2011b).

Table 12. Fetal skeletal findings in a developmental toxicity study in rats

Finding	No. of findings per dose level of bicyclopyrone			
	0 mg/kg bw per day	100 mg/kg bw per day	500 mg/kg bw per day	1000 mg/kg bw per day
Number of fetuses examined	144	132	136	139
Pelvic girdle malpositioned (caudal)	5	19**	54**	61**
Sternebrae offset ossification sites	0	3	3	11**
Sternebrae bipartite ossification	0	2	1	6*
Costal cartilages asymmetrically aligned at sternum	3	8	8	14**
Unossified cervical vertebral body 1	18	11	5**	12
Unossified cervical vertebral body 2	10	43**	49**	87**
Unossified cervical vertebral body 3	5	35**	39**	87**
Unossified cervical vertebral body 4	6	25**	25**	71**
Unossified cervical vertebral body 5	0	20**	20**	56**
Unossified cervical vertebral body 6	1	9**	10**	48**
Unossified cervical vertebral body 7	0	2**	3	20**
Unossified caudal vertebrae, some	1	3	4	35**
Sternum incompletely ossified sternebra 1	0	0	1	2
Sternum incompletely ossified sternebra 2	0	9**	5*	12**
Sternum incompletely ossified sternebra 3	0	1	0	4
Sternum incompletely ossified sternebra 4	0	2	0	2
Sternum incompletely ossified sternebra 5	13	17	11	28*
Sternum incompletely ossified sternebra 6	0	3	5*	17**
Supernumerary, one rib, left	2	4	22**	27**
Supernumerary, one rudimentary rib, left	15	58**	77**	79**
Supernumerary, one rib, right	1	4	19**	27**
Supernumerary, one rudimentary rib, right	19	53**	68**	73**
Long costal cartilage 11, left	2	10*	12**	17**
Long costal cartilage 11, right	2	11**	15**	14**
Supernumerary costal cartilage, left	1	2	12**	13**
Supernumerary costal cartilage, right	1	4	9*	13**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test) *Source*: Gerspach (2011b)

Rabbit

The developmental toxicity of bicyclopyrone was investigated in pregnant New Zealand White rabbits (four dose range-finding studies, including one study in non-pregnant rabbits and one main study) and in pregnant Himalayan rabbits (one dose range-finding study and two main studies).

In a dose range-finding oral toxicity study, non-pregnant female New Zealand White rabbits (n = 2/group) were dosed by gavage with bicyclopyrone (purity 94.5%; batch SEZ3AP006) in 0.5% weight per volume (w/v) aqueous CMC at 50, 200 or 500 mg/kg bw per day for seven days. The rabbits were killed on day 8. Clinical observations, body weights, feed consumption, plasma concentrations of bicyclopyrone and macroscopic examinations were undertaken.

One animal at 500 mg/kg bw per day was euthanized due to its clinical condition (moribund, cold, few faeces) and loss of body weight, after four daily doses. The second rabbit showed little effect of treatment other than an initial reduction in body weight and feed consumption. There was some indication of an effect on body weight at 200 mg/kg bw per day but no effect on clinical condition or feed consumption. No treatment-related effects on clinical condition, body weight or feed consumption were seen in rabbits at

50 mg/kg bw per day. The extent of systemic exposure ($AUC_{0–last}$) and the maximum plasma concentrations of bicyclopyrone increased with dose over the range from 50 to 500 mg/kg bw per day. $AUC_{0–last}$ for bicyclopyrone was similar following repeated dosing. Exposure to bicyclopyrone increased the plasma tyrosine levels and, on repeated dosing, the plasma tyrosine concentrations remained at an elevated state.

As this was a dose range-finding study, no NOAEL was determined (Fox, 2007).

In a dose range-finding developmental toxicity study, time-mated, female New Zealand White rabbits ($n = 10$/group) were dosed by gavage with bicyclopyrone (purity 94.5%; batch SEZ3AP006) in 0.5% w/v aqueous CMC at 0, 100, 200 or 500 mg/kg bw per day on gestation days 5–29 (inclusive). The day of mating was designated gestation day 1. All rabbits were killed on gestation day 30 except those at 500 mg/kg bw per day, which were euthanized prematurely due to toxicity. Clinical observations, body weights, feed consumption, plasma concentrations of bicyclopyrone, number of corpora lutea and gravid uterus weight in all dams were recorded. The number and position of implantations, number of live fetuses, number of intrauterine deaths (early/late), fetal weight, fetal sex, external and visceral observations, and skeletal (bone and cartilage) observations were also recorded.

Two rabbits at 500 mg/kg bw per day died or were euthanized due to their poor clinical condition on day 8. Three of the remaining rabbits at 500 mg/kg bw per day had clinical signs of toxicity, including pinched-in sides, reluctance to move, irregular breathing and subdued behaviour. As it was clear that the dose level of 500 mg/kg bw per day could not be tolerated for the duration of the study, all surviving rabbits at this dose level were euthanized on gestation day 7 or 8. Macroscopic changes in the stomachs of these rabbits included ulcerations, red spot/s and area/s and sloughing off of the mucous layer.

The dose levels of 100 and 200 mg/kg bw per day were associated with lower maternal body weights, but there was no dose–response relationship and no effect on maternal feed consumption or clinical condition (Table 13). Systemic exposure of the rabbits to bicyclopyrone was confirmed from plasma analysis. Average concentrations of bicyclopyrone in plasma increased in proportion with dose and were similar on gestation days 17 and 29.

At 100 or 200 mg/kg bw per day, there was no effect on the number, growth or survival of the fetuses in utero and no evidence of any effect on the type or incidence of fetal abnormality. There was, however, a treatment-related increase in the incidence of three specific skeletal variations: lengthened costal cartilage on rib 10, longer rib 13 (extra rib) attached to the vertebral column and 27 pre-pelvic vertebrae.

No NOAEL for maternal toxicity could be determined as lower maternal body weights and body weight gains were observed in all dose groups. The LOAEL was 100 mg/kg bw per day, the lowest dose tested.

No NOAEL for embryo/fetal toxicity could be identified as the three specific skeletal variations (lengthened costal cartilage on rib 10, longer rib 13 (extra rib) attached to the vertebral column and 27 pre-pelvic vertebrae) were observed in all treatment groups (Moxon, 2007).

Table 13. Maternal body weight in a dose range-finding developmental toxicity study in rabbits

Day	Weight (g) per dose level of bicyclopyrone		
	0 mg/kg bw per day	**100 mg/kg bw per day**	**200 mg/kg bw per day**
3	3509	3328	3360
6	3432	3367**	3407
8	3450	3386*	3394
14	3594	3518	3510
20	3726	3566**	3576**
26	3826	3663**	3683**
30	3911	3760**	3766*

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test)

Source: Moxon (2007)

In a second dose range-finding developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% w/v aqueous high viscosity CMC, was administered by gavage to time-mated female New Zealand White rabbits (Hra:(NZW)SPF; $n = 8$/group) once daily from gestation days 7 through 28. Dose levels were 10, 50, 250 and 400 mg/kg bw per day administered at a dose volume of 10 mL/kg. A concurrent control group of time-mated females ($n = 8$) received the vehicle on a comparable regimen. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and feed consumption were recorded at appropriate intervals. Blood samples for plasma analysis of bicyclopyrone, the metabolite NOA454598 and L-tyrosine were collected from all females on gestation day 7 and from the surviving females on gestation days 17 and 28 at approximately two hours after dosing. On gestation day 29, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the number 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 were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

Maternal toxicity – mortality, moribundity and abortion, leading to group euthanasia – was observed at 250 and 400 mg/kg bw per day. Mean body weight losses and/or lower mean body weight gains with corresponding reduced feed consumption (differences from the control group not statistically significant) were noted in these same groups (Table 14). Euthanasia of the 250 and 400 mg/kg bw per day groups precluded evaluation of intrauterine parameters and fetal morphology. No macroscopic treatment-related changes were noted in females at 250 and 400 mg/kg bw per day found dead, killed in extremis or that aborted, or in females at 10 and 50 mg/kg bw per day that survived to the scheduled necropsy.

Intrauterine growth and survival at 10 and 50 mg/kg bw per day were similar to the control group. Mean litter proportions of thirteenth full rib(s) and 27 pre-pelvic vertebrae were higher at 50 mg/kg bw per day than in controls. Fetal morphology at 10 mg/kg bw per day was similar to the control group.

Analysis of plasma samples collected during gestation revealed consistent dose-proportional increases in the plasma levels of parent bicyclopyrone throughout gestation, with plasma levels of the NOA454598 metabolite generally present at approximately 5% of parent levels. Plasma tyrosine levels measured on gestation day 7 were elevated at all dose levels, reaching a plateau even at the low dose of 10 mg/kg bw per day. Subsequent analyses determined that plasma tyrosine levels continued to increase at 250 mg/kg bw per day (the higher-dose animals having been euthanized prior to further collection).

Table 14. Maternal body weight gain and feed consumption in a dose range-finding developmental toxicity study in rabbits

Parameter Days	Measure per dose level of bicyclopyrone				
	0 mg/kg bw per day	**10 mg/kg bw per day**	**50 mg/kg bw per day**	**250 mg/kg bw per day**	**400 mg/kg bw per day**
Body weight gain (g)					
0–4	−41	6	−71	0	−3
0–7	74	99	27	89	137
7–10	29	−17	66	24	−34
10–13	18	35	20	31	−69
13–21	41	106	103	5	–
21–29	55	31	57	–	–
Feed consumption (g/rat per day)					
4–7	147	150	146	148	163
7–8	167	148	167	141	123
10–13	139	122	138	132	102
13–21	110	123	120	117	–
7–29	117	118	122	–	–

bw: body weight

Source: Sawhney Coder (2012a)

The NOAEL for maternal toxicity was 50 mg/kg bw per day based on excessive maternal toxicity (mortality, moribundity, abortion, decreased body weight and reduced feed consumption) at the LOAEL of 250 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on two specific skeletal variations: thirteenth (extra) rib and 27 pre-pelvic vertebrae at 50 mg/kg bw per day (Sawhney Coder, 2012a).

In a third dose range-finding developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% w/v aqueous high viscosity CMC, was administered by gavage to time-mated, young adult female New Zealand White rabbits (Hra:(NZW)SPF; $n = 10$/group) once daily from gestation day 7 through 28. Dose levels were 100, 150 and 200 mg/kg bw per day administered at a dose volume of 10 mL/kg. A concurrent control group composed of 10 time-mated females received the vehicle on a comparable regimen. The females were approximately six months of age at the initiation of dose administration. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and feed consumption were recorded. Blood samples for plasma analysis were collected from all females on gestation days 7, 17 and 28 at approximately two hours following dose administration. On gestation day 29, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the number 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 were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All females survived to the scheduled necropsy on gestation day 29; there were no treatment-related macroscopic findings. A mean body weight loss (with no corresponding reduction in feed consumption) was noted in the 200 mg/kg bw per day group during gestation days 21–29, resulting in a slightly lower mean body weight gain when the entire treatment period (gestation days 7–29) was evaluated. The differences were not statistically significant. Mean net body weights, net body weight change and gravid uterine weights in this group were similar to the control group values. There were no treatment-related effects on mean body weight parameters or feed consumption in the 100 and 150 mg/kg bw per day groups. Mean fetal body weight in the 200 mg/kg bw per day group was 6.4% lower (not statistically significant) than control group value. This value was also slightly lower than the minimum mean value in the historical control data from the same laboratory. There was a dose-related trend for this parameter in the 150 and 200 mg/kg bw per day groups.

Low incidences of external, visceral and skeletal malformations were observed in all groups, including the control group, and were considered unrelated to test substance administration. Statistically significant increases in skeletal developmental variations (13th full rib[s] and 27 pre-pelvic vertebrae) were observed on all treatment groups and were considered treatment related. At 150 and 200 mg/kg bw per day, higher numbers of an extra site of ossification ventral to cervical centrum no. 2 were also observed.

Plasma concentrations of bicyclopyrone in general increased proportionally with increasing dose and were comparable across gestation days 7, 17 and 28, though there was significant variability in individual values within each dose group. Plasma concentrations of the NOA454598 metabolite were generally present at approximately 5% of parent bicyclopyrone levels at all dose levels and time points. Analysis of tyrosine levels measured on gestation days 7, 17 and 28 revealed plasma tyrosine levels that were elevated to essentially comparable levels at all dose levels and time points.

The NOAEL for maternal toxicity was 150 mg/kg bw per day based on reduced body weight and body weight gain at the LOAEL of 200 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity could not be identified; the LOAEL was 100 mg/kg bw per day, the lowest dose tested, based on two specific skeletal variations: 13th (extra) rib and 27 pre-pelvic vertebrae (Sawhney Coder, 2012b).

In a dose range-finding developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED), in 0.5% w/v aqueous high viscosity CMC, was administered by gavage once

daily to mated young adult female Himalayan rabbits (n = 10/group) at dose levels of 0, 10, 50, 250 mg/kg bw per day from gestation day 5 to 27. A standard dose volume of 4 mL/kg bw with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone. All surviving females were killed on gestation day 28, and the fetuses were removed by caesarean section. Dams and fetuses were examined in accordance with international recommendations.

Animals were examined and/or observed for signs of toxicity and mortality twice daily. Ophthalmoscopy was carried out once prior to treatment and once towards the end of the gestation period. Body weights were recorded daily from gestation day 0 to 28. Feed consumption was recorded at the following intervals: gestation days 0–5, 5–8, 8–11, 11–14, 14–17, 17–20, 20–23, 23–26 and 26–28. At the scheduled necropsy on gestation day 28, females were killed by an intravenous injection of sodium pentobarbital and the fetuses removed by caesarean section, in random order. Necropsy, 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. The uteri (and contents) of all females with live fetuses were weighed during necropsy to enable the calculation of the corrected body weight gain. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All female animals survived until the scheduled necropsy. No clinical signs or ophthalmoscopic effects were noted in any of the dams. There were no treatment-related effects on body weight or feed consumption. At 250 mg/kg bw per day, preimplantation loss was statistically significantly lower. Based on this finding, the implantation rate was statistically significantly higher,. The postimplantation loss was statistically significantly increased to 37.1% of implantation sites (37.1% embryonic resorptions). Thus, the mean number of total fetuses was statistically significantly decreased to 62.9% of implantation sites. At 10 and 50 mg/kg bw per day, preimplantation loss, implantation rate, postimplantation loss and the number of living fetuses, respectively, was not influenced by bicyclopyrone treatment. At necropsy, one low-, one mid- and one high-dose dam were each noted to have crateriform elevations or depressions with increasing diameters at the pylorus area, which were interpreted as beginning ulcerations. All other macroscopic findings were considered to be within the range of normal background alterations.

There were no effects on fetal sex ratios or mean fetal body weight (Table 15). External examination of fetuses did not find any treatment-related findings. Treatment-related increases in the overall incidence of visceral abnormalities were observed at 250 and 50 mg/kg bw per day. At 250 mg/kg bw per day, the majority of affected fetuses had interventricular septal defect (eight fetuses in five litters), and at 50 mg/kg bw per day, 3/4 fetuses with abnormalities had interventricular septal defects. Dose-dependent skeletal abnormalities were noted at 50 and 250 mg/kg bw per day as multiple misshapen, supernumerary or fused cervical vertebrae; fused cervicothoracic vertebrae; absent thoracic vertebrae; fused thoracolumbar vertebrae; scoliosis; and interrupted or short ribs. These were considered treatment related. Dose-dependent bone variations were noted, mostly outside the range of historical reference data at 250 mg/kg bw per day. These were considered likely related to the test item. Skeletal examination (stage of development) of fetuses revealed a mostly dose-dependent increase in ossification of sternebra 5, but incompletely ossified or unossified sternebra 1, dose-dependent increase in supernumerary ribs, and decrease in additional ossifications of humerus, femur and tibia bones. These findings were considered likely treatment related. At 250 mg/kg bw per day, one fetus was noted with severely dilated lateral ventricles of the brain, bilateral (internal hydrocephaly), which was also considered treatment related. Dose-dependent cartilage abnormalities considered treatment related were noted at 50 mg/kg bw per day (one fetus) and at 250 mg/kg bw per day (11 fetuses in seven litters). Common cartilage variations were dose dependent and, at 250 mg/kg bw per day, outside the historical control data. Corresponding to the skeletal examination, the incidence was also considered likely to show treatment-related effects. Increases in the incidence of one variation (pelvic girdle displacement (caudal), also known as 27 pre-pelvic vertebrae) were noted in all dose groups.

Table 15. Selected findings in fetuses in a developmental toxicity gavage study in rabbits

Finding	Number of fetuses per dose level of bicyclopyrone [a]			
	0 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day	250 mg/kg bw per day
Number of fetuses (litters) examined	54 (8)	63 (10)	57 (10)	44 (10)
Visceral abnormalities				
Heart misshapen, malrotated, right ventricular chamber small with no apparent arterial outlet, left ventricular chamber large	0 (0)	1 (1)	0 (0)	0 (0)
Heart interventricular septal defect (total)	1 (1)	0 (0)	3 (3)	8** (5)
Visceral variations				
Heart small interventricular septal defect	0 (0)	0 (0)	1 (1)	2 (2)
Heart interventricular septum muscle lesion	0 (0)	0 (0)	0 (0)	1 (1)
Heart interventricular septum membraneous region abnormal surface appearance or small diverticulum	0 (0)	1 (1)	4 (2)	4* (3)
Heart atrium enlarged slight	0 (0)	0 (0)	1 (1)	0 (0)
Examination of head abnormalities				
Brain lateral ventricle bilateral dilated, severe	0 (0)	0 (0)	0 (0)	1 (1)
Skeletal abnormalities				
Cervical vertebra fused to exoccipital	0 (0)	0 (0)	0 (0)	1 (1)
Cervical vertebra supernumerary	0 (0)	0 (0)	0 (0)	1 (1)
Cervical vertebra supernumerary ventral arch (partial or complete)	0 (0)	0 (0)	1 (1)	8** (5)*
Cervical vertebral body/arch misshapen/ fused	0 (0)	0 (0)	1 (1)	9** (5)*
Cervicothoracic vertebrae additional cervical hemivertebra; absent thoracic hemivertebra and rib	0 (0)	0 (0)	0 (0)	1 (1)
Thoracolumbar vertebrae additional hemivertebra partially fused to adjacent vertebral body; thoracolumbar scoliosis	0 (0)	0 (0)	0 (0)	1 (1)
Rib and/or costal cartilage absent, interrupted or short	0 (0)	0 (0)	0 (0)	5* (4)
Skeletal/cartilage variations				
Cervical vertebral body/arch incompletely ossified (includes isolated ossification site)	0 (0)	0 (0)	1 (1)	9** (4)*
Cervical vertebral body/arch additional ossification site	0 (0)	0 (0)	1 (1)	11** (5)*
Sternebrae offset and/or fused	1 (1)	0 (0)	6 (5)	6* (6)
Pelvic girdle displaced (caudal)	0 (0)	6* (5)*	11** (6)**	33** (10)**
Ventral plate reduced in size/absent/interrupted	1 (1)	3 (2)	2 (2)	15** (7)*
Costal cartilage branched/fused	1 (1)	0 (0)	0 (0)	8** (7)*
Costal cartilages asymmetrically aligned at sternum	0 (0)	1 (1)	2 (2)	6** (5)*
Costal cartilage 8 connected to sternum	0 (0)	0 (0)	0 (0)	4* (3)

(*continued on p 33*)

Finding	Number of fetuses per dose level of bicyclopyrone [a]			
	0 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day	250 mg/kg bw per day
Bone examinations = ossification stage/supernumerary ribs				
Incompletely ossified sternebra 5	10 (6)	11 (5)	8 (4)	2 (2)
Unossified sternebra 5	8 (4)	12 (6)	1 (1)	3 (2)
Incompletely ossified sternebra 1	0 (0)	0 (0)	0 (0)	6** (4)
Unossified sternebra 1	0 (0)	0 (0)	0 (0)	1 (1)
Supernumerary rib left	2 (1)	2 (2)	27** (10)**	32** (10)**
Supernumerary rib right	2 (1)	4 (3)	16** (9)**	32** (8)*
Additional ossification humerus left	46 (8)	29** (10)	38* (10)	24**(10)
Additional ossification humerus right	47 (8)	26** (9)	34** (10)	17** (7)
Additional ossification femur left	41 (8)	34*(9)	33*(9)	20** (6)
Additional ossification femur right	39 (8)	39 (10)	36 (9)	19** (6)
Additional ossification tibia left	5 (3)	2 (2)	3 (2)	1 (1)
Additional ossification tibia right	5(3)	3 (2)	2 (1)	0 (0)

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test)

[a] Results presented as number of fetuses with the finding and, in parentheses, the number of litters with the finding.

Source: Whitlow (2012a)

The NOAEL for maternal toxicity was 50 mg/kg bw per day based on an increased incidence of preimplantation loss at the LOAEL of 250 mg/kg bw per day.

No NOAEL for embryo/fetal toxicity could be determined as one specific skeletal variation (27 pre-pelvic vertebrae) was observed in all dose groups. The LOAEL was 10 mg/kg bw per day, the lowest dose tested (Whitlow, 2012a).

In a developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% w/v aqueous high viscosity CMC, was administered by gavage once daily to time-mated young adult, female New Zealand White rabbits (Hra:(NZW)SPF; $n = 25$/group) from gestation day 7 through day 28. Dose levels were 10, 50 or 200 mg/kg bw per day administered at a dose volume of 10 mL/kg. A concurrent control group ($n = 25$) received the vehicle on a comparable regimen. The females were approximately 5.5 months of age at treatment initiation.

All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and feed consumption were recorded at appropriate intervals. Blood samples for plasma analysis of parent and tyrosine concentrations were collected from all surviving females at approximately 6 hours following dose administration on gestation day 28. A laparohysterectomy was performed on each surviving female on gestation day 29. The uterus, placenta 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.

A dose of 200 mg/kg bw per day exceeded the maximum tolerated dose (MTD). Significant maternal toxicity manifested at this high-dose level, and seven females were found dead (including one that was found dead after an abortion) or were euthanized from gestation day 22 to 28 following feed consumption of ≤10 g/day for at least four days prior to death or euthanasia and body weight losses (as much as 639 g from the gestation day 7 body weight). Two females at 200 mg/kg bw per day delivered on gestation day 29 following slight reductions in feed consumption or body weight loss. Mean maternal body weight gains for this group were lower (although not statistically significantly) than the control group during gestation days 13–21 and a mean body weight loss was noted during gestation days 21–29 (Table 16). Although mean feed consumption values and mean body weights did not differ remarkably from control group values, the effects on body weight gains often occurred in conjunction with low

feed consumption and decreased defecation when evaluated on an individual animal basis. Mean gravid uterine weight, net body weight and net body weight gain for surviving females were unaffected at this dose level. No treatment-related macroscopic findings were observed at 200 mg/kg bw per day. There were no treatment-related effects on survival or clinical or macroscopic findings for maternal animals at 10 and 50 mg/kg bw per day. Mean maternal body weight and feed consumption parameters were also unaffected at 10 and 50 mg/kg bw per day.

The measured bicyclopyrone concentrations were below the LOQ in samples taken from control animals. In dosed animals, they were 116–31 000, 1090–112 000 and 9770–325 000 ng/mL at dose levels of 10, 50 and 200 mg/kg bw per day, respectively. The measured *L*-tyrosine concentrations were below the LOQ in control animals. In dosed animals, they were 29.7–150, 40.6–92.0 and 51.1–157 ng/mL at 10, 50 and 200 mg/kg bw per day, respectively.

Table 16. Maternal body weight gain and feed consumption in a developmental toxicity study of bicyclopyrone in rabbits

Days	Weight per dose level of bicyclopyrone			
	0 mg/kg bw per day	**10 mg/kg bw per day**	**50 mg/kg bw per day**	**200 mg/kg bw per day**
Body weight (g)				
0	3766	3735	3685	3695
4	3705	3692	3647	3662
7	3820	3803	3770	3788
8	3816	3793	3751	3779
9	3825	3798	3765	3804
12	3843	3813	3792	3831
Body weight gain (g)				
0–7 (pretreatment)	53	68	86	93
7–8	−4	−11	−20	-10
7–9	5	−5	−5	15
7–12	24	9	22	42
7–17	90	96	111	49
7–22	141	120	154	94
7–29	105	139	129	87
Feed consumption (g/rat per day)				
4–7	153	158	162	163
7–10	148	148	149	157
7–29	112	115	112	118

bw: body weight *Source*: Sawhney Coder (2012c)

A low incidence of skeletal costal cartilage anomalies (fused, bifurcated and/or malpositioned costal cartilage) was observed at 200 mg/kg bw per day (four fetuses from three litters compared to zero fetuses in the control group). Two fetuses from two litters at 50 mg/kg per day also had costal cartilage anomalies. Although these values were at the upper level of the historical control incidence, an association with the test substance cannot be excluded. Treatment-related, statistically significant increases in the number of skeletal variations were observed in all treatment groups compared to the control group. These included increased incidences of 13th full rib(s), 27 pre-pelvic vertebrae and an extra site of ossification ventral to cervical centrum no. 2. Other skeletal developmental variations observed in the treatment groups occurred similarly in the control group, in single fetuses and/or in a manner that was not dose related (Table 17).

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on excessive maternal toxicity (mortality, moribundity, abortion, decreased body weight and reduced feed consumption) at the LOAEL of 200 mg/kg bw per day.

No NOAEL for embryo/fetal toxicity could be determined as two specific skeletal variations, the thirteenth (extra) rib and 27 pre-pelvic vertebrae, were observed at all dose levels. The LOAEL was 10 mg/kg bw per day, the lowest dose tested (Sawhney Coder, 2012c).

Table 17. Skeletal anomalies in fetuses in a developmental toxicity gavage study in rabbits with bicyclopyrone

Finding	Number of animals with the finding per dose level of bicyclopyrone [a]			
	0 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day	200 mg/kg bw per day
Number of fetuses (litters) examined	212 (23)	183 (23)	211 (23)	156 (18)
Skeletal anomalies				
Costal cartilage anomaly	0 (0)	0 (0)	2 (2)	3 (2)
Skeletal variations				
13th full rib	51 (17)	112** (21)	149** (23)*	147** (18)*
27 pre-pelvic vertebrae	10 (7)	53** (15)*	92** (21)**	110** (18)**
Extra site of ossification ventral to cervical centrum no. 2	0 (0)	3 (3)	4 (1)	4* (1)

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Fisher exact test)

[a] Results presented as number of fetuses with the finding and, in parentheses, the number of litters with the finding.

Source: Sawhney Coder (2012c)

In a developmental toxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED), in 0.5% w/v aqueous high viscosity CMC, was administered by gavage to young mated female Himalayan rabbits (n = 22/group) at dose levels of 0, 10, 50 or 250 mg/kg bw per day. A standard dose volume of 4 mL/kg bw with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone.

The animals were observed for mortality twice daily. Clinical signs were assessed daily for treatment-related effects and/or symptoms of ill health. Body weights were recorded daily from gestation day 0 to 28. Feed consumption was recorded at the following intervals: gestation days 0–3, 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24 and 24–28. On the last day of treatment (gestation day 27), blood samples (approximately 2 mL) were collected from all females. The samples were taken from each of the females six hours after administration. The blood samples were subdivided and prepared accordingly for plasma level determination of bicyclopyrone and L-tyrosine and for haematological and clinical chemistry assessments. At scheduled kill on gestation day 28, the fetuses were removed by caesarean section. Any female euthanized prior to the scheduled kill was macroscopically examined with emphasis on the uterus and its contents. Necropsy, 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. The uteri (and contents) of all females with live fetuses were weighed during necropsy on gestation day 28 to enable the calculation of the corrected body weight gain. The liver weight of the dams was recorded. Examination of dams and fetuses was performed in accordance with international recommendations.

There were no unscheduled deaths or obvious signs of maternal toxicity in any dose group. There were no significant effects on body weight gain or feed consumption. At 50 mg/kg bw per day, a single dam aborted her litter. At 250 mg/kg bw per day, a few maternal animals had macroscopic findings of the stomach wall. Measurement of haematology and clinical chemistry parameters did not indicate any toxicologically significant changes.

Bioanalytical measurements in blood samples taken at six hours after the last dose administration (gestation day 27) confirmed dose-dependent exposure of dams with bicyclopyrone. Blood concentrations of bicyclopyrone increased supralinearly with dose, although variability in the blood concentrations makes a quantitative dose relationship highly uncertain. Measurements indicated a dose-dependent increase of tyrosine levels, with a possible indication of saturation of tyrosine concentrations at higher doses of bicyclopyrone.

Treatment-related fetal effects were noted at all dose levels. At 250 mg/kg bw per day, fetal body weights were significantly decreased and the overall incidences of external, visceral and skeletal abnormalities (malformations) were significantly increased compared to controls. Visceral examination revealed a number of abnormalities at low incidences, except for two that were outside the historical control range: defects of the muscular portion of the interventricular septum and urogenital abnormalities consisting primarily of unilateral missing kidney and ureter. Skeletal effects included increases in the incidence of cervical vertebral abnormalities. The single finding of cleft palate at 250 mg/kg bw per day could be considered incidental, but is in agreement with a statistically significant increased incidence in the other developmental study in Himalayan rabbits; hence a relation to treatment cannot be excluded. Increases in a number of visceral and skeletal variations were also observed in this dose group. Visceral examination indicated an increase in the litter incidence of variations of interventricular septum in the perimembraneous region classified as small septal defects, diverticulae or an abnormal appearance of the surface. Other visceral changes at this dose level were increased fetal incidences of a number of variations common to this strain of rabbit. Skeletal variations noted in this dose group included increases in rib and cartilage variations, cervical vertebral ossification changes, pelvic displacement (reflecting the presence of an extra (27th) pre-pelvic vertebra and supernumerary ribs. Statistically identified increases in the overall incidence of visceral abnormalities seen at 10 and 50 mg/kg bw per day were primarily evident as unilateral missing kidney and ureters. The incidence of missing kidneys was not dose dependent, occurring in 3, 2 and 4 litters in the 10, 50 and 250 mg/kg bw per day dose groups, respectively, but was outside the historical control range. In a subsequent study, the presence of unilateral missing kidney and ureter was observed in 2/88 maternal animals. In addition, unilateral missing or interrupted uterine horn was observed in 3/88 females in this study, as well as the subsequent study. These findings strongly support that there is a genetic predisposition to this condition unrelated to exposure to bicyclopyrone.

Fetal variations at 50 mg/kg bw per day that were considered treatment-related included an increased incidence of interventricular septum variations (not statistically identified on a litter basis) and skeletal changes consisting of malpositioned pelvic girdle (27 pre-pelvic vertebrae), supernumerary ribs and costal cartilage changes (Table 18). At 10 mg/kg bw per day, there were statistical increases in the fetal incidence of interventricular septum variations, although the litter incidence was not increased. However, these effects on the septum were not seen consistently in the other developmental study in Himalayan rabbits (see Table 19): they were either seen in similarly high incidences in controls or not at all at the high dose levels up to 250 mg/kg bw per day. As a result, these cardiac variations were considered not treatment related.

Table 18. Treatment-related variations and abnormalities in fetuses and litters in a developmental toxicity study of bicyclopyrone in rabbits

Finding	No. with the finding per dose level of bicyclopyrone [a]			
	0 mg/kg per day	**10 mg/kg per day**	**50 mg/kg per day**	**250 mg/kg per day**
No. of fetuses (litters) examined	123 (21)	129 (21)	106 (19)	115 (21)
External abnormalities				
Palate cleft	0 (0)	0 (0)	0 (0)	1 (1)
Visceral abnormalities				
Heart muscular interventricular septal defect	0 (0)	0 (0)	0 (0)	3 (3)
Urogenital structure(s) absent or misshapen/ malpositioned (total)	0 (0)	3 (3)	4* (4)*	5* (5)*
Kidney and ureter absent	0 (0)	3 (3)	2 (2)	4 (4)
Ovary/uterine horn or testis/vas deferens absent or misshapen/malpositioned	0 (0)	3 (3)	3 (3)	5* (5)*

Finding	No. with the finding per dose level of bicyclopyrone [a]			
	0 mg/kg per day	10 mg/kg per day	50 mg/kg per day	250 mg/kg per day
Visceral variations				
Heart interventricular septum variations (total)	6 (5)	17* (8)	21** (10)	42** (19)**
Heart perimembraneous region				
– Small septal defect	3 (3)	7 (4)	3 (3)	18** (13)**
– Diverticulum	1 (1)	0 (0)	3 (2)	8* (8)*
– Abnormal surface appearance	2 (2)	10* (7)*	14** (8)*	15** (10)**
Skeletal abnormalities				
Cervical vertebral irregularities (total)	1 (1)	1 (1)	4 (3)	19** (9)**
Vertebrae 2, 3 body/odontoid process/arch absent /misshapen / fused / not fused dorsal / small / split /supernumerary	1 (1)	1 (1)	4 (3)	19** (9)**
Vertebrae 4, 5, 6 body/arch absent / fused / interrupted / not fused dorsal / small / split	0 (0)	0 (0)	1 (1)	5* (3)
Ribs and/or costal cartilage irregularities (total)	2 (2)	0 (0)	4 (4)	16** (7)
Thoracic rib/costal cartilage 1, 2 absent / branched /interrupted / malpositioned / partially duplicated /short	1 (1)	0 (0)	3 (3)	12** (7)*
Other thoracic ribs/costal cartilages absent / fused /interrupted / misshapen / supernumerary	0 (0)	0 (0)	1 (1)	6* (2)
Skeletal variations				
Cervical vertebra incompletely ossified (includes isolated ossification site) (total)	2 (2)	0 (0)	1 (1)	18** (10)**
Vertebrae 1–3 body/arch incompletely ossified (includes unilateral ossification), unossified or isolated ossification site	1 (1)	0 (0)	1 (1)	8* (6)*
Vertebra 2 odontoid process unossified	0 (0)	0 (0)	0 (0)	13** (8)**
Pelvic girdle malpositioned caudal (total)	3 (2)	3 (3)	22** (11)**	79** (21)**
27 pre-pelvic vertebrae (total)	3 (2)	3 (3)	21** (10)**	79** (21)**
Costal cartilages asymmetrically aligned at sternum	1 (1)	3 (3)	6* (6)*	10** (9)**
Costal cartilage 8 (false rib) connected to sternum	0 (0)	0 (0)	0 (0)	4 (4)

bw: body weight; no.: number; *: $P < 0.05$; **: $P < 0.01$ (Fisher exact test)

[a] Results presented as number of fetuses with the finding and, in parentheses, the number of litters with the finding.

Source: Whitlow (2012b)

The NOAEL for maternal toxicity was 50 mg/kg bw per day based on macroscopic findings in the stomach wall of females at the LOAEL of 250 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day based on two specific skeletal variations: costal cartilage asymmetrically aligned at sternum and 27 pre-pelvic vertebrae, and slight increases in several other skeletal abnormalities at the LOAEL of 50 mg/kg bw per day (Whitlow, 2012b).

In another oral developmental toxicity study in Himalayan rabbits, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) in 0.5% w/v aqueous high viscosity CMC, was administered by gavage to mated young adult female Himalayan rabbits ((SPF): Crl:CHBB(HM); $n = 22$/group) at dose levels of 0, 1, 10 or 250 mg/kg bw per day. A standard dose volume of 4 mL/kg bw with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone. Animals were observed for viability/mortality twice daily. Clinical signs were assessed daily for signs of reaction to the

treatment and/or symptoms of ill health. Body weights were recorded daily from gestation day 0 to 28. Feed consumption was recorded at the following intervals: gestation days 0–3, 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24 and 24–28. On the last day of treatment (gestation day 27), blood samples were collected from all females one hour and six hours after dose administration for analysis of bicyclopyrone and tyrosine levels. At scheduled kill on gestation day 28, the fetuses were removed by caesarean section. Dams and fetuses were examined in accordance with international recommendations.

Two dams at 250 mg/kg bw per day were killed on gestation day 22 after showing a general weak condition, decreased activity and prostrate position. All dams at 1 and 10 mg/kg bw per day and in the control group survived until scheduled necropsy. Feed consumption was not affected by treatment with the test item. There were no statistically significant differences in body weight or body weight gain in any dose group. However, cumulative body weight gain in dams at 250 mg/kg bw per day was consistently lower than controls throughout most of the treatment period. At 250 mg/kg bw per day, postimplantation loss was statistically significantly increased (32% of implantation sites) due to an increased number of embryonic resorptions, and the number of fetuses at caesarean section was consequently reduced. At necropsy, the two high-dose dams that were euthanized had macroscopic findings indicative of irritations in the stomach (red and dark red foci).

Bioanalytical measurements in blood samples taken on the last day of treatment (gestation day 27) confirmed the dose-dependent exposure of dams with bicyclopyrone and indicated the dose-dependent increase of tyrosine levels.

There was no effect on fetal sex ratios. Mean body weight of fetuses was statistically significantly lower at 250 mg/kg bw per day. A treatment-related increase in the overall incidence of abnormalities was observed at 250 mg/kg bw per day (Table 19). At external examination, jaw and/or palate cleft was observed (three fetuses of three litters); visceral examination revealed heart muscle and/or perimembraneous interventricular septal defects and misshapen internal musculature in the septum. At 250 mg/kg bw per day, visceral variations attributed to treatment included increased incidences of malpositioned oesophagus and aortic arch supernumerary branch. Urogenital findings (absent kidney and ureter, malpositioned kidney and dilated ureter, absent uterine horn and misshapen ovary) were noted in all treatment groups. However, the presence of two dams with absent kidney in the current study and with missing uterus horn in this study indicated an underlying genetic cause rather than an effect of the test item. The overall incidence of abnormalities at 1 and 10 mg/kg bw per day was not increased compared to controls. At 250 mg/kg bw per day, cleft palate in three litters, of which one case was associated with lip and upper jaw cleft, and eye abnormalities in two litters were confirmed.

Based on litter evaluation, an overall increase in skeletal abnormalities was observed at 250 mg/kg bw per day (Table 19). These effects were primarily cervical vertebral irregularities (mostly absence, fusions, malformation of vertebral body and/or arches of vertebrae 2 or 3) and abnormalities affecting ribs 1 or 2 and/or costal cartilage (short, interrupted, fused). The incidence of bone and cartilage variations was also increased in this dose group, consisting of cervical vertebrae structural variations (absent transverse foramen, supernumerary site ventral) and ossification irregularities (incomplete or no ossification, isolated ossification site) and caudal displacement of the pelvic girdle. At 250 mg/kg bw per day, increases in full supernumerary thoracolumbar ribs and incompletely ossified right and left pubis were considered related to treatment. Cartilage variations observed at 250 mg/kg bw per day included increased incidences of right and/or left costal cartilage 1 not reaching sternum; decreased incidence of right and/or left costal cartilage 7 not reaching sternum; and increased incidence of supernumerary costal cartilages. At 10 mg/kg bw per day, a slight but statistically significant increase in the incidence of supernumerary ribs and costal cartilage variations was observed.

Table 19. Treatment-related findings in fetuses and litters in a developmental toxicity study of bicyclopyrone in rabbits

Finding	No. with the finding per dose level of bicyclopyrone			
	0 mg/kg per day	**1 mg/kg per day**	**10 mg/kg per day**	**250 mg/kg per day**
Number of fetuses (litters) examined	148 (22)	106 (20)	114 (20)	85 (18)
External abnormalities				
Jaw and/or palate cleft	0 (0)	0 (0)	0 (0)	3* (3)*
Visceral abnormalities				
Heart: interventricular septal defect / over-riding aorta /single ventricular chamber (total)	2 (2)	3 (3)	1 (1)	6* (5)
Heart: perimembraneous / muscular septal defect	0	1 (1)	1 (1)	4* (3)
Heart: Muscular septal defect	1 (1)	1 (1)	0	2 (2)
Urogenital(total)	0 (0)	1 (1)	2 (1)	6** (6)**
Kidney and ureter absent	0 (0)	1 (1)	0 (0)	2 (2)
Kidney severely malpositioned caudal (pelvic kidney) and small with or without malrotation	0 (0)	0 (0)	2 (1)	0 (0)
Kidney malpositioned caudal, renal pelvis and ureter severely dilated	0 (0)	0 (0)	0 (0)	1 (1)
Uterine horn absent or threadlike and/or ovary misshapen	0 (0)	1 (1)	0 (0)	4* (4)*
Visceral variations				
Heart interventricular septum variations (total)	23 (15)	8* (6)*	12 (10)	26** (13)
Heart perimembraneous region: Small septal defect	13 (12)	4 (4)*	6 (6)	19** (10)
Heart perimembraneous region: Diverticulum	4 (4)	2 (2)	0	0
Heart perimembraneous region: abnormal surface appearance	6 (6)	2 (2)	6 (5)	7 (6)
Oesophagus malpositioned (right-sided)	1 (1)	1 (1)	1 (1)	4 (3)
Aortic arch supernumerary branch	3 (3)	3 (3)	2 (2)	14** (11)**
Skeletal abnormalities				
Cervical vertebral irregularities(total)	0 (0)	1 (1)	1 (1)	22** (13)**
Vertebra 1 body/arch fused (to exoccipital or adjacent vertebra) / interrupted / misshapen / short	0 (0)	0 (0)	0 (0)	3* (3)
Vertebrae 2, 3 body/odontoid process/arch absent / fused / hemicentric / interrupted / misshapen / small / supernumerary ventral arch or partial ventral arch	0 (0)	0 (0)	1 (1)	21** (12)**
Thoracic rib/costal cartilage 1, 2 fused / interrupted / short	0 (0)	0 (0)	0 (0)	8** (6)**
Skeletal variations				
Cervical vertebra small structural variation (total)	4 (4)	4 (4)	9 (8)	23** (11)**
Vertebral arch 1 transverse foramen absent	4 (4)	4 (4)	9 (8)	20** (11)**
Vertebra 2 supernumerary site ventral	0 (0)	0 (0)	0 (0)	6** (4)**
Cervical vertebra ossification irregularity (total)	0 (0)	0 (0)	3 (3)	16** (10)**

(*continued on next page*)

Finding	No. with the finding per dose level of bicyclopyrone			
	0 mg/kg per day	1 mg/kg per day	10 mg/kg per day	250 mg/kg per day
Vertebra 2 odontoid process incompletely ossified or unossified	0 (0)	0 (0)	1 (1)	12** (8)**
Vertebral arch 2 isolated ossification site	0 (0)	0 (0)	0 (0)	9** (5)*
Pelvic girdle malpositioned caudal (total)	2 (2)	0 (0)	6 (3)	64** (17)**
Bone examination				
Full supernumerary thoracolumbar ribs right	7 (7)	4 (4)	16* (10)	72** (18)**
Full supernumerary thoracolumbar ribs left	4 (4)	6 (5)	13 (8)	70** (18)**
Incompletely ossified pubis right	0 (0)	1 (1)	2 (1)	9** (6)**
Incompletely ossified pubis left	0 (0)	0 (0)	2 (1)	13** (7)**
Cartilage variations				
Costal cartilage 1 not reaching sternum right	0 (0)	1 (1)	11**(8)**	13** (9)**
Costal cartilage 1 not reaching sternum left	0 (0)	1 (1)	3 (3)	10** (6)**
Costal cartilage 7 not reaching sternum right	39 (13)	27 (14)	26 (12)	3** (2)**
Costal cartilage 7 not reaching sternum left	48 (15)	35 (16)	22* (11)	3** (3)**

bw: body weight; no.: number; *: $P < 0.05$; **: $P < 0.01$ (Fisher exact test)

[a] Results presented as number of fetuses with the finding and, in parentheses, the number of litters with the finding.

Source: Whitlow (2012c)

The NOAEL for maternal toxicity was 10 mg/kg bw per day based on mortality, decreased body weight gain and signs of stomach irritation at the LOAEL of 250 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1 mg/kg bw per day based on increases in supernumerary ribs and costal cartilage at the LOAEL of 10 mg/kg bw per day (Whitlow, 2012c).

2.6 Special studies

(a) Acute neurotoxicity

In an acute dose range-finding oral neurotoxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was administered by gavage to Crl:CD(SD) rats ($n = 3$/sex per group) at doses of 0 (control) or 2000 mg/kg bw in 0.5% w/v aqueous CMC. All animals were observed twice daily for mortality and moribundity. Clinical observations and body weights were recorded at regular intervals. Modified FOB parameters were evaluated for all animals approximately 1, 2, 3, 4, 5, 6, 7 and 8 hours after dose administration on study day 0 and once daily on study days 1–7. On study day 8, all animals were killed by carbon dioxide inhalation and discarded without macroscopic examination.

All animals survived to the scheduled kill. No remarkable clinical findings were noted. There were no treatment-related effects on body weights or body weight changes. None of the modified FOB parameters evaluated were affected by dose administration (Beck, 2009).

In an acute neurotoxicity study, a single dose of bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED), in 0.5% w/v aqueous CMC, was administered by gavage to Crl:CD(SD) rats ($n = 10$/sex per group) at dose levels of 20, 200 and 2000 mg/kg bw. A concurrent control group received the vehicle on a comparable regimen. Animals were approximately 6 weeks old at initiation of dose administration. The dose volume was 10 mL/kg bw for all groups.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily. Individual body weights were recorded weekly. FOB and locomotor activity data were recorded for all animals prior to treatment initiation, at time of peak effect (approximately one hour following dose administration) and on study days 0, 7 and 14. A subgroup of rats ($n = 5$/sex per group)

was anaesthetized on study day 15 and perfused in situ, with brain weights were recorded. Specified tissues from the central and peripheral nervous systems of control and high-dose animals (n = 5/sex per group) systems underwent neuropathological evaluation. The remaining animals were killed on study day 15 and discarded without macroscopic examination.

All animals survived to the scheduled kill. No treatment-related clinical findings were noted during the daily observations. Mean body weights and body weight gains were unaffected by bicyclopyrone administration. No treatment-related effects were apparent in the FOB evaluations. At 2000 mg/kg bw, decreased locomotor activity was observed in both males and females at the time of peak effect on study day 0 only, and occurred in the absence of treatment-related effects on FOB parameters and neuropathology.

The NOAEL was 2000 mg/kg bw, the highest dose tested (Beck, 2012a).

(b) Subacute neurotoxicity

In a 28-day preliminary neurotoxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was fed continuously in the diet to three groups of Crl:CD(SD) rats (n = 8/sex per group) at target concentrations of 0, 500, 2500 or 5000 ppm (equal to 0, 50, 240 and 471 mg/kg bw per day for males and 0, 53, 259 and 505 mg/kg bw per day for females, respectively). A concurrent control group was provided the basal diet on a comparable regimen. Animals were approximately six weeks old at the initiation of dose administration. Detailed physical examinations were performed weekly. Individual body weights and feed consumption were recorded weekly. All animals were necropsied on study day 28.

No treatment-related clinical findings were noted at any concentration. Lower mean body weights and body weight gains (up to 32% and 30%, respectively) were noted in males and females at 5000 ppm after four weeks of treatment. Males and females at 2500 ppm also had lower mean body weight gains (16% and 17%, respectively) than control animals during the first week of treatment. No treatment-related findings were noted at 500 ppm. Males at 5000 ppm had corresponding decreased feed consumption (up to 12% in week 1). During study week 0–1, mean feed efficiencies for the 2500 and 5000 ppm males and females were lower than the control group.

Doses of 50, 5000 and 50 000 ppm were proposed for the 90-day neurotoxicity study (Beck, 2012b).

In a 90-day dietary neurotoxicity study, groups of rats (Crl:CD(SD); n = 12/sex per group) were fed bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) admixed in their diet at concentrations of 0, 50, 500 or 5000 ppm (equal to 0, 4, 35 and 336 mg/kg bw per day for males and 0, 4, 42 and 415 mg/kg bw per day for females, respectively) for 13 weeks. General cage-side observations were made for all animals prior to study start and daily throughout the study. Detailed open field evaluations of clinical signs were performed in a randomized order once prior to treatment initiation and once weekly thereafter. FOB assessments, including quantitative assessments of landing foot splay, sensory perception and muscle weakness, were performed in all animals in a randomized order once prior to treatment initiation and in weeks 2, 4, 8 and 13. Locomotor activities were assessed after each FOB evaluation. Feed consumption values were recorded weekly throughout the study. Body weights were recorded once prior to treatment initiation on day 1 and weekly thereafter. Ophthalmic examinations were performed for all animals prior to treatment initiation and during week 11. During study week 13, a subgroup of rats (n = 5/sex per group) were anaesthetized and perfused in situ; brain weights were recorded and neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on rats in the control and 5000 ppm groups. The remaining animals were euthanized by carbon dioxide inhalation during study week 13 and discarded without macroscopic examination.

For the 5000 ppm group, reduced body weights, lower body weight gains (with corresponding decreased feed consumption for males) and lower cumulative body weight gains were noted. In addition, lower brain weights (−11.3% compared to control) were noted in high-dose males and ocular keratitis was observed in low- and mid-dose males and high-dose males and females.

Group mean brain weight for males was statistically significantly lower than the concurrent control at all dose levels (−7.5% at 50 and 500 ppm, −11.3% at 5000 ppm, compared to control; see Table 20). The effects at 50 and 500 ppm were considered to be within the normal biological range.

The greater than 10% decrease at 5000 ppm was still within the laboratory historical control data, while 4/5 brain weights from perfused concurrent control group males were higher than the maximum mean value in the historical control database. The greater than 10% decrease in brain weight at 5000 ppm was therefore considered unlikely to be treatment-related.

Table 20. Intergroup comparison of rat brain weights in a 90-day dietary neurotoxicity study

Sex parameter	Weight per dose level of bicyclopyrone				Historical control week 13, 10 studies July 2005–Oct 2008[a]	
	0 ppm	50 ppm	500 ppm	5000 ppm	Mean	Range
Males						
Brain weight (g)	2.38	2.20*	2.20*	2.11**	2.2	2.08–2.29
Final body weight (g)	530	557	529	523	553.7	497.9–642.5
Relative brain weight (g/100 g final bw)	0.453	0.398	0.421	0.411	–	–
Females						
Brain weight (g)	2.06	2.03	2.00	2.00	2.0	1.96–2.06
Final body weight (g)	308	286	317	264	289.2	266.8–309.7
Relative brain weight (g/100 g final bw)	0.683	0.716	0.637	0.756	–	–

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test)

[a] The experimental phase of the bicyclopyrone study was June/July 2009. *Source*: Beck (2012c)

No NOAEL for systemic toxicity could be determined as the increased incidence of ocular findings were observed at all dose levels. The LOAEL was 50 ppm (equal to 4 mg/kg bw per day).

The NOAEL for subchronic neurotoxicity was 5000 ppm (equal to 336 mg/kg bw per day), the highest dose tested (Beck, 2012c).

(c) Immunotoxicity

In a 28-day immunotoxicity study, bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) was administered ad libitum in the diet for a minimum of 28 consecutive days to three groups of Crl:CD1(ICR) female mice (approximately 7 weeks old) at dietary concentrations of 50, 500 or 5000 ppm (equal to 10.6, 107 and 1192 mg/kg bw per day). A vehicle control group was given the untreated diet. A positive control group was dosed with cyclophosphamide (CPS) via intraperitoneal injection (50 mg/kg per day, dose volume 10 mL/kg per day) for four consecutive days. The vehicle and positive control groups were offered the basal diet on a regimen comparable to that of the treatment groups. In addition, all mice were immunized with an intravenous injection of sheep red blood cells on study day 24, approximately 96 hours prior to the scheduled necropsy. Each group consisted of 10 females. All animals were observed twice daily for mortality and moribundity. Clinical examinations, body weights and feed consumption were recorded throughout the study. All animals were killed on day 28. Complete necropsies were conducted on all animals. A bone marrow smear (right femur) and the liver, mesenteric lymph nodes, Peyer's patches (gut-associated lymphoid tissue; GALT), spleen and thymus were collected from all animals. The liver, spleen and thymus were weighed. Spleens were placed in EBSS/HEPES buffer and spleen cell suspensions prepared. Spleen cell counts were performed and the number of specific immunoglobulin M (IgM) antibody-forming cells directed towards the sheep red blood cells were determined.

All animals survived to the scheduled necropsy. There were no treatment-related effects on body weights, feed consumption, clinical observations or macroscopic findings. There were no treatment-related effects on absolute, adjusted or relative spleen or thymus weights or spleen cell numbers. There were no effects attributed to bicyclopyrone on the specific activity or total activity of splenic IgM antibody-forming cells to the T cell–dependent antigen sheep red blood cells.

Treatment-related higher liver weights were noted in all bicyclopyrone dose groups. At 50 and

500 ppm groups, statistically significantly higher mean adjusted liver weights (12% and 14% higher, respectively) were noted compared to the vehicle control. At 5000 ppm, mean absolute and adjusted liver weights were statistically significantly higher (23% and 25%, respectively) than the vehicle control group. These increases were consistent with the results of previous studies and were attributed to bicyclopyrone exposure. The increase in liver weight could be considered adaptive and not adverse if no concomitant liver effects were observed. However, since neither clinical chemistry nor detailed histopathology were performed, concomitant liver effects could not be assessed and the increase in liver weight (by >15%) at 5000 ppm cannot be discounted as adaptive rather than adverse.

Mean absolute and adjusted spleen and thymus weights were lower in the cyclophosphamide-treated (positive control) group than in the vehicle control group. In addition, cyclophosphamide administration resulted in statistically significantly lower spleen cell numbers (56% lower), specific activity (100% lower) and total spleen activity (100% lower) of IgM antibody-forming cells compared to the vehicle control group. These effects are consistent with the known immunosuppressant effects of cyclophosphamide and validate the sensitivity of the assay.

The NOAEL for immune suppression was 5000 ppm (equal to 1192 mg/kg bw per day), the highest dose tested. A conservative NOAEL for systemic toxicity was 500 ppm (equal to 107 mg/kg bw per day) based on liver effects at the LOAEL of 5000 ppm (equal to 1192 mg/kg bw per day) (Eapen, 2012).

(d) Mechanistic studies

Five mechanistic studies were submitted. These were all non-guideline studies, and only some were performed in accordance with GLP. The studies are used in the statement on the MOA and human relevance provided in Appendix 1.

Mouse

A 28-day dietary toxicity study in mice was performed to investigate the effects of 2-[2-chloro-3-ethoxy-4-(methylsulfonyl)benzoyl]-5-methylcyclohexane-1,3-dione (R243604) and bicyclopyrone (triketone herbicides) when fed to mice. Groups of C57BL/10J$_f$CD-1 mice (n = 5/sex per dose) were fed diets containing 0 (control), 10, 100, 1000 or 7000 ppm R243604 or bicyclopyrone (purity 100%; batch AED-2120/06 and AED 2120) for 28 consecutive days. A positive control, SC-0774 (a CYP inducer) was also fed to groups of five male and five female C57BL/10J$_f$AP/Alpk mice and Crl:CD-1™(ICR)BR mice at dose levels of 3500 ppm (C57 mice only) and 7000 ppm to evaluate possible strain differences. Clinical observations, body weights and feed consumption were measured throughout the study, and all the animals underwent ophthalmoscopy in week 4 of the study. Samples of urine were taken pre-study and during week 4 for nuclear magnetic resonance (NMR) analysis to monitor phenolic acid metabolites, HPPA, 4-hydroxyphenyllactate (HPLA) and 4-hydroxyphenylacetate (HPAA). At the scheduled kill, the animals were necropsied. Cardiac blood samples were taken to determine plasma tyrosine and/or plasma triketone levels. Selected organs were weighed and specified tissues and abnormalities were taken for subsequent histopathological examination. Samples of liver were taken for HPPD, TAT and P450 enzyme analysis.

Body weight and feed consumption effects for animals receiving SC-0774 at 7000 ppm and R243604 at 7000 ppm could not be sustained and the groups were terminated prematurely. However, bicyclopyrone was tolerated up to 7000 ppm, although it was associated with reduced body weight gain. Plasma tyrosine and triketone levels were increased, compared to the controls, in animals receiving R243604, bicyclopyrone or SC-0774 in the diet at all levels tested. There were no significant differences in effects on plasma tyrosine with any of the test substances. For the plasma triketones, the greatest increases were seen in mice receiving R243604, closely followed by those receiving SC-0774, with those receiving bicyclopyrone having the smallest increases. Phenolic acids were present in the urine of all of the groups dosed with R243604, bicyclopyrone and SC-0774, while none was present in the controls (Noakes, 2007).

Rat

An in vitro study was performed to evaluate the effect of bicyclopyrone at 0, 0.1, 10 or 100 µmol/L on rat thyroid peroxidase activity. A pooled thyroid gland microsomal preparation from rats (n = 5) was assayed for thyroid peroxidase activity by determining the mono-iodination of L-tyrosine. As a

positive control, the effect of 6-propyl-2-thiouracil (PTU; 1 and 10 µmol/L) on rat thyroid peroxidase activity was also determined.

Treatment with bicyclopyrone had no significant effect on rat thyroid peroxidase activity at the concentrations tested. Treatment with 6-propyl-2-thiouracil at 10 µmol/L resulted in 100% inhibition of thyroid peroxidase activity, whereas treatment with 1 µmol/L had no significant effect.

Bicyclopyrone is not an inhibitor of rat thyroid peroxidase activity in vitro (Lake, 2012).

In order to elucidate the MOA for thyroid hypertrophy/hyperplasia observed in a combined chronic toxicity and carcinogenicity study in rats, a 28-day non-guideline toxicity study was performed. Male Han Wistar Crl:WI (Han) rats (n = 75/group) were fed diets containing bicyclopyrone (purity 94.5%; batch SEZ3AP006/MILLED) at 0, 5, 500 or 5000 ppm for up to 28 days. Another group of male Han Wistar rats (n = 30) were fed diets containing 1200 ppm phenobarbital, which acted as a positive control. Viability, clinical observations, body weights and feed consumption were assessed at predetermined intervals from prior to treatment initiation until scheduled kill. Blood samples were collected for thyroid function testing from all animals at scheduled kill and, except for the phenobarbital-treated animals, tyrosine analysis. Animals were killed at predetermined intervals throughout the treatment period and necropsied. The thyroid gland and liver were collected and weighed from all animals. These organs were processed to slides for histopathological examination. With the exception of the phenobarbital-treated animals, the biochemistry of the liver was also analysed.

Treatment resulted in dose- and time-dependent increases in tyrosine; decreases in tetraiodothyronine (thyroxine; T_4); increases in liver weight; and, at 5000 ppm, increased incidence of hepatocellular centrilobular hypertrophy. In addition, treatment at 500 or 5000 ppm resulted in dose- and/or time-dependent increases in hepatic UDPGT activity, decreases in triiodothyronine (T_3), and increased incidences of thyroid follicular cell hypertrophy. Mean body weight gain was lower throughout the treatment period for animals at 5000 ppm, and lower mean feed consumption was observed throughout the treatment period for animals at 500 or 5000 ppm.

Treatment with 1200 ppm phenobarbital resulted in time-dependent decreases in T_4; increases in thyroid-stimulating hormone (TSH); increases in liver and thyroid weights; and increased incidences of both hepatocellular centrilobular hypertrophy and thyroid follicular cell hypertrophy. This is consistent with previous findings, demonstrating the suitability of phenobarbital as a positive control with respect to thyroid-function testing.

These data can be used as part of a weight-of-evidence approach to explain the MOA for the increased incidence of thyroid follicular cell hyperplasia observed in male Han Wistar rats in a two-year carcinogenicity study (Donald, 2012).

In a non-GLP, 14-day dietary toxicity study to investigate the dose response of bicyclopyrone and tyrosine in the blood of rats, male HsdRccHan:WIST rats were fed diets containing bicyclopyrone (purity 94.5%; batch SEZ3AP006) at concentrations of 0 (control), 0.5, 1, 2.5, 5 or 10 ppm (no feed conversion data provided) for 14 consecutive days. Clinical observations, body weights and feed consumption were measured throughout the study. At the end of the treatment period, the animals were killed and macroscopically examined. Cardiac blood samples were taken for analysis of plasma tyrosine and bicyclopyrone.

There were no deaths, clinical abnormalities or effects on body weight or feed consumption. There was a clear dose–response relationship in bicyclopyrone and tyrosine plasma concentrations relative to dietary concentration of bicyclopyrone (Table 21). For a 20-fold increase in dose from 0.5 ppm to 10 ppm, plasma concentration of bicyclopyrone increased 11.4-fold and of tyrosine increased 9.7-fold (Pinto, 2007).

Table 21. Plasma concentrations of bicyclopyrone and tyrosine in a 14-day dietary toxicity study in rats

	Plasma concentrations per dietary concentration of bicyclopyrone					
Mean plasma concentrations	0 ppm	0.5 ppm	1 ppm	2.5 ppm	5 ppm	10 ppm
Bicyclopyrone (ng/mL)	< 5	17.5	12.9	72.5	108	200
Tyrosine (nmol/mL)	134	295	306	655	930	1697

ppm: parts per million

Source: Pinto (2007)

A 28-day dietary toxicity study in rats was performed to investigate the effects of R243604 and bicyclopyrone (both triketone herbicides) when fed to rats. Groups of Alpk:APfSD (Wistar-derived) rats ($n = 5$/sex per dose) were fed diets containing R243604 or bicyclopyrone (purity 98%; batch no. ZNB0354/05) at 50, 500, 2000 or 5000 ppm for 28/29 consecutive days. Clinical observations, body weights and feed consumption were measured throughout the study. Ophthalmoscopy was conducted pre-study and prior to scheduled kill. Urine samples were collected on day 1 and during week 4 for NMR analysis. Cardiac blood samples were taken to analyse plasma tyrosine and plasma triketone levels. At scheduled necropsy, selected organs were weighed and specified tissues collected. Samples of liver were collected to evaluate enzyme activity (HPPD, TAT and CYP [for bicyclopyrone only]). The livers were also examined histopathologically. The results are presented in Table 22.

Table 22. Comparison of effects of the triketones R243604 and bicyclopyrone in a 28-day dietary toxicity study in rats

Parameter	R243604	Bicyclopyrone
Mortalities	There were no deaths (rats at 5000 ppm were euthanized after 8 days of treatment due to the marked effect on body weight).	There were no deaths.
Clinical observations	Eye opacities were seen in some animals of both sexes at 50 or 500 ppm. There were no other compound-related clinical changes.	Eye opacities were seen in some animals of both sexes at 50, 500, 2000 or 5000 ppm. There were no other compound-related clinical changes.
Body weights	Body weights in males at 5000 ppm were markedly lower than in controls after 8 days treatment, and the animals were euthanized. Body weights in males at 500 and 2000 ppm were lower than in controls at study end. No effects were seen in males at 50 ppm or in any treated female group.	Body weights in males at 5000 ppm were lower than in controls at study end. No effects were seen in males at lower dose levels or in any treated female group.
Feed consumption	Feed consumption was slightly lower in both sexes at 500, 2000 and 5000 ppm than in controls. There was no effect at 50 ppm.	Feed consumption was slightly lower in males at 5000 ppm than in controls. There were no effects in other male groups or in female groups.
Ophthalmoscopy	Effects in eyes were seen in some animals of both sexes at 500 and 2000 ppm and in 2 females at 50 ppm. Animals at 5000 ppm were not examined.	Effects in eyes were seen in some animals of both sexes at 500, 2000 and 5000 ppm and in one male at 50 ppm.
Organ weights	Liver and spleen weights were lower in both sexes than in controls, and kidney weights were lower in females only at 2000 ppm. There were no other organ weight changes in treatment groups of either sex.	Epididymis and liver weights were lower s in males at 5000 ppm than in controls. There were no other organ weight changes in treatment groups of either sex.
Macroscopic findings	There was an increased incidence in eye opacities in all treatment groups.	There was an increased incidence in eye opacities in all treatment groups.

(*continued on next page*)

Parameter	R243604	Bicyclopyrone
Microscopic findings	Glycogen depletion in the liver was seen in some males and females at 5000 ppm and in some females at 500 and 2000 ppm. Macroscopically abnormal eyes showed the presence of keratitis. There were no other compound-related effects.	Glycogen depletion in the liver was seen in some males and females at 5000 ppm and in some females at 50, 500 and 2000 ppm. Macroscopically abnormal eyes showed the presence of keratitis. There were no other compound-related effects.
Phenolic acids (NMR analysis)	Phenolic acids were present in the urines at all dose levels. There was no evidence of a dose–response relationship. Predominance of HPPA or HPLA was not observed.	Phenolic acids were present in urine at all dose levels. There was no evidence of a dose–response relationship except in females at 50 ppm, where the secretion of phenolic acids was reduced compared to other female treatment groups. Predominance of HPPA or HPLA was not observed.
Plasma triketones	There was a non-linear, dose-related increase in both sexes.	There was a non-linear, dose-related increase in both sexes.
Plasma tyrosine	There was a slight non-linear, dose-related increase in both sexes but the effect was greater in males than females.	There was a slight non-linear, dose-related increase in both sexes but the effect was greater in males than females.
Liver HPPD activity	Liver HPPD activity was undetectable in males and minimal in females at 5000 ppm. In all other treatment groups, activity was minimal in both sexes.	Liver HPPD activity was reduced to minimal levels in all treatment groups in both sexes.
Liver TAT activity	In animals of both sexes, liver TAT activity was only slightly increased in all treatment groups.	In animals of both sexes, liver TAT activity was only slightly increased in all treatment groups. Liver CYP activity was assessed in animals given bicyclopyrone only. Both males and females at 2000 or 5000 ppm showed a minimal to slight induction of CYP2B and CYP3A isoenzymes. In males at all dose levels, there was a slight induction of microsomal UDPGT.

CYP: cytochrome P450; HPPA: 4-hydroxyphenylpyruvate; HPLA: 4-hydroxyphenyllactate; HPPD: 4-hydroxyphenylpyruvate dioxygenase; NMR: nuclear magnetic resonance; ppm: parts per million; R243604: 2-[2-chloro-3-ethoxy-4-(methylsulfonyl)benzoyl]-5-methylcyclohexane-1,3-dione; TAT: tyrosine aminotransferase; UDPGT: uridine diphosphoglucuronosyltransferase

Source: Botham, Stevens & Williams (2015)

Systemic toxicity, clinical signs of toxicity, body weights and feed consumption were greater in animals given R243604, to the extent that animals at 5000 ppm of this compound were euthanized after 8 days treatment. The level of R243604 in the plasma was markedly higher than that of bicyclopyrone. Effects considered to be due the tyrosinaemia induced by this group of compounds were generally similar, although effects on some parameters were slightly more marked in animals given R243604. CYP analysis indicated that bicyclopyrone had only a minimal to slight effect as a P450 enzyme inducer (Botham, Stevens & Williams, 2015).

Comparison of results for mice and rats

The administration of R243604 in the diet, for 28 days, completely inhibited the enzyme HPPD in both male and female rats at doses above 100 ppm. In comparison, HPPD was not completely inhibited in the male mouse at a lower level (10 ppm) of dietary R243604. The administration of bicyclopyrone in the diet, for 28 days, completely inhibited the enzyme HPPD in both male and female rats at doses above 100 ppm. At a lower level of dietary bicyclopyrone (10 ppm), HPPD was not completely inhibited.

(e) MOA studies and relevance to human risk assessment

Bicyclopyrone is an inhibitor of the enzyme HPPD in both plants and animals. In plants, this MOA results in the inhibition of carotenoid biosynthesis. In animals, HPPD is the second enzyme in the catabolic pathway of tyrosine. The action of TAT, the first enzyme in the tyrosine catabolic pathway, is reversible. Any inhibition of HPPD results in the excretion of HPPA and its metabolites in the urine and a build-up of the substrate of TAT, tyrosine.

Details on the MOA and possible human relevance are described in Appendix 1.

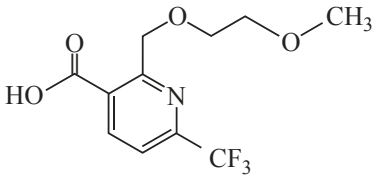
Of the toxicities observed after administration of bicyclopyrone to test animals, the ocular effect (corneal opacity) was highly correlated with the elevated blood tyrosine levels. Other toxicities may be associated with chemically induced tyrosinaemia, but other mechanisms may also be involved. However, based on the absence of such effects in other test animals and humans, it could be argued that the perturbations of the thyroid noted in male rats at bicyclopyrone concentrations of 500 ppm and above is possibly of limited or no relevance to human risk assessment.

A full human relevance MOA for bicyclopyrone according to the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI/HESI) framework was not available. Based on the available database it should be considered that bicyclopyrone could be able to inhibit human HPPD and increase plasma tyrosine concentrations, but since humans have a significantly higher TAT activity than rats, humans are unlikely to exhibit the toxicities seen in rats. However, some ocular effects were seen in dogs, and no information is available on TAT activity in dogs.

(f) Studies on metabolites or impurities

A number of bicyclopyrone metabolites were noted at levels above 10% total radioactive residue (TRR) or 0.01 mg/kg in crops and livestock, including the structurally related metabolites determined to be SYN503780 (Fig. 4, Table 22) and CSCD686480.

Figure 4. Structure of SYN503780



SYN503780 is also an intermediate used in the manufacture of bicyclopyrone. Only a limited amount of toxicology data on SYN503780 are available. The studies available are screening studies used for determining potential handling hazards and are not full guideline studies. However, the data do suggest that SYN503780 would not be acutely toxic by the oral and dermal routes, is not sensitizing and would not be a mutagen (Table 23).

Table 23. Summary of acute toxicity and genotoxicity studies with SYN503780

Test	Test species	Guideline	Result	Reference
Oral toxicity	Rat	Not applicable Screening study, 2 animals/sex	LD_{50} > 2000 mg/kg bw	Pooles (2009a)
Dermal toxicity	Rat	Not applicable Screening study, 2 animals/sex	LD_{50} > 2000 mg/kg bw No sign of dermal irritation	Pooles (2009b)

(*continued on next page*)

Test	Test species	Guideline	Result	Reference
LLNA	Mouse	Not applicable Screening study with 1 concentration only (50% w/w), 4 animals/group	No sign of dermal sensitization	Pooles (2009c)
Ames test	*Salmonella typhimurium*	Not applicable Screening study 2 bacterial strains only (TA100 and TA98)	Not mutagenic	Thompson & Bowles (2009)

bw: body weight; LD_{50}: median lethal dose; LLNA: local lymph node assay; w/w: weight per weight

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Bicyclopyrone was initially manufactured and formulated in 2014 at the Syngenta site in Münchwilen, Switzerland, with approximately 20 tonnes of technical active ingredient being produced. Full-scale production of the technical material moved to the Syngenta site at Monthey, Switzerland, in 2014. The main formulation types are liquid emulsion concentrate, soluble concentrate and ZC (capsule suspension and suspension concentrate) formulations. The majority of the formulation, fill and pack activities take place at the Syngenta site in St. Gabriel, United States of America, with small volumes handled at the San Louis Potosi, Mexico, site. Additional handling is carried out by third-party companies located in the USA and Australia.

Since 2002 Syngenta's occupational health group has maintained a database of incidents involving chemical exposure of workers. A query in September 2016 for bicyclopyrone resulted in zero records of adverse health effects reported from the handling of bicyclopyrone during synthesis or formulation activities. Medical surveillance of manufacturing plant personnel and monitoring studies

3.2 Reports of adverse effects from product end users

Syngenta has kept detailed records of exposure and poisoning incidences on marketed products for many years. Incident data in Syngenta are collected in two different databases. Reports on cases reported in the USA and Canada are collected in the PROSAR database; all other cases are reported in the Adverse Health Incident Database (AHI-DB). There have been no reported incidences of adverse effects following the use of products containing bicyclopyrone.

Comments

Biochemical aspects

In metabolism studies conducted in rats, bicyclopyrone was rapidly absorbed (>80%). T_{max} in blood and plasma were 1–2 hours at the low and high doses (2 and 200 mg/kg bw, respectively) and 2–6 hours in tissues. Independent of dose and route (oral or intravenous) of administration, radioactivity declined rapidly in a biphasic pattern. The half-lives of the first phase were 1–3 hours in blood and plasma. The majority of administered radioactivity was excreted in the urine within 24 hours (>63%) and excretion was nearly complete by seven days after a single dose (98–99%). There was no evidence of bioaccumulation following repeated dosing. Tissue distribution was independent of sex, dose or route of administration. The highest levels of radioactive residues were found in the liver and kidney (up to 4% and 0.4%, respectively). Absorption, pharmacokinetics and total elimination were independent of sex, dose or route of administration. However, males tended to have slightly higher biliary and faecal elimination compared to females (Hurst, 2009, 2010; Hurst & Stow, 2009, 2010; Kendrick & Goodwin, 2010).

The levels of radioactivity in the liver following administration of the 200 mg/kg bw dose were only approximately three times higher than those following administration of the 2 mg/kg bw dose, despite the 100-fold increase in dose.

Bicyclopyrone is not extensively metabolized, with unchanged parent being the principal radioactive component independent of dose or route. The principal routes of biotransformation were via oxidative phase I reactions, namely hydroxylation and *O*-demethylation. Minor routes involved glycine conjugation and cleavage between the pyridinyl and bicyclo rings (each accounting for less than 0.5% of the dose). A quantitative sex difference was apparent in the metabolism of bicyclopyrone; males transformed a higher proportion of parent compound into metabolites than did females. The major component present in the liver was the parent compound (Hardwick & Swalwell, 2012).

Toxicological data

The oral and dermal median lethal dose (LD_{50}) for bicyclopyrone in rats was greater than 5000 mg/kg bw (Arcelin, 2007a,b). The inhalation median lethal concentration (LC_{50}) was greater than 5.21 mg/L in rats (Decker, 2008). Bicyclopyrone caused no skin irritation and slight eye irritation in rabbits (Arcelin, 2007c,d). It caused no sensitization in the mouse local lymph node assay (LLNA) (Pooles, 2008).

Bicyclopyrone inhibits the liver enzyme HPPD, which is involved in the catabolism of tyrosine. The observed ocular effects reported in experimental animals (corneal opacity, keratitis, absent pupillary reflex) are highly correlated with the elevated blood tyrosine levels (tyrosinaemia). Other developmental, thyroid and liver effects may be associated with chemically induced tyrosinaemia, although other mechanisms may also be involved. Severe ocular effects were seen in rats as early as 4 weeks after administration of bicyclopyrone; in dogs, the ocular effects were less severe and seen only after 13 weeks at higher dose levels. No ocular effects were observed in mice. This species-specific sensitivity for ocular opacity and keratitis is related to differences in tyrosine clearance. A metabolic pathway to remove tyrosine from the blood involves the liver enzyme TAT. In contrast to rats, mice and humans are unlikely to achieve the levels of plasma tyrosine necessary to produce ocular opacities because murine and human TAT activity is much greater than in rats. Although no data on TAT activity in dogs and rabbits are available, since the ocular effects in dogs were far less severe than in rats and only occur at higher dose levels and after prolonged elevated tyrosine levels, it can be assumed that dogs also have a more efficient metabolic process for handling excess tyrosine than do rats.

In a 90-day oral toxicity study, mice were administered bicyclopyrone in the diet at 0, 100, 3500 or 7000 ppm (equal to 0, 15.4, 543 and 1130 mg/kg bw per day for males and 0, 20.8, 809 and 1340 mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 15.4 mg/kg bw per day) based on increased liver weights at 3500 ppm (equal to 543 mg/kg bw per day) (Shearer & Wood, 2009).

In a 90-day oral toxicity study, rats were administered bicyclopyrone in the diet at 0, 500, 2000 or 5000 ppm (equal to 0, 51.2, 208, 503 [analytical grade bicyclopyrone] and 518 mg/kg bw per day [technical grade bicyclopyrone] for males and 0, 50.5, 202, 495 [analytical grade bicyclopyrone] and 500 mg/kg bw per day [technical grade bicyclopyrone] for females, respectively). No NOAEL could be identified as ocular toxicity (opacity and keratitis) was observed in males and females at 500 ppm (equal to 50.5 mg/kg bw per day) (Rattray, 2003).

In another 90-day oral toxicity study, rats were administered bicyclopyrone in the diet at 0, 2.5, 10, 2500 or 5000 ppm (equal to 0, 0.18, 0.72, 183 and 363 mg/kg bw per day for males and 0, 0.22, 0.88, 229 and 442 mg/kg bw per day for females, respectively). The NOAEL was 10 ppm (equal to 0.72 mg/kg bw per day) based on ocular toxicity (opacities, absent pupillary reflex, keratitis) at 2500 ppm (equal to 183 mg/kg bw per day) (Shearer & Robertson, 2009).

In a 90-day oral toxicity study, dogs were administered bicyclopyrone at 0, 5, 25 or 125 mg/kg bw per day by oral capsule. The NOAEL was 125 mg/kg bw per day, the highest dose tested. Macroscopic and microscopic examinations found no changes in neurological tissues (Jackson, 2009).

In a one-year oral toxicity study, dogs were administered bicyclopyrone at 0, 2.5, 25 or 125 mg/kg bw per day by oral capsule. Persistent corneal opacity at 25 and 125 mg/kg bw per day was reported from week 13 onwards. Dorsal ganglia chromatolysis and swelling of some neurons was noted at all dose levels without a clear dose–response effect. In addition, degeneration of sciatic nerve and spinal nerve roots was observed in slightly increased incidences in treated animals compared to controls. The relevance of these findings in the absence of any clinical neurotoxicity signs is unknown. As these minimal neurological effects could potentially be treatment related, the LOAEL was 2.5 mg/kg bw per day, the lowest dose tested (Braun, 2010).

In an 80-week carcinogenicity study in mice, bicyclopyrone was administered in the diet at 0, 70, 1700 or 7000 ppm (equal to 0, 8.7, 233 and 940 mg/kg bw per day for males and 0, 9.2, 242 and 1027 mg/kg bw per day for females, respectively). The NOAEL for bicyclopyrone was 1700 ppm (equal to 233 mg/kg bw per day) based on decreases in body weight and body weight gain and less efficient feed utilization in males and females treated at 7000 ppm (equal to 940 mg/kg bw per day). There were no tumours considered to be related to treatment with bicyclopyrone (Robertson & Perry, 2012a).

In a 104-week combined chronic toxicity and carcinogenicity study in rats, bicyclopyrone was administered in the diet at 0, 5, 500, 2500 or 5000 ppm (equal to 0, 0.28, 28.4, 141 and 280 mg/kg bw per day for males and 0, 0.35, 35.8, 178 and 368 mg/kg bw per day for females, respectively, in the carcinogenicity part of the study; the doses in the chronic toxicity study were slightly higher). At 500 ppm (equal to 28.4 mg/kg bw per day) and above, ocular alterations (opacity, keratitis and regenerative hyperplasia of the cornea in males and females, and squamous cell carcinoma and papilloma of the cornea in males only) and focal follicular cell hyperplasia of the thyroid gland in males were observed. No NOAEL could be identified as increased incidences of thyroid hyperplasia were observed after two years at the lowest dose, 5 ppm (equal to 0.28 mg/kg bw per day). The NOAEL for carcinogenicity was 5 ppm (equal to 0.28 mg/kg bw per day) based on increased incidences of squamous cell carcinoma and papilloma of the cornea in males only at 500 ppm (equal to 28.4 mg/kg bw per day) and above (Robertson & Perry, 2012b).

Several mechanistic studies indicated that bicyclopyrone did not inhibit rat thyroid peroxidase activity in vitro (Lake, 2012). However, in vivo bicyclopyrone administration in rats resulted in increased levels of tyrosine, decreased T_3 and T_4, increased thyroid follicular cell hypertrophy, increased liver weight, increased hepatocellular centrilobular hypertrophy and increased hepatic UDPGT activity (Donald, 2012). Moreover, dietary treatment of rats for 14 days resulted in a clear dose-dependent increase in both bicyclopyrone and tyrosine plasma concentrations. When the bicyclopyrone dose in rats was increased 20-fold, from 0.5 ppm to 10 ppm, the achieved plasma concentration of bicyclopyrone and tyrosine increased 11.4-fold and 9.7-fold, respectively (Pinto, 2007).

The thyroid effects were observed mainly in male rats and to a lesser extent in female rats; male rats have higher tyrosine levels than female rats and male and female mice, suggesting a relationship between thyroid effects and tyrosine plasma concentrations. The relevance of these effects for humans cannot be excluded; however, since the thyroid effects were not observed in mice or dogs, and humans have a more efficient tyrosine clearance mechanism than do rats, humans are unlikely to reach tyrosine levels at which these thyroid effects in rats were observed.

The Meeting concluded that bicyclopyrone is carcinogenic in rats, but not in mice.

Bicyclopyrone did not induce gene mutations in bacteria or mammalian cells in vitro (Booth, 2006; Callander, 2007; Sokolowski, 2010); nor was it clastogenic in human lymphocytes in vitro (Fox, 2006). It was not clastogenic and did not induce DNA repair in vivo (Clay, 2007; Honarvar, 2008). Bicyclopyrone 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 bicyclopyrone is unlikely to be genotoxic.

Ocular tumours have been seen with some but not all HPPD inhibitors, but a progression from corneal damage/repair to ocular tumours has not been demonstrated. Inhibition of HPPD and increase of plasma tyrosine concentrations, as demonstrated for rats and mice in mechanistic studies (Noakes, 2007; Botham, Stevens & Williams, 2015), could potentially occur in humans. The tumours were only observed in male rats, which have higher plasma tyrosine levels than have female rats and male and female mice, suggesting a relation with tyrosine plasma concentrations. Although the human relevance of these ocular tumours cannot be excluded, the plasma tyrosine levels associated with ocular tumours in rats are unlikely to be achieved in humans because of significantly higher TAT activity.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that squamous cell carcinoma and papilloma of the cornea in male rats only were observed at high tyrosine levels unlikely to occur in humans, the Meeting concluded that bicyclopyrone is unlikely to pose a carcinogenic risk to humans from low levels in the diet.

In a two-generation reproductive toxicity study, bicyclopyrone was administered to rats in the diet

at 0, 25, 500 or 5000 ppm (equal to 0, 1.9, 38.4 and 377 mg/kg bw per day for males and 0, 2.1, 42.2 and 410 mg/kg bw per day for females, respectively). No NOAEL for parental toxicity could be identified as effects on eyes, liver, thyroid and kidney occurred at the lowest dose tested, 25 ppm (equal to 1.9 mg/kg bw per day). The NOAEL for offspring toxicity was 25 ppm (equal to 1.9 mg/kg bw per day) based on effects on eyes, liver, thyroid and kidney at 500 ppm (equal to 38.4 mg/kg bw per day). The NOAEL for reproductive toxicity was 5000 ppm (equal to 377 mg/kg bw per day), the highest dose tested (Davies & Penn, 2012).

In a developmental toxicity study in rats, bicyclopyrone was administered by oral gavage at 0, 100, 500 or 1000 mg/kg bw per day on gestation days 6–20. No NOAEL could be identified for maternal toxicity as transiently reduced lower maternal body weights and body weight gains were observed at 100 mg/kg bw per day, the lowest dose tested. No NOAEL could be identified for embryo/fetal toxicity as skeletal effects consisting of increases in caudal displacement of the pelvic girdle (27 pre-pelvic vertebrae), supernumerary ribs, ossification delays and cartilage changes were observed at 100 mg/kg bw per day, the lowest dose tested. No increases in malformations were observed (Gerspach, 2011b).

In a developmental toxicity study in New Zealand White rabbits, bicyclopyrone was administered orally by gavage from gestation days 7 through 28 at 0, 10, 50 or 200 mg/kg bw per day. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on excessive maternal toxicity (mortality, moribundity, abortion, decreased body weight and reduced feed consumption) at the LOAEL of 200 mg/kg bw per day. No NOAEL could be identified for embryo/fetal toxicity as two specific skeletal variations (13th [extra] rib and 27 pre-pelvic vertebrae) were observed at 10 mg/kg bw per day, the lowest dose tested (Sawhney Coder, 2012c).

In a developmental toxicity study in Himalayan rabbits, bicyclopyrone was administered orally by gavage at 0, 10, 50 or 250 mg/kg bw per day. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on macroscopic findings of irritation in the stomach wall, a possible local effect, at the LOAEL of 250 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day based on two specific skeletal variations: costal cartilage asymmetrically aligned at the sternum and 27 pre-pelvic vertebrae, and slight increases in several other skeletal abnormalities at the LOAEL of 50 mg/kg bw per day. At 250 mg/kg bw per day, increases in visceral and skeletal malformations were observed, including heart muscular interventricular septal defects and several cervical vertebral irregularities (e.g. absence, misshapen, fused, supernumerary) (Whitlow, 2012a).

In another developmental toxicity study in Himalayan rabbits, bicyclopyrone was administered orally by gavage at 0, 1, 10 or 250 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day based on mortality, decreased body weight gain and signs of stomach irritation at the LOAEL of 250 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1 mg/kg bw per day based on increases in supernumerary ribs and costal cartilage variations at the LOAEL of 10 mg/kg bw per day. At 250 mg/kg bw per day, increases in external, visceral and skeletal malformations were observed, including jaw and/or palate cleft, heart muscular and/or perimembraneous interventricular septal defects and several cervical vertebral irregularities (e.g. absence, misshapen, fused) (Whitlow, 2012c).

For rabbits, an overall maternal NOAEL of 50 mg/kg bw per day was identified based on maternal toxicity, including mortality, at 200 mg/kg bw per day.

The Meeting concluded that bicyclopyrone is teratogenic in rabbits but not in rats.

In an acute neurotoxicity study in rats, bicyclopyrone was administered orally by gavage at 0, 20, 200 or 2000 mg/kg bw. The NOAEL was 2000 mg/kg bw, the highest dose tested (Beck, 2012a).

In a 90-day dietary neurotoxicity study, rats were fed bicyclopyrone at 0, 50, 500 or 5000 ppm (equal to 0, 4, 35 and 336 mg/kg bw per day for males and 0, 4, 42 and 415 mg/kg bw per day for females, respectively). No NOAEL for systemic toxicity could be identified; the LOAEL was 50 ppm (equal to 4 mg/kg bw per day) based on the increased incidence of ocular findings at all dose levels. The NOAEL for neurotoxicity was 5000 ppm (equal to 336 mg/kg bw per day), the highest dose tested (Beck, 2012c).

The Meeting concluded that bicyclopyrone is not neurotoxic in rats, but could not exclude the possibility of weak neurotoxicity in the dog.

In a 28-day immunotoxicity study in female mice, bicyclopyrone was administered in the diet

at 0, 50, 500 or 5000 ppm (equal to 0, 10.6, 107 and 1190 mg/kg bw per day). No effects were observed in spleen or thymus weights, spleen cell counts or splenic IgM antibody-forming cells. The NOAEL for immunotoxicity was 5000 ppm (equal to 1192 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 500 ppm (equal to 107 mg/kg bw per day) based on increased liver weights at the LOAEL of 5000 ppm (equal to 1190 mg/kg bw per day) (Eapen, 2012).

The Meeting concluded that bicyclopyrone is not immunotoxic.

Toxicological data on metabolites and/or degradates

Some acute toxicity screening data available for SYN503780 indicated that it would not be acutely toxic by the oral and dermal routes, was not sensitizing, and was not mutagenic in a screening Ames test (Pooles, 2009a,b,c; Thompson & Bowles, 2009). No toxicological data were available on any other metabolites of bicyclopyrone.

Following application of bicyclopyrone to crops (maize, soya bean and sugar cane), a large number of structurally similar metabolites were detected. The majority of these metabolites were either desmethyl dihydroxylated bicyclopyrone isomers or desmethyl monohydroxylated bicyclopyrone isomers (free and glycoside conjugated forms).

The Meeting noted that the majority of the metabolites are structurally related to bicyclopyrone and fall into one of two groups. The first group comprises compounds that can be hydrolysed to SYN503780; the second group comprises compounds that can be hydrolysed to CSCD686480. All crop fractions also contained numerous other metabolites, all individually less than 10% total radioactive residue (TRR), which would also belong to either of these two groups. Common moiety methods are available to cover these two groups of metabolites.

Based on screening toxicity tests on SYN503780, the detection of several of the quantified metabolites at relatively high levels in the rat and the structural similarities between all the metabolites, the Meeting concluded that these structurally related compounds are unlikely to be more toxic than bicyclopyrone.

The Meeting concluded that the acceptable daily intake (ADI) and acute reference dose (ARfD) of bicyclopyrone cover all the structurally related metabolites of SYN503780 and CSCD686480 described above.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisoning in humans is available.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.003 mg/kg bw for bicyclopyrone on the basis of a LOAEL of 0.28 mg/kg bw per day for thyroid hyperplasia in the two-year carcinogenicity study in rats, and using a safety factor of 100 ($4 \times 0.83 \times 10 \times 3$).

The Meeting considered that the MOA of bicyclopyrone, inhibition of the liver enzyme HPPD leading to impaired clearance of tyrosine, is relevant to humans. However, humans are less sensitive than rats due to more efficient tyrosine clearance by the liver TAT enzyme. The thyroid effect seen in the two-year rat study is therefore considered less relevant to human risk assessment, and a safety factor for interspecies variation lower than the usual one of 10 (4×2.5 for kinetic and dynamic factors, respectively) might be used.

Based on the available data and information indicating at least three-fold higher activity of the human TAT compared to rat TAT (Henderson et al., 1981; Botham, Stevens & Williams, 2015), a factor of 2.5/3 = 0.83 could be proposed for toxicodynamic interspecies differences. The resulting interspecies

safety factor would be 4 × 0.83 = 3.3. The intraspecies safety factor would remain at 10, which results in a safety factor of 33.

To convert from a LOAEL to a NOAEL an additional safety factor is needed. There was only a slight dose–response in thyroid hyperplasia without an increase in severity and at the low dose without any other thyroid effects such as weight changes or concomitant hypertrophy. Therefore, the Meeting considered that a factor of 3 was adequate and that the total safety factor to be applied to the LOAEL would be 33 × 3 = 100, resulting in the ADI of 0.003 mg/kg bw per day.

This ADI provides a margin of exposure of nearly 10 000 to the ocular tumours in male rats.

This ADI was supported by the LOAEL of 2.5 mg/kg bw per day in the one-year toxicity study in dogs, based on minimal possible neurological effects.

The Meeting established an ARfD of 0.01 mg/kg bw for bicyclopyrone for women of childbearing age on the basis of the overall NOAEL of 1 mg/kg bw per day for skeletal variations in rabbit fetuses, and using a safety factor of 100.

The Meeting concluded that it was not necessary to establish an ARfD for bicyclopyrone for the remainder of the population in view of its low acute oral toxicity and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

The Meeting concluded that the ADI and ARfD for bicyclopyrone could be applied to all structurally related metabolites of SYN503780 and CSCD686480, expressed as bicyclopyrone.

Levels relevant to risk assessment of bicyclopyrone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighty-week study of carcinogenicity [a]	Toxicity	1700 ppm, equal to 233 mg/kg bw per day	7000 ppm, equal to 940 mg/kg bw per day
		Carcinogenicity	7000 ppm, equal to 940 mg/kg bw per day [b]	–
Rat	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	–	5 ppm, equal to 0.28 mg/kg bw per day [c]
		Carcinogenicity	5 ppm, equal to 0.28 mg/kg bw per day	500 ppm, equal to 28.4 mg/kg bw per day
	Two-generation studies of reproductive toxicity [a,d]	Reproductive toxicity	5000 ppm, equal to 377 mg/kg bw per day [b]	–
		Parental toxicity	–	25 ppm, equal to 1.9 mg/kg bw per day [c]
		Offspring toxicity	25 ppm, equal to 1.9 mg/kg bw per day	500 ppm, equal to 38.4 mg/kg bw per day
	Developmental toxicity study [d,e]	Maternal toxicity	–	100 mg/kg bw per day [c]
		Embryo/fetal toxicity	–	100 mg/kg bw per day [c]
Rabbit	Developmental toxicity studies [d,e]	Maternal toxicity	50 mg/kg bw per day	200 mg/kg bw per day
		Embryo/fetal toxicity	1 mg/kg bw per day	10 mg/kg bw per day
Dog	One-year study of toxicity [f]	Toxicity	–	2.5 mg/kg bw per day [c]

[a] Dietary administration. [b] Highest dose tested. [c] Lowest dose tested. [d] Two or more studies combined.
[e] Gavage administration. [f] Capsule administration.

Estimate of acceptable daily intake (ADI) applies to bicyclopyrone and all structurally related metabolites of SYN503780 and CSCD686480, expressed as bicyclopyrone

0–0.003 mg/kg bw

Estimate of acute reference dose (*ARfD*) *applies to bicyclopyrone and all structurally related metabolites of SYN503780 and CSCD686480*, *expressed as bicyclopyrone*

0.01 mg/kg bw (women of childbearing age)

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; information on MOA and human relevance of thyroid effects; information on TAT activity of dogs and rabbits compared to that of rats and humans

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; extensive (> 80%)
Dermal absorption	No data
Distribution	Highest residues in liver and kidneys
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Largely complete within 24 hours; primarily via urine
Metabolism in animals	Mostly excreted unchanged; slight sex difference with males forming more metabolites
Toxicologically significant compounds in animals and plants	Bicyclopyrone and metabolites structurally related to SYN503780 and CSCD686480
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.21 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Slightly irritating
Mouse, dermal sensitization	Non-sensitizing (LLNA)
Short-term studies of toxicity	
Target/critical effect	Eye
Lowest relevant oral NOAEL	0.72 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	50 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of carcinogenicity	
Target/critical effect	Thyroid
Lowest relevant oral NOAEL	< 0.28 mg/kg bw per day (rat)
Carcinogenicity	Ocular tumours in rats at 28.4 mg/kg bw per day, possibly not relevant to humans[a] Not carcinogenic in mice[a]
Genotoxicity	No evidence of genotoxicity in vitro or in vivo[a]
Reproductive toxicity	
Target/critical effect	Eye, liver, thyroid, kidney
Lowest relevant parental NOAEL	< 1.9 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	1.9 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	377 mg/kg bw per day (rat)[b]

Developmental toxicity	
Target/critical effect	Maternal mortality, body weight, fetal skeletal abnormalities
Overall maternal NOAEL	50 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	1 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw (rat) [b]
Subchronic neurotoxicity NOAEL	336 mg/kg bw per day (rat) [b]
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	NOAEL: 1 192 mg/kg bw per day (mouse) [b]
Studies on toxicologically relevant metabolites	SYN503780: screening studies do not indicate acute toxicity (oral, dermal), skin-sensitizing or mutagenic properties
Mechanistic/mode of action studies	HPPD inhibition is plausible for humans. The ocular tumours and thyroid hyperplasia could be due to the male rat–specific high tyrosine levels; however, human relevance cannot be excluded. Humans have a significantly higher TAT activity than do male rats and are unlikely to exhibit the toxicities seen in rats
Human data	No adverse effects in manufacturing personnel

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

[b] Highest dose tested.

Summary

	Value	Study	Safety factor
ADI[a]	0–0.003 mg/kg bw	Two-year study of toxicity (rat)	100
ARfD[a,b]	0.01 mg/kg bw	Developmental studies in rabbits	100

[a] Applies to bicyclopyrone and all structurally related metabolites of SYN503780 and CSCD686480, expressed as bicyclopyrone.

[b] Applicable to women of childbearing age.

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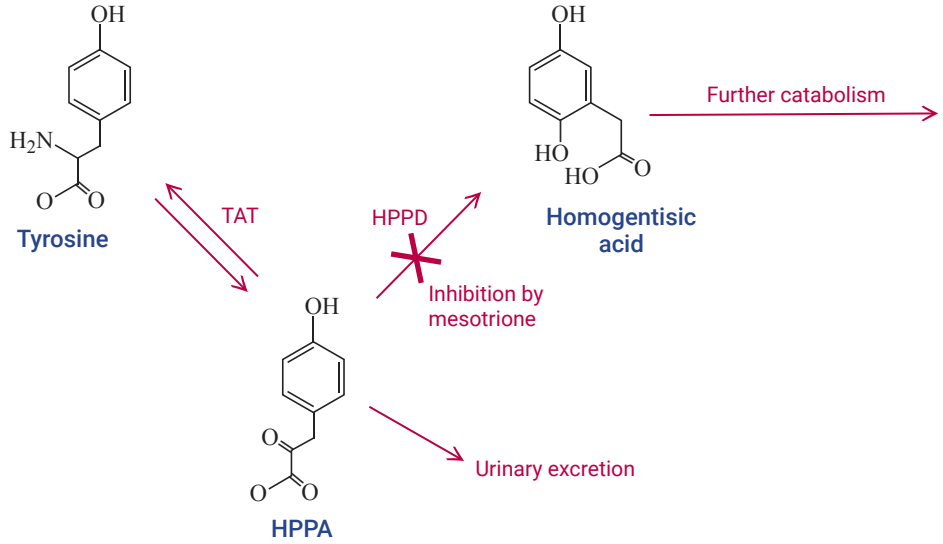
Appendix 1: Mode of action and relevance to human risk assessment

Bicyclopyrone is an inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) in both plants and animals. In plants, this mode of action (MOA) results in the inhibition of carotenoid biosynthesis; in animals, HPPD is the second enzyme in the catabolic pathway of tyrosine. The action of tyrosine aminotransferase (TAT), the first enzyme in the tyrosine catabolic pathway, is reversible, and any inhibition of HPPD results in excretion of HPPA and its metabolites in the urine and a build-up of tyrosine, the substrate of TAT (Fig. A1).

Figure. A1. Catabolic pathway of tyrosine

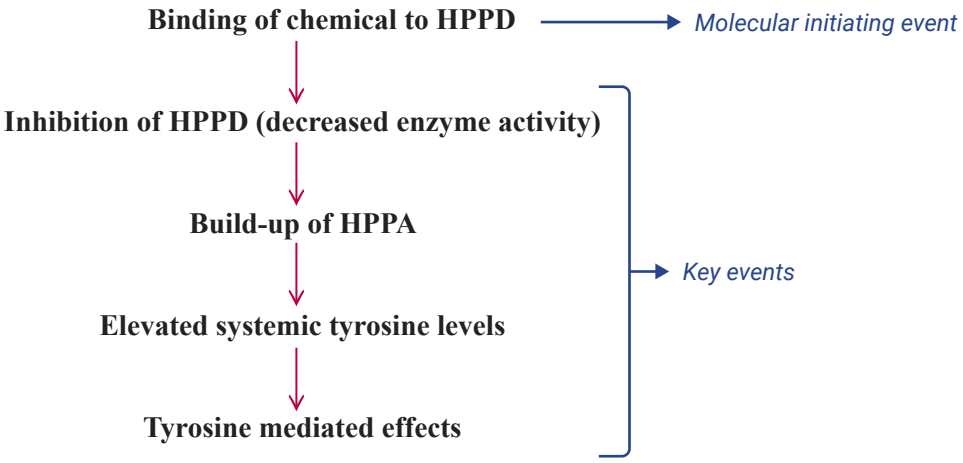


HPPA: 4-hydroxyphenylpyruvate; HPPD: 4-hydroxyphenylpyruvate dioxygenase

Source: Redrawn from Syngenta (2017)

A proposed MOA for HPPD inhibition leading to an elevation in plasma tyrosine and, in rats, associated toxicities, is shown in Fig. A2.

Figure. A2. Key events related to the proposed MOA for HPPD inhibitors



HPPA: 4-hydroxyphenylpyruvate; HPPD: 4-hydroxyphenylpyruvate dioxygenase

Source: Redrawn from Syngenta (2017)

Evidence for the effect of bicyclopyrone in the key steps in the MOA

Key event 1 – inhibition of HPPD

Bicyclopyrone has been shown to inhibit HPPD in both rats and mice. Data shown are taken from 28-day investigative studies in the rat (Botham, Stevens & Williams, 2015) and the mouse (Noakes, 2007), with both reports summarized in section 2.6 (d). At dose levels of 50, 500, 2000 or 5000 ppm of bicyclopyrone, liver HPPD activity in the rat was reduced to minimal levels in both sexes, although there was slight evidence for a dose–response relationship in females (Table A1).

Table A1. Group mean liver HPPD activity in rats after 28 days of bicyclopyrone treatment

	HPPD activity (µL oxygen/min per mg protein) per dose level of bicyclopyrone				
	0 ppm	**50 ppm**	**500 ppm**	**2000 ppm**	**5000 ppm**
Males	0.072 ± 0.034	0.011 ± 0.007	0.004 ± 0.006	0.004 ± 0.005	0.001 ± 0.003
Females	0.278 ± 0.126	0.035 ± 0.019	0.012 ± 0.004	0.008 ± 0.003	0.004 ± 0.005

HPPD: 4-hydroxyphenylpyruvate dioxygenase; ppm: parts per million

Source: Botham, Stevens & Williams (2015)

At dose levels of 10, 100, 1000 and 7000 ppm of bicyclopyrone, liver HPPD activity in the mouse was reduced in both sexes. Levels at 10 ppm were at the limit of detection for this assay. Levels at 100 ppm and above were below the limit of detection and are indistinguishable from each other. HPPD is completely inhibited at dose level of 100 ppm and above in the mouse (Table A2).

Table A2. Group mean liver HPPD activity in mice after 28 days of bicyclopyrone treatment

	HPPD activity (µL oxygen/min per mg protein) per dose level of bicyclopyrone				
	0 ppm	**10 ppm**	**100 ppm**	**1000 ppm**	**7000 ppm**
Males	0.058 ± 0.023	0.015 ± 0.013	0.008 ± 0.011	0.000 ± 0.000	0.005 ± 0.007
Females	0.176 ± 0.038	0.035 ± 0.021	0.017 ± 0.010	0.014 ± 0.013	0.013 ± 0.010

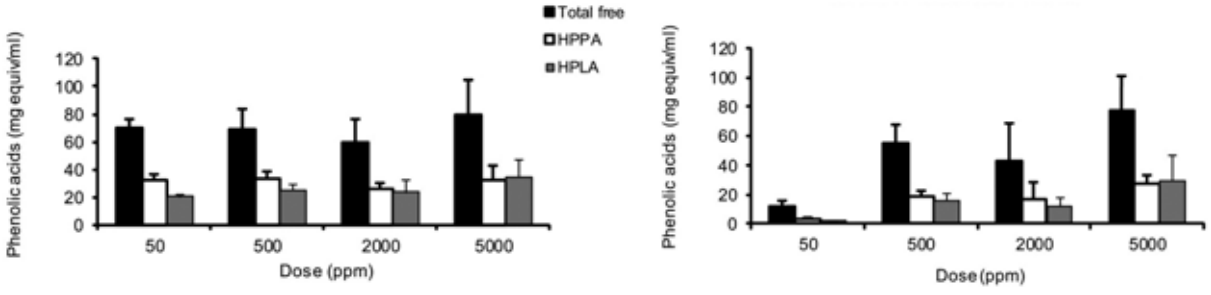
HPPD: 4-hydroxyphenylpyruvate dioxygenase; ppm: parts per million

Source: Noakes (2007)

Key event 2 – build-up of HPPA

Phenolic acids were present in the urine of rats at all dose levels following administration of bicyclopyrone (Fig. A3). These were not detected in the urine of control animals (Botham, Stevens & Williams, 2015).

Figure. A3. Phenolic acid excretion in male and female rats treated with bicyclopyrone for 28 days



HPAA: 4-hydroxyphenylacetate; HPLA: 4-hydroxyphenyllactate; HPPA: 4-hydroxyphenylpyruvate; NOA-449280: bicyclopyrone; ppm: parts per million

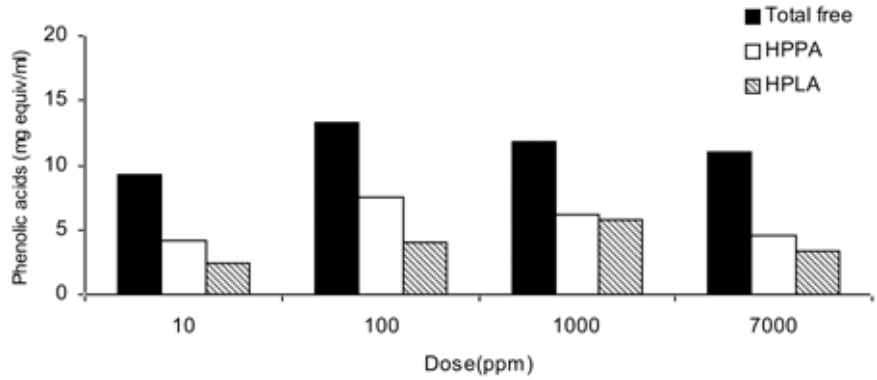
Total free = HPPA + HPLA + HPAA

Source: Botham, Stevens & Williams (2015)

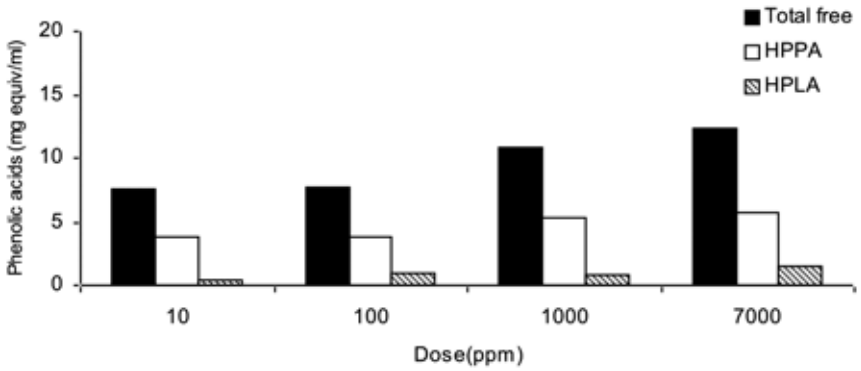
In mice, phenolic acids were also present in the urine at all dose levels following administration of bicyclopyrone; these were also not detected in the urine of control animals. Despite a certain amount of variability in the level of phenolic acid excretion, comparable levels were observed for each dosed group (Fig. A4) (Noakes, 2007).

Figure. A4. Phenolic acid excretion in the urine of male and female mice treated with bicyclopyrone

Male rats treated with NOA-449280



Male rats treated with NOA-449280



HPAA: 4-hydroxyphenylacetate; HPLA: 4-hydroxyphenyllactate; HPPA: 4-hydroxyphenylpyruvate; NOA-449280: bicyclopyrone

Total free = HPPA + HPLA + HPAA

Source: Noakes (2007)

Key event 3 – elevated systemic tyrosine levels

Administration of bicyclopyrone results in elevated concentrations of tyrosine in the plasma in both rats and mice.

Concentrations were double that of controls at doses of bicyclopyrone of 0.5 and 1 ppm in male rats but rose rapidly at higher dose levels, reaching a maximum between 10 and 50 ppm bicyclopyrone (Table A3; Fig. A5; Fig. A6). In females, maximum concentrations of tyrosine in plasma were reached at 50–500 ppm bicyclopyrone (Botham, Stevens & Williams, 2015; Noakes, 2007). The maximum concentration of tyrosine in plasma was higher in males than females, a finding characteristic of HPPD inhibitors in rats and attributable to the more efficient clearance of excess tyrosine in female rats (Lewis & Botham, 2013).

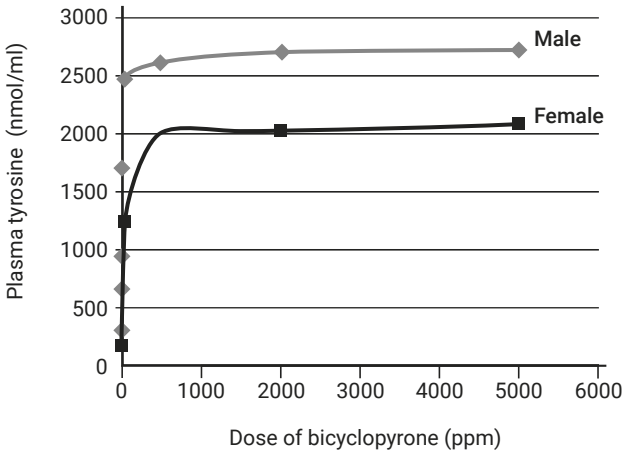
Table A.3. Plasma tyrosine concentrations in rats following administration of bicyclopyrone in diet

	Tyrosine concentrations (nmol/mL)				
	0 ppm	**50 ppm**	**500 ppm**	**2000 ppm**	**5000 ppm**
Males	185 ± 39.3	2470 ± 154	2610 ± 185	2710 ± 263	2720 ± 703
Females	147 ± 19.3	1230 ± 145	1930 ± 170	2010 ± 371	2070 ± 132

ppm: parts per million

Source: Botham, Stevens & Williams (2015)

Figure. A5. Plasma tyrosine concentrations in rats following administration of bicyclopyrone in diet



Source: Syngenta (2017)

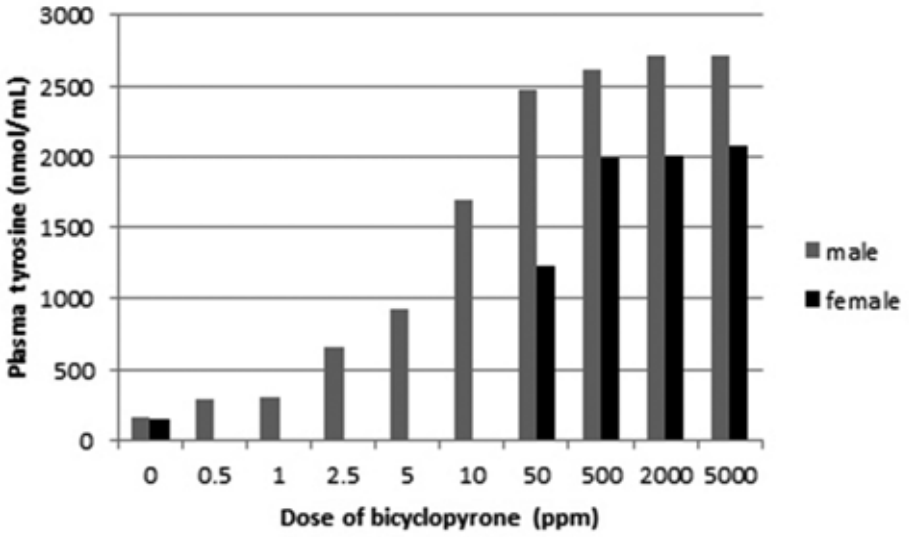
In mice, plasma tyrosine concentrations were similar in males and females following administration of bicyclopyrone but were lower than concentrations seen in rats (Table A4; Fig. A6).

Table A4. Plasma tyrosine concentrations in mice following administration of bicyclopyrone in diet

	Plasma tyrosine concentrations (nmol/mL) per dose level of bicyclopyrone				
	0 ppm	**10 ppm**	**100 ppm**	**1000 ppm**	**7000 ppm**
Males	151 ± 46	817 ± 99	879 ± 63	994 ± 141	883 ± 109
Females	145 ± 16	682 ± 57	989 ± 59	1095 ± 112	1127 ± 136

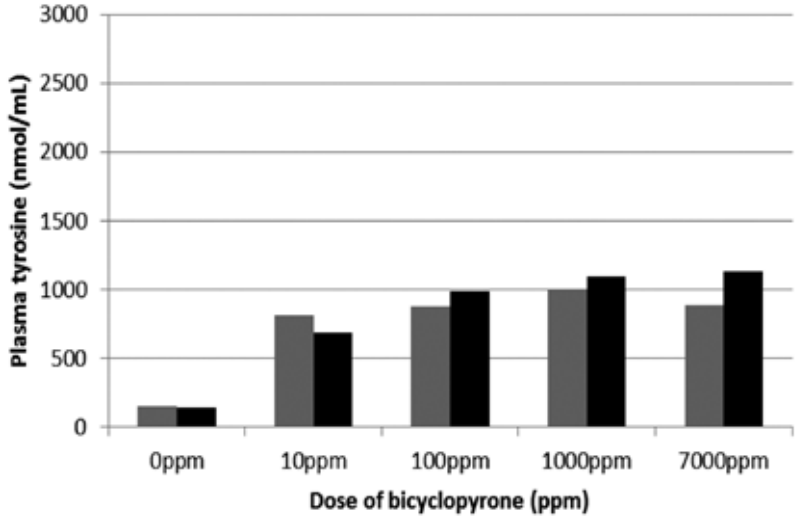
Source: Noakes (2007)

Figure. A6. Plasma tyrosine concentrations in rats following bicyclopyrone administration



Source: Pinto (2007); Botham, Stevens & Williams (2015)

Figure. A7. Plasma tyrosine concentrations in mice following bicyclopyrone administration



Source: Noakes (2007)

Key event 4 – Tyrosine mediated effects

Inhibition of HPPD has been shown to result in a different spectrum of toxicities in rats and mice as a consequence of the different degrees of tyrosinaemia seen in both species (Lewis & Botham, 2013). Bicyclopyrone shows the same species difference in key toxicities (Table A5).

Table A5. Toxicities in the rat and mouse following bicyclopyrone administration

Effect	Presence	
	Rat	Mouse
Corneal opacity	+	−
Thyroid proliferation	+	−
Glomerulonephropathy	+	−
Liver weight increase	+	+/−
Kidney weight increase	+	+/−
Body weight decrease	+	+/−
Minor modulation in the rate of normal ossification	+	ND

ND: not done

Source: Syngenta (2017)

As with other HPPD inhibitors, the most notable target organ of bicyclopyrone in the rat was the eye. Ocular opacity, keratitis and/or regenerative hyperplasia of the cornea were usually noted at dose levels of 500 ppm in repeated-dose toxicity studies in rats, but in the two-generation study, ocular effects were already observed, mainly in males, at 25 ppm (equal to 1.9 mg/kg bw per day). In males, low incidences of corneal squamous cell tumours were noted at 500 ppm in the chronic toxicity study. Bicyclopyrone at limit dose (1 g/kg bw per day) for up to 80 weeks had no effect on the eyes of mice.

Increased incidence of thyroid follicular cell hypertrophy was noted in male rats at 500 ppm and above after 52 weeks of dosing bicyclopyrone. After 104 weeks, an increased incidence of thyroid follicular cell hyperplasia was noted at the same dose levels. In contrast, no differences from control animals were noted in the thyroid of mice dosed at limit dose (1 g/kg bw per day) for up to 80 weeks. To elucidate the MOA for the observed thyroid hypertrophy/hyperplasia, a 28-day non-guideline toxicity study was performed. In this study, dietary dosing of bicyclopyrone at 500 ppm and above to male rats resulted in markedly elevated tyrosine concentrations, decreased levels of T_3 and T_4 and increased incidences of thyroid follicular cell hypertrophy (see section 2.6(d); Donald, 2012). Bicyclopyrone

does not affect thyroid peroxidase activity (Lake, 2012, see section 2.6(d)). According to the hypothesis presented here, conditions in the thyroid mimic a mild iodine deficiency in which reduced synthesis of T_3 and T_4 results in compensatory increases in thyroid function and follicular cell proliferation. The increase in thyroid follicular cell hypertrophy/hyperplasia at 500 ppm and above supports the hypothesis of mild thyroid perturbation due to elevated tyrosine, which is consistently seen upon histopathological examination only after prolonged administration of bicyclopyrone.

Human relevance

Ocular, kidney and liver effects can be observed as a result of a build-up of tyrosine in humans with tyrosinaemia, a genetic disorder characterized by disruptions in the multistep process that breaks down tyrosine.

There are three types of tyrosinaemia: type I, the most severe form of this disorder, is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase. It is characterized by signs and symptoms that begin in the first few months of life. Tyrosinaemia type I can lead to liver and kidney failure, softening and weakening of the bones (rickets) and increased risk of liver cancer (hepatocellular carcinoma). Tyrosinaemia type II, caused by an enzymatic deficit in TAT, can affect the eyes, skin and mental development. Signs and symptoms often begin in early childhood. About 50% of individuals with tyrosinaemia type II have some degree of intellectual disability. Tyrosinaemia type III is the rarest of the three types. The characteristic features of this type include intellectual disability, seizures and periodic loss of balance and coordination (intermittent ataxia) (https://ghr.nlm.nih.gov/condition/tyrosinemia). Interventions for those with tyrosinaemia type II include dietary restriction of tyrosine and phenylalanine, which leads to resolution of the ocular and cutaneous lesions (Mascai et al., 2001).

For mesotrione, another HPPD inhibitor, the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI/HESI) framework was used to describe the human relevance of the MOA ("Mesotrione", Annex 1, reference 133). Human data from volunteer studies with mesotrione and clinical trials and over 2000 patient years of experience with nitisinone (NTBC) that demonstrate that these HPPD inhibitors cause an increase in plasma tyrosine concentration. It was concluded that the key events seen predominantly in rats are plausible in humans, although there were significant species differences in the level of plasma tyrosine that can accumulate. The MOA for mesotrione-related adverse effects seen predominantly in rats depends on the sustained elevation of tyrosine. Two factors determine whether humans would sustain high plasma tyrosine levels: (1) residence time of the HPPD inhibitor in the body; and (2) the efficiency of clearance of tyrosine by TAT.

The differences in mesotrione toxicity profile are attributable to differences in the steady-state plasma tyrosine concentrations under conditions of complete HPPD inhibition, which have been shown to depend on innate TAT activity. TAT activity in humans is much higher than that in the rat but similar to that in male mice; thus, at equivalent doses of the potent HPPD inhibitor nitisinone (NTBC), plasma tyrosine concentrations in mice and humans are much lower than those seen in rats (Table A6).

Table A6. Comparison of innate hepatic TAT activity in rats, mice and humans

	Hepatic TAT activity (nmol HPPA/min per mg protein)		
	Rat	**Mouse**	**Human[a]**
Males	1.7 ± 0.2	7.8 ± 1.5	7.17 ± 1.17
Females	3.3 ± 0.5	10.5 ± 1.9	no data

HPPA: 4-hydroxyphenylpyruvate; TAT: tyrosine aminotransferase

[a] Henderson et al. (1981).

Source: JMPR ("Mesotrione", Annex 1, reference *133*)

For bicyclopyrone, two 28-day investigative studies performed in rats and mice also measured TAT activity. The TAT activities from these studies differ slightly from the values provided above; however, they still indicate that rats have a lower TAT activity than mice (Tables A7 and A8).

Table A7. Liver TAT activity in a 28-day study with Alpk:ApfSD (Wistar-derived) rats

	Hepatic TAT activity (nmol HPPA/min per mg protein)				
	0 ppm	**50 ppm**	**500 ppm**	**2000 ppm**	**5000 ppm**
Males	2.279 ± 0.195	3.549 ± 0.772	3.610 ± 0.405	3.649 ± 1.094	3.205 ± 0.728
Females	2.430 ± 0.618	2.768 ± 0.335	3.426 ± 1.413	3.005 ± 0.715	3.344 ± 0.614

HPPA: 4-hydroxyphenylpyruvate; ppm: parts per million; TAT: tyrosine aminotransferase

Source: Botham, Stevens & Williams (2015).

Table A8. Liver TAT activity in a 28-day study with C57 mouse

	Hepatic TAT activity (nmol HPPA/min per mg protein)				
	0 ppm	**10 ppm**	**100 ppm**	**1000 ppm**	**7000 ppm**
Males	3.476 ± 0.561	4.495 ± 0.554	4.349 ± 1.117	4.805 ± 0.328	3.952 ± 0.611
Females	4.547 ± 0.865	4.240 ± 0.539	4.440 ± 0.802	5.455 ± 0.938	5.043 ± 0.786

HPPA: 4-hydroxyphenylpyruvate; ppm: parts per million; TAT: tyrosine aminotransferase

Source: Noakes (2007)

The data provided in Tables A6 and A7 indicate a 3–4 times higher activity of the human TAT compared to rat (Henderson et al., 1981; Botham, Stevens & Williams, 2015).

Based on the available data, it was concluded that the MOA and key events for mesotrione-related adverse effects had been identified and, on a qualitative basis, were plausible in humans. Given the quantitative factors (kinetics and dynamics) of this MOA (short half-life and the significant differences in TAT activity between humans and rats), humans are unlikely to exhibit the toxicities seen in rats. Nonetheless, mesotrione at some relatively high-dose level may raise tyrosine levels in humans, but certainly not to an extent or for a duration that is likely to cause adverse effects. In addition, the available data lead to the conclusion that for tyrosine-related toxicities, the mouse is the most appropriate model to use to assess risk of mesotrione in humans ("Mesotrione", Annex 1, reference 133).

Mechanistic studies with HPPD inhibitors have led to the conclusion that rats are uniquely sensitive to the effects of HPPD inhibitors and that the ocular keratitis and regenerative hyperplasia observed in these animals are directly linked to the resulting highly elevated plasma tyrosine (in excess of 2000 nmol/mL). These mechanism are not relevant to humans (Carmichael et al., 2011). The United States Environmental Protection Agency (USEPA, 2007: http://www.fluoridealert.org/wp-content/pesticides/EPA-HQ-OPP-2006-1026-0008.pdf) recognized that the data available for HPPD inhibitors support the conclusion that the high levels of plasma tyrosine seen in male rats and the consequent corneal damage is unlikely to be seen in humans. The use of the HPPD inhibitor nitisinone (Orfadin®) to treat tyrosinaemia type I in children and the knowledge of plasma tyrosine concentrations in the various genetic tyrosinaemia types has confirmed that inhibition/lack of HPPD activity results in tyrosinaemia in humans comparable to that seen in mice following administration of HPPD inhibitors (Lewis & Botham, 2013).

Of the toxicities observed after administration of bicyclopyrone to test animals, the ocular effect (corneal opacity, including ocular tumours) seems to be due to the elevated blood tyrosine levels. Ocular tumours have been seen with some but not all HPPD inhibitors and a progression from corneal damage/repair to ocular tumours has not been demonstrated directly. However, the tumours were only observed in male rats, which have higher tyrosine levels than female rats, and in both male and female mice, suggesting a relation to tyrosine plasma concentrations. While the human relevance of these ocular tumours cannot be excluded, as humans have a significantly higher TAT activity than rats, they are thus unlikely to reach the plasma tyrosine levels associated with these ocular tumours in male rats.

Although the other toxicities, like perturbation of the thyroid function and developmental and reproductive effects may be associated with chemically induced tyrosinaemia, other mechanisms may also be involved. However, thyroid effects were not observed in mice, dogs and rabbits treated with bicyclopyrone. The administration of the pharmaceutical HPPD inhibitor, nitisinone, to adult humans

(with tyrosinaemia) in the absence of dietary restriction did not cause any perturbation of thyroid function during three years of dosing despite measured plasma tyrosine concentrations of up to 1500 nmol/mL (Introne et al., 2011). The thyroid effects were observed mainly in male rats and to a lesser extent in female rats; male rats have higher tyrosine levels than female rats and male and female mice, suggesting an association with tyrosine plasma concentrations. The relevance of these thyroid effects for humans cannot be excluded, however, since the thyroid effects were not observed in humans, mice or dogs, and since humans have a more efficient tyrosine clearance mechanism than rats, humans are unlikely to reach tyrosine levels that induce these thyroid effects in rats.

The developmental and reproductive effects in pregnant rats and rabbits also seems to be associated with plasma tyrosine concentration (Lewis & Botham, 2013). The human TAT activity is compared to rat and mouse TAT activity; however, there is no information on the relevance of the rabbit developmental effects to human, since there is no comparison of rabbit and human TAT activity. Therefore, the relevance of the developmental effects to humans cannot be excluded.

In conclusion, for bicyclopyrone, a human relevance MOA according to the International Programme on Chemical Safety (IPCS) framework was not made available by the sponsor. Inhibition of HPPD and increase of plasma tyrosine concentrations, as demonstrated for rats and mice in mechanistic studies, could potentially occur in humans. Ocular tumours were only observed in male rats; while these ocular tumours have been seen with some but not all HPPD inhibitors, a progression from corneal damage/repair to ocular tumours has not been demonstrated. The thyroid effects were observed mainly in male rats and to a lesser extent in female rats. Male rats have higher tyrosine levels than female rats and male and female mice, suggesting a relation between the effects (ocular tumours, thyroid) and tyrosine plasma concentrations. The relevance of these effects for humans cannot be excluded; however, as the thyroid effects and ocular tumours were not observed in mice or dogs and as humans have a more efficient tyrosine clearance mechanism than do rats, humans are unlikely to reach tyrosine levels at which these ocular tumours and thyroid effects in rats were observed.

Appendix 1 References

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Chlormequat chloride

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Explanation

Chlormequat chloride is the International Organization for Standardization-approved common name for 2-chloroethyltrimethylammonium chloride (International Union of Pure and Applied Chemistry name), with Chemical Abstracts Service number 999-81-5. Chlormequat, also called chlorocholine-chloride, belongs to the group of quaternary ammonium compounds that are used as plant growth regulators acting as an inhibitor of the biosynthesis of gibberellins.

Chlormequat chloride was evaluated by Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1970, 1972, 1994, 1997 and 1999 (Annex 1, references 14, 18, 71, 80 and 86). In 1972 an acceptable daily intake (ADI) of 0–0.05 mg/kg bw was established on the basis of the no-observed-adverse-effect level (NOAEL) in a study of reproductive toxicity in rats. In 1994, this ADI was withdrawn as the database was considered inadequate by contemporary standards. In 1997, a number of new studies were evaluated and an ADI of 0–0.05 mg/kg bw was established based on the NOAEL of 4.7 mg/kg bw in a one-year dog study.

In 1999 the compound was considered again solely to determine an acute reference dose (ARfD), which was set at 0.05 mg/kg bw based on a one-year dog study with a NOAEL of 150 ppm (equal to 4.7 mg/kg bw per day), as the clinical signs found were considered to be possibly due to a single dose.

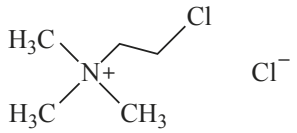
In 2017, the Meeting evaluated new studies/analyses on rodent reproductive and developmental toxicity, acute dog toxicity and case reports on human deaths by suicide using chlormequat chloride.

The studies evaluated by the previous JMPR meetings (1997 and 1999) are included in this assessment and are re-evaluated according to current JMPR policies and procedures.

Most of the unpublished studies evaluated were performed by laboratories that were certified for good laboratory practice (GLP) and that complied, where appropriate, with the relevant Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TGs) or similar guidelines of the European Union or United States Environmental Protection Agency (USEPA), unless otherwise indicated. Minor deviations from these protocols were not considered to affect the reliability of the studies.

Absorbed and translocated throughout the plant, chlormequat chloride acts as a growth retardant, and is used predominantly on cereals to reduce unwanted longitudinal shoot growth, without lowering plant productivity. It is also used in the cultivation of grapes, pears, potatoes and tomatoes.

Figure 1. Structure of chlormequat chloride



Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion of chlormequat chloride was studied in rats following a single oral low dose, a single oral high dose repeated for 14 days and a single oral low dose repeated for 14 days followed by a radioactive dose. In addition, absorption and excretion of chlormequat chloride was studied following intravenous and intraperitoneal administration in rats and intramuscular administration in rhesus monkeys.

1.1 Absorption, distribution and excretion

(a) Oral route

Several studies on the absorption, distribution and excretion of chlormequat chloride have been published. The level of detail in these papers did not permit a complete evaluation by previous JMPR meetings. The main study is from 1989, and there appear to be no additional recent rodent in vivo studies.

In the first study, 61% of an unspecified oral dose of ^{14}C-labelled chlormequat chloride administered in 0.8 mL of water to two male rats was excreted in the urine within four hours, and 96% was eliminated within 47 hours; faecal excretion accounted for 2.3%, and less than 1% was expired as ^{14}C-labelled carbon dioxide. The remainder was found in the tissues, with the largest amounts in the carcass (0.25%), intestines (0.11%) and liver (0.08%). Analysis of urine samples by four different thin-layer and paper chromatographic systems showed that all of the radiolabel was on chlormequat chloride (Blinn, 1967).

In the second study, rats were given a single oral dose of 60 mg of ^{15}N-labelled chlormequat chloride or 2 mg of the labelled compound daily for 100 days. After the single dose, the amount of compound in the brain decreased quickly, but there was considerable accumulation in the kidneys over the 20 days of the investigation. After continuous repeated-dose administration, chlormequat chloride was found primarily in the heart and diaphragm (Bier & Ackermann, 1970).

In the third study, several different treatment experiments were conducted on small groups of male and female rats (1–10/sex per dose level and sampling point). The animals were treated intravenously with a single dose of 0.1 mg/kg bw or orally by gavage with a repeated low dose of 0.5 mg/kg bw or a single high dose of 30 mg/kg bw of ^{14}C-labelled chlormequat chloride (radiochemical purity 96.8%). Urine, faeces, organs, bile and expired air were collected from all animals at various intervals up to 168 hours after treatment; 82–103% of the radiolabel was recovered. Most was excreted during the first 24 hours. More than 85% of the radiolabel was excreted in urine and less than 6% in faeces; less than 1% was eliminated as volatile compounds. The maximum level in the blood was reached about two hours after oral administration of either the low or the high dose. Less than 1% of the radiolabel was eliminated in bile during the first 24 hours. The maximum level in the bile was reached 2–5 hours after oral administration. At scheduled kill 168 hours post dosing, very low levels of radioactivity were found in the kidneys, followed by the liver, the heart and the gastrointestinal tract. These data indicate that chlormequat chloride has no potential for accumulation (Giese & Hoffmann, 1989).

For the repeated low-dose study, at two hours after the last of the seven daily doses of 0.5 mg/kg bw, the highest amounts of radioactivity were found in the kidneys, followed by the liver, the heart and the gastrointestinal tract, at levels much lower than in the high-dose study. At scheduled kill (168 hours post dosing), virtually no radioactivity remained. Faeces and bile did not play a major role in the elimination. There were no significant differences in absorption and excretion between the high and low dose levels. Excretion after a 14-day pretreatment was comparable to that after a single oral dose.

The blood maximum concentration (C_{max}) increased less than proportionally with dose, whereas the area under the concentration–time curve (AUC) was linear over the tested dose range, indicating that chlormequat chloride has no potential for accumulation.

(b) Dermal route

A single study of the absorption, distribution and excretion following dermal application of ^{14}C-labelled chlormequat chloride (purity >95%) was conducted in male Wistar rats (4/dose level per sampling point) according to OECD Draft Guideline "Percutaneous absorption: in vivo method" (1996) and in accordance with GLP. Dose levels were 7.5, 1.0 or 0.1 mg/cm^2 (corresponding to about 75, 10 and 1.0 mg/animal or about 250, 30 and 3 mg/kg bw). The exposure durations were one hour (scheduled kill at one hour), four hours (scheduled kill at four hours), 10 hours (scheduled kill at 10 hours), 24 hours (scheduled kill at 24 hours) and 10 hours (scheduled kill at 72 hours). For the low dose level, an additional data point at 10 hours exposure (scheduled kill at 24 hours) was included. At the respective kill times, excreta, blood cells, plasma, liver, kidneys, carcass and treated and untreated skin were collected. During the study the animals were maintained in metabolism cages.

The dermal absorption of chlormequat chloride, when administered at the commercial formulation concentration after a 10-hour exposure and a 72-hour collection period, was 3.26%. When administered at 1/7.5th of the commercial formulation concentration (corresponding to a dermal exposure as derived from application of the Predictive Operator Exposure Model [UK POEM]), dermal absorption after a 10-hour exposure and a 72-hour collection period was 2.23%. When administered at 1/66th of the commercial formulation concentration (corresponding to the spray dilution), dermal absorption after a 10-hour exposure and a 24-hour collection period was 1.30% (Leibold & Hoffmann, 2001).

1.2 Biotransformation

(a) Oral administration

Only chlormequat chloride and two other compounds, which may have been other salts of chlorocholine, were reported in the urine of rats that received 200 mg/kg bw of chlormequat chloride orally. Choline itself was not identified (Bronisz & Romanowski, 1968).

A summary provided to the 1997 JMPR described several different treatment experiments, including blood plasma level profile experiments, balance experiments, distribution experiments and biliary excretion experiments, conducted on small groups of rats (0–10 rats/sex per dose level and sampling point), following range-finding studies. The rats were given a single intravenous dose of 0.1 mg/kg bw or a single oral dose of 0.5 mg/kg bw (with or without pretreatment with unlabelled chlormequat chloride [purity 96.8%] for 14 days) or 30 mg/kg bw ^{14}C-labelled chlormequat chloride (radiochemical purity 96.6% and 98.1% using two different solvents). Urine, faeces, bile, liver, kidney, gastrointestinal tract, brain, muscle, spleen, bone, lungs, heart, fat, testes and uterus were examined for radiolabel up to 168 hours after treatment. Expired air from two males was examined within 0–24 hours.

A total of 81% of the dose of radiolabel was excreted during the test period. More than 85% of the radiolabel was found in urine and less than 5% in faeces. Chlormequat chloride was excreted mainly unmetabolized. Traces of a very polar, but unidentified, metabolite were found in faeces (Giese & Kohl, 1989).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with chlormequat chloride administered orally, dermally or by inhalation are summarized in Table 1. Clinical signs of toxicity seen after treatment with chlormequat chloride were consistent with activation of nicotinic and muscarinic acetylcholine receptors, and included salivation, writhing, chromodacryorrhoea, decreased activity, tremors, diuresis and piloerection. Death generally occurred within 24 hours of treatment; animals that survived recovered within 48 hours. The findings at necropsy were not consistent with or related to treatment.

Table 1. Acute toxicity of chlormequat chloride

Species	Strain	Sex	Route	TG/GLP status	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L air)	Reference
Mouse	NR	NR	Oral	Pre GLP	NR	215–1020 [a]	Oettel (1965)
Mouse	NR	M	Oral	Pre GLP	NR		Levinskas & Shaffer (1962)
Mouse	NR	NR	Oral	Pre GLP	NR		Ignatiev (1967)
Mouse	ddY	M, F	Oral	No	98.0	544	Hattori (1981)
Mouse	Swiss albino	M, F	Oral	TG 401 (1987)	63.5	629	Suresh (1991a, 2002 amendment)
Mouse	NMRI	M, F	Oral	TG 401 (1982)	98	589	Munk & Freisberg (1975a)
Mouse	NR	NK	IP	NR	NR	60–68	Shaffer (1970)
Mouse	NR	NR	SC	No	98.0	88–92	Hattori (1981)
Rat	SD	NK	Oral	Pre GLP	NR	433	Oettel (1965)
	Wistar					660	
Rat	Albino	M	Oral	Pre GLP	NR	670 [a]	Levinskas & Shaffer (1962)
Rat	NR	NK	Oral	Pre GLP	NR		Ignatiev (1967)
Rat	NR	NK	Oral	NR	NR		Stefaniak (1969)

Species	Strain	Sex	Route	TG/GLP status	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L air)	Reference
Rat	SD	M, F	Oral	No	98.1	M: 524 F: 564 M + F: 544	Hattori (1981)
Rat	Wistar	M, F	Oral	TG 401 (1981)	63.5	534	Suresh (1991b, 2002 amendment)
Rat	SD	M, F	Oral	Pre GLP	98	807	Munk & Freisberg (1975b)
Rat	Crl CD(SD) BR	M, F	Oral	USEPA (FIFRA) 81-1 (1982)	66.1	522	Lowe & Fischer (1990a)
Rat	CRJ-SD	M, F	IP	No	98.1	53–75	Hattori (1981)
Rat	SD	M, F	IP	Pre GLP	98	62.1	Munk & Freisberg (1975c)
Rat	NR	M, F	SC	No	98.1	113–118	Hattori (1981)
Rabbit	Mixed breed	NR	Oral	No	98	115	Kirsch (1975)
Rabbit	NR	NR	Oral	Pre GLP	NR	~75	Oettel (1965)
Rabbit	NR	M	Oral	Pre GLP	99	81	Levinskas & Shaffer (1962)
Rat	SD	M, F	Dermal	No	99	>4000	Gelbke & Freisberg (1978)
Rat	SD	M, F	Dermal	No	98.1	>5000	Hattori (1981)
Rat	Wistar	M, F	Dermal	TG 402 (1987) Yes	63.5% w/w solution in water	>4540	Suresh (1991c, 2002 amendment)
Rabbit	NZW	M, F	Dermal	TG 402 (1987) Yes	66.1% w/w solution in water	1250	Fischer et al. (1990)
Rabbit	NZW	NR	Dermal	No	750 g/L solution in water	>1875	Kynoch & Lloyd (1978)
Rabbit	Albino	M	Dermal	No	12% aqueous solution	440	Levinskas & Shaffer (1962)
Rat	SD	NR	Inhalation	No	99	>5.2	Klimisch & Zeller (1979)
Rat	SD	M, F	Inhalation	TG 403 (1981) Yes	66.1 Solution in water	>4.57	Hershman (1990)
Rat	SD	NR	Inhalation	TG 403 (1981) Yes	720 g/L solution in water, 63.5% w/w	>2.51	Suresh (1991d, 2002 amendment)
Hamster	NK	NR	Oral	Pre GLP	99	1070	Levinskas & Shaffer (1966)

Species	Strain	Sex	Route	TG/GLP status	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L air)	Reference
Guinea-pig	NR	NK	Oral	Pre GLP	NR	210	Oettel (1965)
Guinea-pig	NR	M	Oral	Pre GLP	99	620	Levinskas & Shaffer (1966)
Cat	NR	NK	Oral	Pre GLP	NR	~10	Oettel (1965)
Cat	NR	M, F	Oral	No	99	50	Levinskas & Shaffer (1966)
Dog	NR	NK	Oral	Pre GLP	NR	~75	Oettel (1965)
Dog	Beagle	M, F	Oral	Pre GLP	99	37 [b]	Levinskas & Shaffer (1966); Levinskas & Shaffer (2000)
Chick	NR	F	Oral	Pre GLP	99	920	Levinskas & Shaffer (1966)
Sheep	NR	NK	Oral	Pre GLP	NR	150–200	Schulz et al. (1970)
Monkey	NR	NK	Oral	Pre GLP	NR	> 800	Costa, Hansen & Woodward (1967)

GLP: good laboratory practice; F: females; IP: intraperitoneal; M: males; NK: not known; NR: not reported; SC: subcutaneous; TG: test guideline; w/w: weight per weight
USEPA: United States Environmental Protection Agency;

[a] Combined LD_{50} from several studies presented in JMPR 1997 (Annex 1, references *80*).

[b] Recalculated using PROBIT.BAS Version 3.0 on 23 February 2000 (Levinskas & Shaffer, 2000).

In summary, the oral median lethal dose (LD_{50}) values of chlormequat chloride were 800–1000 mg/kg bw in rats, mice, hamsters, guinea-pigs, sheep and monkeys and 10–80 mg/kg bw in rabbits, cats and dogs.

The dermal LD_{50} was greater than 4000 mg/kg bw in rats and 440 mg/kg bw in rabbits.

The rat inhalation median lethal concentration (LC_{50}) was greater than 2.51 mg/L.

The sponsor further suggests that on the basis of human poisoning cases with products containing chlormequat chloride, and on the premise that large quantities are gulped and not sipped by persons attempting suicide, the active ingredient should be classified as toxic if swallowed ($50 < \text{oral } LD_{50} < 300$ mg/kg bw) (Frericks M, BASF SE, Ludwigshafen, Germany; personal communication, 10 May 2017, received 26 August 2017).

Rat

In a study in Sprague Dawley rats (5/sex per dose) with chlormequat chloride (purity 99%), two dose groups of 2000 and 4000 mg/kg bw were tested. The test substance, prepared in water (50% at the low dose and 80% at the high dose), was applied to about 42 cm^2 on the dorsal flank under occlusive conditions for 24 hours. Clinical signs seen soon after application were slight apathy, staggering, spastic movements and impaired general condition. Local signs of irritation were reversible.

The median LD_{50} was determined to be greater than 4000 mg/kg bw for male and female animals (Gelbke & Freisberg, 1978).

In another old and poorly described study (numbers of animals not stated and procedures not described), the dermal LD_{50} in CRJ-SD rats was quoted as being greater than 5000 mg/kg bw for males and females. The purity of chlormequat chloride was described as 98%, and the test compound was diluted with water (Hattori, 1981).

In a third study, chlormequat chloride 720 g/L (active ingredient 63.5%) was tested for acute dermal toxicity in Wistar rats. The dermal LD_{50} was determined to be greater than 4540 mg/kg bw for males and females (Suresh, 1991c, 2002 amendment).

Chlormequat chloride (active ingredient 66.1%) was tested for dermal toxicity in New Zealand White albino rabbits (5/sex per dose; zero males and five females at highest dose). The test substance was applied to the shaven skin (about 10% of the body surface) under occlusive conditions at doses of 312.5, 625, 1250, 2500 or 5000 mg/kg bw. Clinical signs and mortality occurred at all dose levels. Mortality was 5/5 at 5000 mg/kg bw, 6/10 at 2500 mg/kg bw (4/5 males, 2/5 females), 6/10 at 1250 mg/kg bw (3/5 males, 3/5 females), 3/10 for the 625 mg/kg bw dose and 2/10 for the lowest dose. The animals that died generally did so within a day of application. Decreased activity, salivation, ataxia and anorexia were seen in the low and higher dose groups. In addition, nasal discharge was seen at 1250 and 2500 mg/kg bw, and salivation was reported at 5000 mg/kg bw. Male rabbits were slightly more sensitive.

The dermal LD_{50} of chlormequat chloride was calculated as follows: for males 964 (range: 509–1825); for females 1621 (range: 856–3069); and for males plus females 1250 (range: 647–2414) mg/kg bw (Fischer et al., 1990).

A study with chlormequat chloride (750 g/L) with comparable test conditions reported that at a dose of 2.5 mL/kg bw, chlormequat chloride was lethal to 1/6 rabbits within 24 hours after application. Systemic toxicity (piloerection, slight lethargy, hunched posture and slight increased respiratory rate) was reversible within 5 days. Slight erythema and/or oedema were observed both in control and treated rabbits, with a slightly longer persistence (up to day 8) in treated rabbits.

The dermal LD_{50} of chlormequat chloride was greater than 1875 mg/kg bw (Kynoch & Lloyd, 1978).

Rabbit

In an old but scientifically acceptable study, 11 male and 12 female mixed breed rabbits (3–4/dose level) were administered chlormequat chloride (purity 98%) at 46.4, 100, 121, 147, 215 or 464 mg/kg bw in water by gavage. There is a lack of information on the breed, sexes and small group size in this study.

The mortality was 0/4, 0/4, 3/4, 3 /4, 4/4, 3/3, respectively, within two hours, and the oral LD_{50} was 115 mg/kg bw. Clinical signs included salivation, isolated cases of prostate and lateral posture, tremors, palmus, diarrhoea and dyspnoea. Findings at necropsy of the decedents included acute dilation of heart (right side) and acute congestive hyperaemia. There were no such findings in the surviving animals (Kirsch, 1975).

Dog

A PROBIT recalculation analysis of the dog single-dose oral toxicity data from a comparative species study by Levinskas & Shaffer (1966) was conducted in 2000. This recalculation provides the lowest experimental LD_{50} of 36.85 mg/kg bw. No further information is available on this study.

(b) Exposure by inhalation

Rat

In an inhalation toxicity study, Sprague Dawley rats (10/sex per dose) were exposed to a liquid aerosol of chlormequat chloride (purity not indicated) at an analytical concentration of 4.57 mg/L (nominal technically maximum achievable concentration 6.75 mg/L).

There were no mortalities. Due to the whole body exposure scenario, wet and damp hair, wet ventral areas, red-brown periocular and muzzle staining were observed for all animals until day 5 and persisted in one male until day 8. Individual animals exhibited body weight losses or no body weight gain from day 7 to 14. No gross pathological findings were observed at scheduled kill on day 14. Absolute and relative organ weights appeared normal.

It was concluded that chlormequat chloride has low acute inhalation toxicity as a liquid aerosol (Hershman, 1990). The four-hour LC_{50} by whole body exposure is above the maximum technically achievable concentration of 4.57 mg/L.

Further acute inhalation toxicity studies in rats similarly have a low acute inhalation toxicity. These include an older liquid aerosol study in Sprague Dawley rats (chlormequat chloride purity 99%) using head-nose exposure (LC_{50} > 5.2 mg/L per 4 hours) (Klimisch & Zeller, 1979).

Chlormequat chloride as a liquid aerosol at 720 g/L water (active ingredient 63.5%) was tested for acute inhalation toxicity in Wistar rats using whole body exposure. The LC_{50} was calculated to be greater than 2.5 mg/L per four hours for males and females (Suresh, 1991d, 2002 amendment).

(c) Dermal irritation

Rabbit

Three studies in rabbits concluded that chlormequat chloride is a non-irritant to rabbit skin after four-hour occlusive exposure, and two studies concluded that chlormequat chloride is a non-irritant to rabbit skin after a 24-hour exposure.

In two studies conducted between 1978 and 1980, which were not in accordance with current OECD TGs, the irritancy of chlormequat chloride to the skin was tested in rabbits for a 24-hour or longer exposure. In the first study, about 0.5 mL of the test material (purity not specified) was applied to intact and abraded sites on Vienna White rabbits (n = 6) and left in place for 24 hours under an occlusive dressing. At the intact sites, erythema and oedema were observed at the end of the application period, but these signs were almost fully reversed within two days. More severe signs of irritation were observed at the abraded sites; these signs were only partly reversible, and superficial necrosis was seen in three animals after three days (Gelbke, 1978).

In the second study, about 500 mg of the same material was tested in the same way in New Zealand White rabbits. Dermal reaction at the treatment sites was limited to very slight or well-defined erythema, which was evident only at the end of the application period. All reaction had resolved completely within 72 hours of treatment (Buch & Gardner, 1980).

In the first of the three studies testing the irritancy of chlormequat chloride over four-hour occlusive exposure (based on current test guidelines), about 0.5 mL of chlormequat chloride (purity 66.1% active ingredient) was applied to intact sites on New Zealand White rabbits (n = 6) and left in place for 4 hours under an occlusive dressing. Dermal reaction at the treatment sites was limited to barely perceptible or slight erythema in three animals one hour after treatment and in one animal at 24 hours. All reactions had resolved completely within 48 hours of treatment (Fischer, Boczon & Scubelek, 1990).

In the second study, chlormequat chloride (active ingredient 63.5%) at 720 g/L did not elicit skin irritation after a four-hour occlusive exposure. There were also no signs of systemic toxicity (Suresh, 1991e, 2002 amendment).

In the third study, Atlas chlormequat chloride 700 was tested for primary skin irritation according to OECD TG 404 in New Zealand White rabbits, and was not irritating to the skin (Guest & Jones, 1988a).

(d) Ocular irritation

The chlormequat chloride test results below indicate that the test substance is not an eye irritant. Although a single study did indicate systemic toxicity, this appears to be an isolated finding as six studies found no systemic toxicity.

Rabbit

The irritancy of chlormequat chloride to the eye was tested in Vienna White rabbits by applying about 0.1 mL of the test material (purity unspecified) to the conjunctival sac of the right eyelid of six rabbits. Conjunctival redness was seen in five rabbits 24 hours after treatment. After 48 hours, conjunctival redness was seen in two rabbits, one of which had a conjunctival discharge. All reactions resolved by 72 hours after treatment (Grundler & Gelbke, 1981a).

The irritancy of chlormequat chloride (a technical material consisting of a 66.1% aqueous solution) was also tested in Vienna White rabbits. About 0.1 mL was applied to the conjunctival sac of

the left eyelid of six rabbits and left for 24 hours. One hour after treatment, slight reactions were seen in all the rabbits. As there was still a conjunctival reaction in one rabbit at the 72-hour reading, this eye was also examined after four days. The study was terminated after complete reversibility of ocular findings either on day 3 (five rabbits) or day 4 (one rabbit) (Lowe & Fischer, 1990b).

A volume of 0.1 mL chlormequat chloride 700, was instilled into the conjunctival sac of the right eye of White Vienna rabbits (two males and one female). The animals were scored for corneal changes, iris effects and conjunctival reaction at 1, 24, 48 and 72 hours after test substance administration. As there were no findings at the 72-hour reading, the study was terminated.

There were no effects on cornea in this study. Iris findings were restricted to two animals with respect to iritis grade: one at the one-hour reading, the other at the 24-hour reading. In addition, mydriasis was noted in one of the two animals, but the third animal had no indication of iritis. The effect was reversible by the next reading interval (24 hours after test substance instillation). Redness and swelling was observed within one hour of the treatment (mainly Grade 2). Reversibility for these findings was noted mainly at the 24-hour reading, but in one animal the findings were reversible at the 72-hour reading. Discharge was only noted 1 hour after treatment (Guest & Jones, 1988b).

Another study instilled a volume of 0.1 mL of chlormequat chloride (purity 63.5%) into the conjunctival sac of the left eye of New Zealand White rabbits (four males and three females). There were no effects on cornea and iris. Shortly after instillation, conjunctival reactions (Grade 1) were observed in two animals; these were reversible within 24 hours. No signs of chemosis were noted in the study and ocular discharge (Grade 1) was noted in all animals one hour after test substance application. Although the compound tested was not an ocular irritant to rabbits, systemic toxicity was observed in the majority of the animals: 2/3 females died within minutes of ocular instillation after showing clinical signs such as salivation and lachrymation; three other animals had identical clinical signs, but survived, whereas two animals did not show any clinical signs indicative of systemic resorption at toxic dose levels.

It was concluded that while chlormequat chloride was not irritating to the rabbit eye, it was resorbed at toxic/lethal dose levels in this study (Suresh, 1991f, 2002 amendment).

Similar results with respect to irritation properties to the eyes have been reported in three further studies, but with no indication of a systemic toxic/pharmacological effect (mydriasis or clinical signs). This includes a study in White Vienna rabbits (6 animals/study) (Grundler & Gelbke, 1981a,b). Chlormequat chloride at 750 g/L was also a non-irritant in albino rabbits (6 animals/study) under comparable test conditions (Kynoch & Liggett, 1978).

(e) Dermal sensitization

Guinea pig

Three skin sensitization studies in the guinea-pig determined that chlormequat chloride was not sensitizing. However, a pretest for one of the studies did show clinical signs and a mortality, which increases the weight of evidence regarding systemic adverse effects.

In the first skin sensitization study, 20 test group animals and 10 control group animals underwent the guinea-pig maximization test (according to TG 406 [1981]). Purity of chlormequat chloride was not indicated. During the dermal induction phase, no skin reaction indicating a sensitizing or irritant effect in control and test animals was observed. A concentration of 50% chlormequat chloride in aqua bidest (double-distilled water) was determined to be the maximum non-irritant concentration under the same test conditions. An intradermal dose of 5% test substance preparation caused systemic toxicity and was finally lethal, while 1% in 0.9% aqueous NaCl solution resp. Freund's adjuvant/0.9% aqueous NaCl solution (1:1) did not cause any test substance-related signs or mortality. In the case of the death of one guinea-pig, a test substance-related effect cannot be ruled out because the animals in the pretest showed clinical signs, and the death occurred during intradermal induction. Gross pathological examination revealed intensified hyperaemia of the lung (Rossbacher & Kirsch, 1992).

Two studies reported that chlormequat chloride was not a skin sensitizer in guinea pigs when tested during dermal induction and the challenge phase in a Buehler test (Ventura & Moore, 1990; Suresh, 1992a; 2002 amendment).

2.2. Short-term studies of toxicity

(a) Oral administration

Mouse

In a four-week oral toxicity study, chlormequat chloride (purity 66.7%) was administered to B6C3F1 mice (5/sex per dose group) at dietary concentrations of 0, 500, 1500 or 3000 ppm (equal to 0, 148, 439 and 885 mg/kg bw per day for males and 0, 223, 618 and 1190 mg/kg bw per day for females, respectively). At the end of the study, a swimming and rotating rod test was performed with the control and high-dose animals.

There were no test substance-related mortalities, no haematological changes and no clinical chemistry changes. No adverse effects were noted in the locomotor activity, swimming and rod tests; nor were effects seen in body weight or feed consumption. While absolute liver weight increased (9%) in males at 3000 ppm, no changes were seen in relative liver weight. While histopathology was not performed, macroscopic examinations did not show any treatment-related changes.

The NOAEL was 3000 ppm (equal to 885 mg/kg bw per day for males and 1190 mg/kg bw per day for females), the highest dose tested (Schilling, 1990).

In a 90-day oral toxicity study conducted according to GLP and TG 408 (1981), chlormequat chloride (purity 67.4%) was administered to B6C3F1xCrlBR mice (10/sex per group) for three months at dietary concentrations of 0, 472, 1408 or 4212 ppm (equal to 0, 120, 370 and 1070 mg/kg bw per day for males and 0, 150, 470 and 1400 mg/kg bw per day for females, respectively). There were no mortalities, no signs of clinical toxicity and no treatment-related effects on body weight or feed consumption. Organ weights and gross and histopathology did not indicate treatment-related effects.

The NOAEL was 4212 ppm (equal to 1070 mg/kg bw per day for males and 1400 mg/kg bw per day for females), the highest dose tested (Schilling & Hildebrand, 1991).

Rat

Two short-term studies of toxicity are described in the 1972 JMPR monograph addendum (Annex 1, reference 82). Although detailed reports were not available for evaluation in 1997, extracts were obtained for the present Meeting.

The summary of the first study states that rats (10 males/group) were fed chlormequat chloride at dietary levels of 0, 500, 1000 or 2000 ppm (equivalent to 0, 25, 50 and 100 mg/kg bw per day) for 29 days. There were no deaths, and no clinical signs of reaction to treatment; body weight gain and feed intake remained unaffected by treatment, and no gross pathological changes were observed at study termination (Levinskas & Shaffer, 1962).

In the second study, rats (20/sex per group) were fed chlormequat chloride at dietary levels of 0, 200, 600 or 1800 ppm (equivalent to 0, 10, 30 and 90 mg/kg bw per day) for 90 days. There were no deaths, no clinical signs of reaction to treatment and no treatment-related changes in blood chemistry. The body weight gain of males fed 1800 ppm was slightly depressed in comparison with that of controls. Slightly increased kidney weights were recorded in treated female rats and slightly increased liver weights in treated males, particularly at 1800 ppm; however, histopathological examination of major organs revealed no treatment-related changes (Levinskas, 1965).

In a four-week oral toxicity study conducted according to TG 407 (1981), Wistar rats (5/sex per group) were fed chlormequat chloride daily at dietary levels of 0, 500, 1500, 3000 or 4500 ppm (equal to 0, 47, 137, 258 and 367 mg/kg bw per day for males and 0, 51, 148, 291 and 418 mg/kg bw per day for females, respectively). The test material was a technical grade formulation of 66.7% purity, but the dietary levels were expressed as pure chlormequat chloride.

Clinical signs of general deterioration in health were seen in males and females at 4500 ppm and, temporarily, in one male and one female at 3000 ppm. Reduced body weight gain and feed intake were seen in animals fed 4500 ppm, and slightly reduced weight gain was seen in those fed 3000 ppm. Serum creatinine levels in males and females at 4500 ppm and in females at 3000 ppm were lower than those of controls. Decreased serum concentrations of total protein (in males) and of urea (in females) were also seen at 4500 ppm. Locomotor activity and swimming tests, gross pathological examinations, organ weights and histopathological examination revealed no consistent reaction to treatment.

The NOAEL was 1500 ppm (equal to 137 mg/kg bw per day) based on reduced body weight gain at 3000 ppm (equal to 258 mg/kg bw per day) (Schilling et al., 1990).

In a four-week oral toxicity study conducted according to TG 407 and TG 408 (1981), chlormequat chloride (purity 63.5%) was administered to Wistar rats (5/sex per dose level) at dietary doses of 0, 100, 1000 or 10 000 ppm (equal to 0, 8.5, 95 and 1210 mg/kg bw per day for males and 0, 9.8, 120 and 1240 mg/kg bw per day for females, respectively). A similarly sized group was treated for four weeks with 10 000 ppm followed by a treatment-free diet for two weeks to assess recovery (equal to 1110 mg/kg bw per day for males and 1140 mg/kg bw per day for females).

There were no test substance-related mortalities in any of the dose groups, but weakness and emaciation were observed at 10 000 ppm (3/5) as well as in females (1/5) at 1000 ppm. While the body weight reduction was reversible to a degree, full recovery was not observed in the two-week post-treatment period. There was a dose-related incidence of urine incontinence in both sexes; nasal discharge and snuffling in females (high dose 4/5; recovery group during treatment 2/5). Clinical chemistry showed a 20% and higher increase in blood urea nitrogen at 10 000 ppm. Sodium was significantly ($P = 0.05$) consistently increased at all dose levels, but the increase was not dose dependent and can therefore be considered incidental; the increase was not reversed in the high-dose recovery group. Total protein (< 10%) and creatinine (< 15%) were decreased at 10 000 ppm. Creatinine also steadily decreased (< 10%; $P = 0.05$; control, 0.48 mg/dL; 1000 ppm, 0.42 mg/dL, 10 000 ppm, 0.39 mg/dL), but these effects were reversible.

At 10 000 ppm, adrenal, ovary and absolute kidney weights were decreased. Relative liver weight was increased at 100 and 10 000 ppm, but it is unlikely that these were treatment related, as there was no dose–response relationship, no effects on absolute organ weights and no histopathological changes.

A higher incidence of hypoplasia of seminal vesicles and prostate was observed at 10 000 ppm; this seemed to originate from low growth seen at the highest dose, but was observed to occur at a low incidence in the recovery group, so appeared to be reversible.

The NOAEL was 100 ppm (equal to 9.8 mg/kg bw per day) based on emaciation and clinical signs of weakness observed in females at 1000 ppm (equal to 1240 mg/kg bw per day) (Suresh, 1991g, 2002 amendment).

In a four-week oral toxicity study, chlormequat chloride (purity not stated) was administered to two groups of Wistar rats: 10/sex per dose received dietary concentrations of 100 and 500 ppm and 20/sex per dose received dietary concentrations of 0 and 2500 ppm (the four concentrations equal 0, 8.3, 41 and 202 mg/kg bw per day for males and 0, 8.8, 45 and 211 mg/kg bw per day for females). A two-week recovery period was included for 10 rats/sex. Starting weights were noted to be highly variable, which affects the quality of the study and the data interpretation.

There were no treatment-related deaths and no signs of clinical toxicity in any of the treatment groups.

The sponsor set the NOAEL at 500 ppm for females (equal to 45 mg/kg bw per day) and 2500 ppm (equal to 202 mg/kg bw per day) for males on the basis of body weight gain and feed consumption in high-dose females, but the Meeting considered that these changes were probably due to natural variability rather than the systemic toxicity of chlormequat chloride.

The NOAEL was 2500 ppm (equal to 202 mg/kg bw per day), the highest concentration tested (Nemec, 1991).

In a 59-day range-finding oral toxicity study that was similar to TG 407, chlormequat chloride (purity 64.05%) was administered to Wistar rats (10/sex per dose level) at dietary doses of 0, 4000, 5500, 7500 or 10 000 ppm (equal to 0, 423, 590, 791 and 1067 mg/kg bw per day for males and 0, 495, 637, 912 and 1200 mg/kg bw per day for females, respectively).

One female at 4000 ppm and three females at 5500 ppm died, but there were no deaths at 7500 or 10 000 ppm, and the cause of death could not be established. Body weights, body weight gain and feed consumption were decreased in the two highest dose groups, but whether this was due to systemic toxicity or unpalatability is unclear.

Full necropsy and histopathology were not carried out (Medhamurthy, 1996).

In a 90-day pre-GLP oral toxicity study, first evaluated by the 1997 Meeting, rats (20/sex per group) were fed chlormequat chloride at dietary levels of 0, 200, 600 or 1800 ppm (equivalent to 0, 10, 30 and 90 mg/kg bw per day). There were no deaths, no clinical signs of reaction to treatment and no treatment-related changes in blood chemistry. The body weight gain of males fed 1800 ppm was slightly depressed in comparison with that of controls. Slightly increased kidney weights were recorded in treated female rats and slightly increased liver weights were recorded in treated males, particularly at 1800 ppm; however, histopathological examination of major organs revealed no treatment-related changes (Levinskas, 1965).

In a 13-week oral toxicity study conducted according to TG 408, chlormequat chloride (purity 97%) was administered to Sprague Dawley CRJ:CD rats (10/sex per group) at dietary levels of 0, 300, 900 or 2700 ppm (equal to 0, 21, 61 and 189 mg/kg bw per day for males and 0, 24, 73 and 220 mg/kg bw per day for females, respectively). Full clinical chemistry, macroscopic and histopathological evaluations were conducted. There were no mortalities or test substance-related clinical signs of toxicity, but there were reductions in feed consumption, body weight (< 10%, $P < 0.05$) and body weight gain (11%, $P < 0.05$) in high-dose males.

A reduction in feed consumption occurred in the last four weeks of the study, with a statistically significantly lower body weight in the final five weeks of treatment; this was considered to be due to systemic toxicity and/or the reduced feed consumption.

Urine analysis and haematological and clinical chemistry evaluations did not show any adverse impacts except for a statistically significant decrease in brain acetylcholinesterase activity in the high-dose males. Reductions in feed consumption, body weight and body weight gain were observed in high-dose males.

The NOAEL was 900 ppm (equal to 61 mg/kg bw per day) based on reduced body weight gain and feed intake in males at 2700 ppm (equal to 189 mg/kg bw per day) (Tanabe & Nagao, 1981).

In a quality-assured 90-day oral toxicity study conducted according to TG 408, Wistar rats (10/sex per group) were administered chlormequat chloride (purity 63.5%) at dietary concentrations of 0, 100, 1000 or 5000 ppm (equal to 0, 6.0, 61 and an estimated 305 mg/kg bw per day for males and 0, 7.9, 89 and an estimated 445 mg/kg bw per day for females, respectively). An additional group (of a similar size) was treated for 90 days with 5000 ppm followed by a treatment-free four-week recovery period.

There were no test substance-related mortalities in any of the dose groups. Clinical signs of urinary incontinence were observed in one high-dose female and two high-dose males but not in the recovery group. In addition, three males were found to have prolapsed penis, but this finding was not clearly treatment related and not reported by other studies.

Body weight gains were markedly reduced at 5000 ppm (from about 60% to 20%) in the first four weeks of treatment, with statistical significance ($P = 0.05$) by week 3, such that body weight lagged behind the other groups for the duration of the treatment. A trend was also evident at 1000 ppm. Reversibility of the body weight reduction was indicated in males but much less so in females. Reduced body weight gain is consistent with observations in the other short-term rat studies. Some reversibility was observed in the males, but there appeared to be confounding with a marginal gain in body weight

in the female terminal group, as opposed to the 5000 ppm recovery group, which lagged behind all the other groups. There were no effects on feed consumption (Suresh, 1992b, 2002 amendment).

There were inconsistent changes in haematological parameters. The only clinical chemistry effects observed were a slightly statistically significant ($P = 0.05$) perturbation in sodium concentration in the highest dose males (control: 146 m equiv./L; 5000 ppm: 141 m equiv./L); and potassium in females (controls: 4.60 m equiv./L; 5000 ppm: 4.20 m equiv./L), which were reversible. These changes may have been due to the high levels of chloride in the diet, and were also observed in the 28-day study conducted at the same laboratory (Suresh, 1991g, 2002 amendment).

There was an absence of histological findings, but relative and absolute liver organ weights (12–15% and 7–10%, respectively) were increased at the highest dose for both males and females; these returned to levels similar to controls for the recovery group, so can be considered an adaptive rather than an adverse toxicological effect.

The NOAEL was 1000 ppm (equal to 61 mg/kg bw per day) based on decreased body weight gain in males at 5000 ppm (approximately 305 mg/kg bw per day) (Suresh, 1992b, 2002 amendment).

In a 90-day oral toxicity study, chlormequat chloride (purity 700g/L, 1.2% HCl stated as an impurity) was administered to rats. The study was mostly compliant with TG 408, but had some deviations that included limited clinical chemistry evaluations (only glucose, blood urea nitrogen, serum alanine aminotransferase and electrophoresis) and limited modifications of the initial highest dose group. Test chemical intake was also not included in the report. Rats were given dietary doses of chlormequat chloride at 0, 500, 1500 or 4500 ppm (equal to 0, 50, 150 and 450 mg/kg bw per day). The 4500 ppm group had their dose raised to 9000 ppm (equivalent to 900 mg/kg bw per day) in the eleventh week, and additional groups (10/sex) were treated at 9000 ppm for seven weeks, followed by a six-week recovery period.

Body weight gain decreased in the two higher dose groups of 4500/9000 ppm. In the recovery phase, the females recovered completely, but the males did not recover within the study time frame. Feed consumption also decreased for these high-dose groups. There were no mortalities or signs of clinical toxicity in any of the groups, but sensitivity to noise was observed in the highest dose groups.

No haematological changes were observed during the treatment period, but haemoglobin, mean cell haemoglobin and mean cell haemoglobin concentration were reduced at the end of the recovery phase; as such, these changes may not be treatment-related. At 9000 ppm, total leucocytes were increased.

As noted, clinical chemistry parameter measurements were limited, but no differences were observed in the results of the tests conducted during the treatment period, bar glucose levels, which were slightly elevated at the end of the recovery phase.

There was an absence of gross or histological findings at all dose levels.

The NOAEL was 1500 ppm (equivalent to 150 mg/kg bw per day) based on decreased body weight gain and feed consumption at 4500 ppm (equal to 450 mg/kg bw per day) (Sterner, 1977a).

Dog

In a four-week range-finding oral toxicity study conducted according to TG 407, chlormequat chloride (purity 67.4%) was administered to beagle dogs (2/sex per dose level) at 0, 270, 404, 539, 674 or 809 ppm (equivalent to 0, 9, 13, 17, 26 and 30 mg/kg bw per day, respectively, for both sexes).

There were no test substance-related mortalities, and feed consumption and body weight were not affected by treatment. Histopathological evaluations were not conducted. In the groups dosed at 404 ppm and above, a slight to moderate salivation was observed in all animals, but the intensity and frequency was variable. This may have been a localized neuropharmacological effect of chlormequat chloride, via the nicotinic and muscarinic acetylcholinesterase activated receptors.

The NOAEL was 270 ppm (equivalent to 9 mg/kg bw) based on the salivation at 404 ppm (13 mg/kg bw per day) (Hellwig, 1993).

In a similar four-week range-finding oral toxicity study, chlormequat chloride (purity 67.4%) was administered to beagle dogs (2/sex per dose level) at 0, 1000 or 1200 ppm (equal to 0, 33 and 42 mg/kg bw per day, respectively, in both sexes).

There were no test substance-related mortalities, and feed consumption and body weight were not affected by treatment. Clinical signs of toxicity included diarrhoea on most days in males and females at 1200 ppm and sporadically in males at 1000 ppm; unsteady gait in one male at 1200 ppm (on days 6 and 7) and in both males at 1000 ppm on day 6; and a high stepping gait on the first day of administration in the other high-dose male.

Salivation ranged from moderate to severe at 1200 ppm and from slight to severe at 1000 ppm, in both sexes. In the high-dose group, other clinical signs in females included lachrymation on most days and hindlimb weakness in one female on day 6. This animal also had abdominal-lateral position, seal-like position of the extremities, paresis of the hind limbs, hypothermia, apathy and high stepping gait on day 7. However, in the following days there were no further observations of this nature for this or the other animals.

Clinical chemistry and haematological assessments and urine analysis did not show any test chemical-related adverse effects. Gross pathology and histopathology were conducted; there were no indications of adverse effects.

No NOAEL could be identified as effects were observed at all dose (Mellert, 1993).

In an older three-week range-finding oral toxicity study, chlormequat chloride was administered to beagle dogs (3/sex per dose level) at 0, 100, 300 or 900 ppm (equal to 0, 3.9, 12 and 34 mg/kg bw per day, respectively, in both sexes). No TG compliance or quality assurance information was provided.

There were no test substance-related mortalities. Feed consumption was not affected by treatment, but there were slight reductions in body weight gain in males at 900 ppm. The slightly reduced body weight gain in males at 300 ppm were considered not treatment related, as there were no overall effects on body weight at study end. Clinical chemistry and haematological assessments and urine analysis did not show any test substance-related adverse effects. Organ weights and gross pathology and histopathology indicated no adverse effects.

The NOAEL was 300 ppm (equal to 12 mg/kg bw) based on body weight changes in male dogs at 900 ppm (equal to 34 mg/kg bw per day) (Chesterman et al., 1976).

In a 90-day oral toxicity study, chlormequat chloride (purity not stated; liquid: 700g/L, 1.2% HCl stated as an impurity) was administered to beagle dogs (3/sex per group) in the diet at concentrations of 0, 100, 300 or 600 ppm, with the top dose level raised to 800 ppm in week 8, 1200 ppm in week 10, 2000 ppm in week 11 and 2500 ppm in week 12 (equivalent to 0, 2.5, 7.5 and 15/20/30/50/62.5 mg/kg bw per day).

There were no treatment-related deaths. Body weight gain decreased in high-dose animals from week 9 onwards. Feed consumption was not affected by treatment. From week 10, increased salivation was observed 3–6 hours after feeding in two animals at 300 ppm and was pronounced in one animal at 1200 ppm and in all animals in the high-dose group (2000 and 2500 ppm) from week 11. Animals dosed with 2500 ppm (weeks 12 and 13) were observed to have a reduced reflex response, apathy and staggering gait; one male had a pronounced lack of coordination and paresis of the hind limbs. These clinical signs appeared 3–6 hours after feeding and were only slightly in evidence 20 hours after feeding.

Urine analysis found increased albumin secretion in high-dose animals at treatment end. There were no consistent organ weight changes in any of the dose groups. Gross and histopathological examinations found no treatment-related changes at any dose level. Haematological and clinical chemistry evaluations found no treatment-related changes. An electrocardiograph (ECG) of control and high-dose animals showed no treatment-related changes.

The NOAEL was 100 ppm (equivalent to 2.5 mg/kg bw per day) based on salivation at 300 ppm (equivalent to 7.5 mg/kg bw per day) (Sterner, 1977b).

In a one-year dietary toxicity study, beagle dogs (5/sex per group) were given technical grade chlormequat chloride (purity 67.4%) mixed in the diet at doses of 0, 150, 300 or 1000 ppm (equal to 0, 4.7, 9.2 and 31 mg/kg bw per day for males and 0, 5.2, 10 and 32 mg/kg bw per day for females, respectively, when corrected for purity; achieved concentrations of chlormequat chloride in the vehicle were equivalent to the nominal concentration [Mueller & Hofer, 2007]). Feed consumption was determined daily and body weight once a week. In addition to clinical, haematological and ophthalmological examinations, urine analysis and neurofunctional examinations were carried out. At study end, all animals underwent gross pathological and histopathological examinations.

Animals at 1000 ppm had diarrhoea, vomiting, salivation, apathy and other severe clinical signs as well as many changes in clinical chemistry and haematological parameters. Two dogs at this dose died. Diarrhoea, vomiting and salivation were also seen at 300 ppm.

The NOAEL was 150 ppm (equal to 4.7 mg/kg bw per day) based on diarrhoea, vomiting and salivation at the LOAEL of 300 ppm (equal to 9.2 mg/kg bw per day) (Mellert et al., 1993).

In an 106–108-day oral toxicity study for which no detailed report was available to the 1997 Meeting, dogs (2/sex per group) were fed chlormequat chloride at dietary levels of 0, 20, 60 or 180 ppm (equivalent to 0, 0.5, 1.5 and 4.5 mg/kg bw per day) for 106–108 days.

There were no deaths and no clinical signs of reaction to treatment. Body weight gain and feed intake were unaffected. Organ weight analysis and histopathological examination at study termination found no treatment-related changes (Levinskas, 1965).

In a two-year oral toxicity study that was not conducted to current scientific standards, beagle dogs (3/sex per group) were fed chlormequat chloride (technical grade purified twice by recrystallization) at dietary levels of 0, 100, 300 or 1000 ppm (equivalent to 0, 2.5, 7.5 or 25 mg/kg bw per day); groups of 10 males and 10 females served as controls.

Excessive salivation and hind limb weakness were seen in some animals at 1000 ppm. One male died after 22 days and one female after 38 days. Blood chemistry and urine analysis found no treatment-related changes other than the presence of chlormequat chloride in the urine of treated animals. At study termination, gross pathology, organ weight analysis and histopathology showed no treatment-attributable changes. The lack of adherence to acceptable scientific standards precluded establishment of a reliable NOAEL (Oettel & Sachsse, 1967).

(b) Dermal application

Rabbit

In a dermal toxicity study, New Zealand White rabbits (10/sex per group) received chlormequat chloride (purity 99%) by repeated occluded dermal applications on shaven skin, 5 days/week for three weeks at doses of 0, 20, 50 or 150 mg/kg bw per day. Reactions to treatment at the application site were limited to erythema during the first two weeks of the study; however, these reactions were no more severe than reactions frequently seen after repeated occluded dermal applications of control compounds. There were no other clinical signs, and body weight gain and feed intake were unaffected by treatment. Investigation of haematological parameters and blood chemistry, gross pathology, organ weights and histopathology showed no reaction to treatment.

The NOAEL for systemic toxicity was 150 mg/kg bw per day. However possible signs of slight and transient local irritation may have been observed at 50 and 150 mg/kg bw. There were no signs of local irritation at a dose of 20 mg/kg bw (Buch & Finn, 1981).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a 78-week toxicity and carcinogenicity study, in accordance with currently accepted scientific standards, CFLP mice (52/sex per group) were fed chlormequat chloride (purity 98.5%) at dietary levels of 0 or 1000 ppm (equivalent to 0 or 150 mg/kg bw per day). Survival was unaffected, and there were no clinical signs of reaction to treatment, except that treated animals gained less weight than controls. Histopathological examination was initially restricted to 10 animals/sex per group, but was extended to all tissues from animals in which a treatment-related effect was seen.

The incidence of benign lung tumours was higher (20/52) in treated males than in controls (10/51), but was within the normal range in untreated mice. The incidences of lung tumours in females and of tumours in all other organs examined in animals of each sex were not statistically significantly higher in the treated group than in controls (Weldon et al., 1971).

In a 110-week toxicity and carcinogenicity study, B6C3F1/CrlBr (VAF) mice (50/sex per group) were given diets containing chlormequat chloride (purity 67.4%) at doses of 0, 150, 600 or 2400 ppm (equal to intakes of chlormequat chloride of 0, 21, 84 and 336 mg/kg bw per day for males and 0, 23, 91 and 390 mg/kg bw per day for females, respectively). Satellite groups comprising 10 mice/sex received chlormequat chloride at the same dietary concentrations for 52 weeks (measured intakes of chlormequat chloride were 0, 23, 89 and 355 mg/kg bw per day for males and 0, 28, 109, and 445 mg/kg bw per day for females, respectively). The animals were inspected daily and more thoroughly weekly for clinical signs. Body weight and feed consumption were recorded weekly during the first 14 weeks of the study and thereafter every four weeks. The animals in the satellite group were killed at 52 weeks and those in the main group at 110 weeks. Blood was drawn from all killed animals and differential blood counts were carried out. The mice were examined postmortem both grossly and histopathologically.

No treatment-related clinical signs were observed. Survival was not affected by the test material at any dose. Animals killed at 52 weeks showed a reduction in weight gain at the highest dose at some times, but the significance of this finding is hard to assess because of the small group sizes and because of confounding as the body weight of the control females was unusually high (mean 41.4 g compared to 38.8 g in the main group; specifically, two females weighed 51.3 and 48.2 g) particularly as no such reduction was seen in the main groups, killed at 110 weeks. No significant differences in feed consumption were seen between groups. Minor intergroup differences in leukocyte counts lacked consistency and are unlikely to be of biological significance.

No treatment-related differences in organ weights were seen between groups. The only organ-specific findings that appeared to be related to treatment were increased incidences of ovarian tubular down growth and of cystic endometrial hyperplasia at the two higher doses. However, the study pathologist considered these to be secondary treatment-related effects of minor toxicological significance, as they are known to belong to a spectrum of age-related spontaneous lesions (Haseman, Hailey & Morris, 1998; Brayton, 2013), and have been explained by the presence of a more sustained and higher level of endogenous estradiol in B6C3F1 mice (Gervais & Attia, 2005). Furthermore, the sponsor rechecked the histological sections to clarify the diagnostic term “tubular downgrowth” (Gröters, 2017) or “ovary surface epithelium” and according to current diagnostic criteria, the ovary finding would be considered to represent “atrophy”, which is a normal age-related background observation in aged mice. The apparent increase in incidence/severity in the high-dose group compared to the controls is likely related to the higher survival of the high-dose group females. There was no treatment-related increase in tumour incidence.

The 1999 JMPR determined the NOAEL to be 150 ppm (equal to 23 mg/kg bw per day) based on these histopathological effects in the uterus and ovaries at 600 ppm (Annex 1, reference 86).

With the re-evaluation of the ovarian histology, the NOAEL was determined to be 2400 ppm (equal to 336 mg/kg bw per day), the highest dose tested (Mellert et al., 1994).

In a 102-week cancer bioassay study, B6C3F1 mice (50/sex per group) were fed diets containing chlormequat chloride (purity 97–98%) at doses of 0, 500 or 2000 ppm (equivalent to 0, 70

and 290 mg/kg bw per day). The dietary levels were set on the basis of the results of an eight-week study with doses of 1200–20 000 ppm designed to provide a statistical estimate of the dose that would depress body weight gain by 10%. The control group consisted of 20 males and 20 females. Organ weights were not determined. Test substance intake was not determined.

Body weight gain remained largely unaffected by treatment, and there were no treatment-related clinical signs. Survival was unaffected by treatment and was adequate for assessment of carcinogenicity, as at least 80% of animals in each group survived until study termination. A trend in hepatocellular carcinomas occurred in the male mice at dose-related incidences ($P = 0.036$), but direct comparisons of the incidences in the individually dosed groups were not significantly higher than the controls (controls: 7/20; low dose: 13/50; high dose: 23/49). The combined incidence of haemangiomas and haemangiosarcomas was slightly increased in treated females (controls: 1/20; in controls, low dose: 4/50; high dose: 5/50), but no tumours occurred in the female mice at incidences statistically significant for a positive dose-related trend or for greater incidences in dosed groups than controls.

The authors concluded that there was no clear evidence for the carcinogenicity of chlormequat chloride in these mice (National Cancer Institute, 1979). This conclusion was further supported by later historical data from the NCI published in the open literature (Haseman, Hailey & Morris, 1998).

Several chronic inflammatory, degenerative or proliferative lesions frequently seen in aged B6C3F1 mice occurred with approximately equal frequency and severity in the dosed and control animals.

The Meeting concluded that there was no clear evidence for the carcinogenicity of chlormequat chloride in these mice.

In an 18-month carcinogenicity study, chlormequat chloride (purity 63.5%) was administered to Swiss albino mice (50/sex per dose) at dietary dose levels of 0, 250, 1000 or 4000 ppm (equal to 0, 20, 79 and 323 mg/kg bw per day for males and 0, 22, 91 and 352 mg/kg bw per day for females, respectively). Animals were examined for clinical signs of toxicity and mortalities once per day, and other parameters were assessed in accordance with test guidelines; however, organ weights were not determined. At nine months and at the end of the administration period, differential white blood counts were measured. After 18 months of treatment, all surviving animals underwent gross pathology and histopathology.

The histopathological evaluation of high-dose animals killed at study termination found statistically significantly higher incidences of a number of findings (see Table 2).

Table 2. Histopathological findings[a] in 18-month carcinogenicity study with chlormequat chloride in mice

Histological finding [a]	**Incidence of finding per dose level** [a]			
	Males		**Females**	
	0 ppm	**4000 ppm**	**0 ppm**	**4000 ppm**
Mucosal cysts in gall bladder	0 / 29	3 / 22	–	–
Cystic glands in trachea	6 / 29	16 /22	–	–
Chronic inflammation in pancreas	0 / 29	5 / 22	–	–
Nephropathy	9 / 29	13 / 22	–	–
Lymphocytic infiltration in the epididymis	2 / 29	6 / 22	–	–
Pituitary cysts	0 / 29	5 / 22	–	–
Lymphocytic infiltration in the stomach	–	–	14 / 22	18 / 20
Lymphoid hyperplasia in mandibular lymph node	–	–	11 /22	9 / 20 [b]

no.: number; ppm: parts per million *Sources*: Suresh (1998, 2002 amendment)

[a] Statistically significantly higher incidences of findings in high-dose group versus concurrent controls. Shown as no. of animals with the finding / no. of animals examined.

[b] Although mentioned in the summary of the report, the incidence is similar to that of the control. An amendment to the report stated that these changes were considered incidental as there was no dose dependency or the incidences were within the historical data range.

There were no treatment-related increases in mortality or signs of clinical toxicity in any of the treatment groups, but survival was just under 50% in some groups (including the female control group).

There were no treatment-related increases in incidences of neoplasia in any of the treatment groups. Furthermore, the limited end-points assessed prevent identification of a NOAEL for nonneoplastic effects (Suresh, 1998, 2002 amendment). In conclusion, chlormequat chloride was not carcinogenic in this study.

Rat

In an 18-month chronic toxicity study according to OECD TG 452 (1981), Wistar rats (20/sex per group) received diets containing chlormequat chloride (purity 67.4%) at nominal concentrations of 0, 281, 937 or 2810 ppm (equal to 0, 12, 43 and 136 mg/kg bw per day for males and 0, 15, 56 and 172 mg/kg bw per day for females, respectively)[1]. The rats were examined daily and more thoroughly once a week. Ophthalmological examinations were carried out at study start and end. Feed consumption and body weight were determined weekly for the first 14 weeks and thereafter every four weeks. Clinical chemistry and haematological assessments and urine analysis were performed 3, 6, 12 and 18 months after the start of the study. The animals were killed after 18 months and examined grossly and histopathologically.

No treatment-related clinical signs were observed in any group, and ophthalmological examination showed no adverse effects. The mortality rate was unaffected by treatment. The body weight gain of males was reduced throughout the study and that of females at the highest dose from week 58 so that by the end of the study the weight of the males was 18% less than that of concurrent controls, while that of the females was 10% less. The feed consumption of males was reduced from week 54 and that of females from time to time. Lower doses had no effect on weight gain or feed consumption. Although some differences in clinical chemical and haematological parameters were seen between groups, they were marginal, sporadic and showed no relation to dose; they were therefore considered not toxicologically significant. At autopsy, the only finding attributable to treatment was decreased body weight gain in high-dose animals compared with controls and consequent differences in the relative weights of the kidney, brain and liver. No treatment-related histopathological changes were found.

The NOAEL was 937 ppm (equal to 43 mg/kg bw per day) based on reduced body weight at 2810 ppm (equal to 136 mg/kg bw per day) (Schilling et al., 1992).

In a two-year carcinogenicity study according to OECD TG 451 (1981), chlormequat chloride (purity 67.4%) was administered to Wistar rats (50/sex per group) at concentrations of 0, 280, 940 or 2800 ppm (equal to 0, 13, 42 and 120 mg/kg bw per day for males and 0, 16, 55 and 170 mg/kg bw per day for females, respectively). The animals were observed daily and inspected weekly. Body weight and feed intake were determined weekly for the first 14 weeks and every four weeks thereafter. At scheduled kill, the survivors were examined grossly and selected tissues underwent histopathological examination. Clinical chemistry and haematological examinations, urine analysis and brain acetylcholinesterase activity was measured in 20 animals/sex per group.

No treatment-related clinical signs were seen, and the mortality rate was not affected. Reduced body weight gain and feed consumption were seen in high-dose males and females. Weight gain was decreased throughout the study, by 14% in males and 22% in females, in comparison with concurrent controls. Feed consumption in males was reduced throughout study; in females, it was reduced during the latter part of the study. Some intergroup differences were seen in clinical chemical and haematological findings, but these were inconsistent and likely unrelated to treatment. Chlormequat chloride did not affect plasma, erythrocyte or brain cholinesterase activity. No treatment-related neoplastic or nonneoplastic histopathological changes were seen.

The NOAEL was 940 ppm (equal to 42 mg/kg bw per day) based on reduced weight gain and feed consumption at the highest dose of 2800 ppm (equal to 129 mg/kg bw per day) (Mellert et al., 1992).

1 Note that test substance intake values were recalculated for the lowest dose of 281 mg/kg as the actual concentration at this dose was 87.5–89.1% of the target. These corrected and recalculated values are provided now in this Meeting report. The original study data had been calculated erroneously on the basis of purity of 72%.

A carcinogenesis bioassay was conducted according to TG 451 but with major deviations: only 20 control animals were used, there were two dose groups instead of three, and organ weights were not determined. As a result, this study can only be used for the determination of the carcinogenic potential. Chlormequat chloride (purity 97–98%) was administered to Fischer F344 rats (50 rats/sex per dose) at either 1500 ppm or 3000 ppm (equivalent to 75 and 150 mg/kg bw per day) for 108 weeks. A concurrent group (20/sex) acted as controls over the same time period.

There was no test substance-related increase in mortality or signs of clinical toxicity in any of the treatment groups. Body weights of treated rats were dose-dependently lower than the controls during the second half of the administration period. The body weights were presented graphically; from this, it was inferred that the mean body weight difference between high-dose and control animals was about 10% towards the end of the study. For low-dose animals, the difference was less than 5%. It seems likely that the very slight effect on body weight at 1500 ppm would not be statistically significant. Histopathological examinations did not find any treatment-related changes; the low numbers of tumours/ lymphomas found were generally similar to those observed in controls or were not statistically significantly different. In female rats, lymphoma or leukaemia occurred in a greater number of dosed than control animals (controls: 3/20 [15%]; low dose: 11/50 [22%]; high dose: 14/50 [28%]), but these differences were not statistically significant. In male rats, dose-related pancreas islet cell adenomas were observed ($P = 0.023$), but in direct comparisons the incidences in the individual dose groups were not significantly higher than those in the control group (controls: 0/19; low dose: 2/47; high dose: 7/49). As the occurrence of pancreatic tumours in treated males and of lymphoma or leukaemia in treated females cannot be definitively correlated with administration of chlormequat chloride, it was concluded that under the conditions of this bioassay chlormequat chloride was not carcinogenic in male and female Fischer F344 rats (National Cancer Institute, 1979).

Given the difficulties in diagnosing pancreatic lesions (Nolte et al., 2016) and considering the quality of the available data, the Meeting concluded that under the conditions of this bioassay, there was no convincing evidence that chlormequat chloride is carcinogenic in male and female Fischer F344 rats up to the highest dose tested (3000 ppm).

2.4 Genotoxicity

The results of tests for the genotoxicity of chlormequat chloride are summarized in Table 3. Chlormequat chloride has been extensively tested for possible mutagenic/genotoxic effects using a variety of end-points in a range of organisms. In bacterial cells, chlormequat chloride was negative in the presence or absence of metabolic activation in numerous tests with one exception (Kennelly, 1984). Chlormequat chloride was negative in a range of appropriate in vitro and in vivo tests with two exceptions: weakly positive results were obtained with the tester strain TA97 in one study with metabolic activation, and with *Escherichia coli* K12 only at pH 9 but not at pH 5–8, which is probably a consequence of the pH value (Sussmuth & Lingens, 1976).

In eukaryotic cells, chlormequat chloride was negative for point mutation and chromosomal aberration. No cytogenetic anomalies were found in human lymphocytes in vitro. There was no indication of DNA damage and repair or of unscheduled DNA synthesis in primary rat hepatocytes and in the SOS chromotest in bacteria (*E. coli* PQ37).

Chlormequat chloride did not induce chromosomal aberration after oral dosing in rats and mice including testing of lethal doses, and was negative for micronuclei in mice. There was no indication of a dominant lethal effect in two strains of male mice (NMRI and ICR/Ha Swiss mice).

Chlormequat chloride was negative in all in vitro and in vivo mutagenicity tests performed covering all three end-points (point mutation, chromosomal aberration and DNA damage and repair). It was negative in the sex-linked recessive lethal test and dominant lethal test.

Table 3. Summary of genotoxicity studies with chlormequat chloride

End-point	Test object	Concentration	Purity (%)	GLP status (Y/N)	Results	Reference
In vitro						
Reverse point mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538	≤10 000 µg/plate	NK	NK	±S9: negative	Hoorn (1983)
Reverse point mutation	*S. typhimurium* strains TA97, TA98, TA100, TA1535	≤5000 µg/plate	NK	NK	−S9: negative +S9: only TA97 weakly positive	Kennelly (1984)
Reverse point mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 *Escherichia coli* WP2*uvrA*	≤5000 µg/plate	66.1	Y	±S9; negative	Traul & Mulligan (1990)
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538	±2500 µL/plate	92.4	NK	±S9; negative	Zeller & Engelhardt (1979)
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538	100–10 000 µL/plate	720 g/L	Y	±S9; negative	Suresh (1993)
Reverse point mutation	*Escherichia coli* K12	4×10^8 to 1.2×10^9/mL	97–98	NK	−S9; negative at pH 5–8, positive at pH 9 (high bacteriotoxicity)	Sussmuth & Lingens (1976)
Reverse point mutation	*E. coli* WP2, WP2uvrA, WP2uvrA pKM 101	100–10 000 µL/plate	720 g/L	Y	±S9; negative	Suresh (1994a)
Reverse mutation	CHO cells, *hprt* locus	≤5000 µg/mL	66.1	Y	±S9; negative	Traul & Johnson (1990)
Reverse mutation	CHO cells	≤5000 µg/mL	NK	Y	±S9; negative highest dose did inhibit mitosis in the presence of S9	Kirkland (1984)

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End-point	Test object	Concentration	Purity (%)	GLP status (Y/N)	Results	Reference
Reverse mutation	CHO V79 cells, *hprt* locus	≤5000 µg/mL	94.5–98.9	Y, OECD TG 476 (1984)	±S9; negative	Debets et al. (1986)
Chromosomal aberration	Human lymphocytes	≤5000 µg/mL	94.5–98.9	NK	±S9; negative	Enninga et al. (1987)
SOS chromotest	*E. coli* PQ37	NK "own results unpublished"	NK	Y, OECD TG 473 (1983)	±S9; negative	Von der Hude et al. (1988)
Unscheduled DNA synthesis	Rat hepatocytes	≤7.5 µg/mL	66.1	Y	Negative	Pant & Law (1990)
Unscheduled DNA synthesis	Rat hepatocytes	≤10 000 nL/mL	72	NK	Negative	Cifone & Myhr (1987)
Unscheduled DNA synthesis	Rat hepatocytes	0.10, 0.33, 1.0, 3.33 or 10 mmol/L	97.1	Y	Negative	Timm (1987)
In vivo						
Dominant lethal mutation	Male NMRI mice	1 × 261 mg/kg bw	99.6	N, but scientifically valid	Negative	Englehardt and Gelbke (1979)
Dominant lethal mutation	ICR/Ha Swiss mice	IP: 26 or 30 mg/kg bw Gavage: 12.5 or 25 mg/kg bw on 5 consecutive days	Unknown (Cycocel)	Presumed N	Negative	Epstein et al. (1972)
Micronucleus formation	Male and female NMRI mice	Oral dose: 0, 8.1, 40.5 or 202.5 mg/kg bw, on 2 consecutive days	94.5–98.9	Y	Negative	Guenard et al. (1983)
Chromosomal aberration	Male and female SD rats: bone marrow cells	Single oral dose: 0, 125, 250 or 500 mg/kg bw	66.1	Y	Negative	Sharma & Caterson (1991)
Chromosomal aberration	Swiss albino mice	Single oral dose: 0, 30, 100, 300 mg/kg bw	720 g/L	Y	Negative	Ponnana (1995)

(*continued on the next page*)

End-point	Test object	Concentration	Purity (%)	GLP status (Y/N)	Results	Reference
Sex-linked recessive lethal mutation	*Drosophila melanogaster*	IP doses of 0.2 µL of control, 1 and 10 mg/mL in 4-day-old males, then mated with females	NK	NK	Negative	Kramers, Knaap & Voogd (1975)
Dominant lethal mutation	*Drosophila melanogaster*	As above	NK	NK	Negative	Kramers, Knaap & Voogd (1975)

bw: body weight; CHO: Chinese hamster ovary; hprt: hypoxanthine–guanine phosphoribosyltransferase; IP: intraperitoneal; N: no; NK: not known; SD: Sprague Dawley; Y: yes

Chlormequat chloride has been adequately tested in a wide range of in vitro and in vivo mutagenicity/genotoxicity assays. The Meeting concluded that the overall weight of evidence indicates that chlormequat is unlikely to be genotoxic.

In view of the lack of genotoxic potential and absence of carcinogenicity in mice and rats, the Meeting concluded that chlormequat is unlikely to pose a carcinogenic risk to humans.

2.5 Reproductive and developmental toxicity

Multigeneration studies

Rat

In an early study conducted between 1965 and 1967 (noted not to be in accordance with currently accepted scientific standards), Wistar rats (20/sex per group) received diets containing chlormequat chloride at 0, 100, 300 or 900 ppm (equivalent to 0, 5, 15 and 45 mg/kg bw per day) throughout three generations.

No abnormalities were seen in the appearance, behaviour, feed intake, body weight gain, fertility, gestation, lactation or viability of the offspring, and no fetal malformations occurred that could be attributed to treatment. Histopathological examination of F_3 rats (10/sex per dose) after nine weeks of treatment revealed the presence of giant cells in the testicular tubules of four rats at 900 ppm and two at 300 ppm. The authors suggested that the cells were an expression of delayed maturation during spermatogenesis; however, the Meeting in 1997 found it difficult to assess the significance of the finding and concluded that the NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day) (Leuschner, Leuschner & Schwerdtfeger, 1967).

In a two-generation study of reproductive toxicity conducted according to OECD TG 416 (1983) and GLP, technical grade chlormequat chloride (purity 67.4%) was administered to Wistar rats (24/sex per group) at dietary levels of 0, 300, 900 or 2700 ppm (equal to 0, 29, 86 and 250 mg/kg bw per day for males and 0, 23, 69 and 230 mg/kg bw per day for females, respectively). At least 70 days after the beginning of treatment, F_0 animals were mated in order to produce an F_{1a} litter, and subsequently re-mated to produce an F_{1b} litter. Animals from the F_1 litter (24/sex) were selected as the F_1 parents and kept on the diet for at least 98 days before they were mated to produce an F_2 litter. The study was terminated after weaning of the F_2 litter. All animals were inspected daily, and the feed consumption of the F_0 and F_1 parents was determined weekly. Pups were observed for the usual parameters and for physiological development and behavioural effects (gripping reflex, acoustic startle and pupillary reflex). A statistically significant reduction in body weight was observed in F_0 and F_1 females at 2700 ppm, and feed consumption was moderately reduced in males and females of both generations. Transient tremor and hypersensitivity (mechanistically considered to be due to the nicotinic acetylcholine receptor leading to a depolarization of the muscle fibre, which after excitation of the muscle, leads to weakness and

tremor) were also observed in F_0 and F_1 females at this dose, mainly during or after the lactation period; this was reversible. Also at this dose, male fertility was reduced, probably due to reduced nutrition levels and reduced feed consumption, fewer pups were delivered by each dam, and the growth and development of the F_{1a}, F_{1b} and F_2 pups were retarded.

Reductions in pup weight gain at the highest dose level were associated with corresponding reduced weight gains of the maternal animals during gestation and lactation; in addition, high-dose females showed clinical signs during the lactation period, which likely negatively affected nursing behaviour. A direct effect on pup development is not apparent from the observations of the two-generation study with chlormequat chloride.

The NOAEL for parental toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced feed consumption and decrease in body weight and body weight gain in female and male parental animals of both generations at 2700 ppm (equal to 230 mg/kg bw per day).

The NOAEL for offspring toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced pup weight gain and retarded development during lactation at 2700 ppm (equal to 230 mg/kg bw per day).

The NOAEL for reproductive toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced numbers of pregnancies and delivered pups at 2700 ppm (equal to 230 mg/kg bw per day) (Hellwig et al., 1993).

In another two-generation study conducted according to GLP and OECD TG 416, dietary doses of chlormequat chloride (purity 63.5%) were given to Wistar rats (30/sex per dose; F_0 animals 7–8 weeks old at study start) as a constant homogeneous addition to the feed at dosages of 0, 10, 100 or 1000 ppm (equal to 0, 0.7, 6.8 and 69.3 mg/kg bw per day for males and 0, 1, 10.2 and 105.4 mg/kg bw per day for females, respectively). The doses were selected based on the results of a 90-day dietary study in the same rat strain using doses of 100, 1000 and 5000 ppm. At least 10 weeks after treatment initiation, F_0 animals were mated to produce the F_1 litter. F_1 pups (30/sex per dose group) were subsequently selected to mate and produce F_2 litters. These animals also received the test substance at identical dose levels. All F_2 pups were killed at weaning.

The parents' and pups' state of health was checked daily, and mating and reproductive performances were examined. All parental animals (F_0 and F_1), weanlings not selected for subsequent mating and all F_2 pups were examined macroscopically for structural abnormalities and pathological changes. Dead and moribund pups were examined for defects. The following organs were collected from dams and sires of all dose groups: ovaries, uterus, vagina, epididymis, seminal vesicles, prostate, coagulation glands, pituitary, adrenals, liver and kidneys. All reproductive organs showing gross lesions and all tissues of animals suspected for infertility were histopathologically examined. Pups were sexed and their physical development was monitored. Body weight and body weight gain in F_0 males and females were not affected prior to and during gestation and lactation. No dose response was noted for body weight development in F_1 sires and dams during the premating period. However, due to the longer duration of reduced body weight (up to week 9 in males and week 4 in females) a treatment-related effect at this dose level cannot be ruled out. During this period, body weight gain was reduced up to week 9 (males) or 4 (females); however, these effects were compensated for during the premating period of 13 weeks. No effect on body weight/ body weight development on parental animals was observed thereafter, during the gestation/lactation phases.

There were no parental mortalities or signs of clinical toxicity, no treatment-related effects on feed consumption and no significant effects upon body weight in any group.

The only effect noted on reproduction was a decrease in live birth index from 100% (control) to 96.8% (high dose) in a dose-dependent manner in F_0 dams. In the F_1 dams, this index was 99.7% (control), 98.1% (low dose), 99.7% (mid dose) and 93.2% (high dose), with statistical significance ($P < 0.05$) at the high dose. The 24-hour survival index was 98% for high-dose F_0 pups and 100% for all other groups of F_0 and F_1 pups. The four-day survival index decreased from 98.3% (control) to 94.1% (high dose) without dose response for F_0 pups, but F_1 pups did not show such an effect. Control values of 98.4% were similar to the high dose of 98.7%. This inconsistency between generations may not be treatment related. As no historical control values were available at the time the study was performed, an effect at 1000 ppm could not be completely ruled out at the time. Subsequent historical control data for 10 studies conducted between 1991 and 1996 indicate that this was within the historical control range.

Mean viable litter size, pup body weight and physical development were not affected. There was no effect on reproductive parameters; macroscopic and histopathological examination of reproductive organs found no effects.

To summarize, chlormequat chloride had a slight effect on body weight during the premating phase, particularly in the F_1 rats at the high dose level of 1000 ppm, but it is not clear whether this was adverse. At this dose level, the live birth index and 4-day survival index were also slightly affected; a nonsignificant trend was apparent for day 14 and 21 survival indices. No other adverse effects on dams, sires and their offspring were evident.

On the basis of historical control data indicating that the decreased live birth index and four-day survival index were within additional historical controls, the parental NOAEL was determined to be 100 ppm (equal to 6.8 mg/kg bw per day) based on reduced body weight gain, clinical signs during lactation (females) and marginal anaemia at 1000 ppm (equal to 69.3 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 69.3 mg/kg bw per day), the highest dose tested. The NOAEL for reproductive toxicity was 1000 ppm (equal to 69 mg/kg bw per day), the highest dose tested (Suresh, 1995, 2002 amendment).

In another two-generation study conducted according to GLP and TG 416, chlormequat chloride (purity 65.8%) was administered in the diet to Wistar rats (25/sex per dose; F_0 animals 5–6 weeks old at study start). The doses were selected based on the results of a four-week range-finding study in the same rat strain and testing facility (Medhamurthy, 1996). The doses were 0, 100 (equal to 8.4–8.5 mg/kg bw per day for males and 9.1–9.6 mg/kg bw per day for females, respectively), 500 (equal to 41.4–43.3 mg/kg bw per day for males and 46.3 and 48.9 mg/kg bw per day for females, respectively) or 2500 ppm (equal to 211.1 and 232.2 mg/kg bw per day for males and 241.3 and 259.0 mg/kg bw per day for females, respectively). The test substance was analysed for purity, homogeneity and stability. Test substance preparations were examined for stability and homogeneity. The test concentrations were verified throughout the study period.

Twelve weeks after treatment initiation, F_0 animals were mated to produce the F_{1a} litter and subsequently re-mated to produce the F_{1b} litter. Of the F_{1b} pups, 20 animals/sex per dose group were mated to produce the F_{2a} and F_{2b} generation. F_1 animals received the test substance for 11 weeks at identical dose levels to the F_0 animals.

The parents' and pups' state of health was checked daily, and mating and reproductive performances were examined. Haematological, biochemical and ophthalmoscopic examinations were performed on F_0 and F_1 parents. Litter size and loss, clinical litter inspection, sex, number of surviving pups and pup weight, physical and behavioural abnormalities were assessed; ophthalmoscopic examination was performed on F_{1b} and F_{2b} animals. At weaning, gross necropsy was conducted on 20 animals/sex per group from F_{1a} and F_{1b} litters. Twelve F_1 females/group were selected for clinical chemistry examinations during and after the lactation period. Gross and histopathological examinations were conducted in control and high-dose parents plus those animals in the low- and mid-dose groups with potentially suspect organs (with special attention paid to the reproductive organs).

There were no treatment-related effects on parental parameters, offspring parameters or reproductive data at the low and mid dose. At the high dose (2500 ppm), feed consumption was not affected, but body weight development was impaired in both sexes in all generations. Clinical signs were seen only during the lactation period; dams showed severe signs of exhaustion – tremor, passivity, piloerection, laboured respiration, increased vocalization. However, these clinical signs were reversible and maternal behaviour was not affected. Clinical chemistry and haematology showed slightly reduced erythrocyte counts, haemoglobin and haematocrit. Slight but statistically significant polychromasia was seen, particularly in females in the first two weeks of lactation; this was more pronounced in the F_1 generation (by 7%). There were no gross pathological findings in parental animals or offspring. However, histological examination of F_{2b} animals at seven weeks of age showed multifocal dystrophic changes in skeletal muscles (especially lumbar and thigh) in all 20 high-dose males and females examined. The muscle sarcoplasm was characterized by multifocal hyaline, vacuolar, granular and necrotic floccular lesions that were sometimes associated with focal fibroblastic proliferation. Calcification of the dystrophic muscle fibres was sometimes observed and no repair process was identified. These dystrophic changes were graded from minimal to marked, and were considered treatment-related. The authors found a reference to a similar dystrophic effect on skeletal muscle in rats that can be due to magnesium deficiency

(Benirschke, Garner & Jones, 1978), but no mechanistic interpretation was offered in the sponsor's report and these effects were not looked for in the other two multigeneration studies submitted. The F_{2b} animals were not examined for clinical chemistry or haematology. In addition, effects on skeletal muscle were not noted in other repeated oral toxicity studies, for example, in the 28-day study in the same rat strain and laboratory (Nemec, 1991) in which the highest dose level tested was also 2500 ppm or in a 90-day feeding study in Wistar rats at dose levels of up to 5000 ppm (Suresh 1992b, 2002 amendment).

No such changes were noted in the low-dose, mid-dose or control animals. There were no pathological findings in the reproductive organs. In spite of the clear effects (clinical signs, body weight development) in dams, reproductive parameters such as fertility, length of gestation and litter losses were comparable to the untreated control. No treatment-related effect was noted in pups with respect to viability and lactation indices; however, body weight and body weight gain was clearly affected at the high dose. Chlormequat chloride caused no effect in parental animals or pups at doses of 100 and 500 ppm. Toxic effects – reduced body weight and body weight gain – were noted in parental animals at 2500 ppm; toxic effects in dams during lactation were reversible thereafter. In addition, polychromasia was noted. Reduced pup weight and gain and dystrophic changes in the skeletal muscle of the offspring were also noted. Viability and lactation indices were not affected (such as reproductive parameters including examination of those organs).

The parental NOAEL was 500 ppm (equal to 41.4 mg/kg bw per day for males and 46.3 mg/kg bw per day for females) based on reduced body weight and body weight gain at 2500 ppm (equal to 211 mg/kg bw per day).

The offspring NOAEL was 500 ppm (equal to 41.4 mg/kg bw per day) based on reduced pup weight and pup weight gain and dystrophic changes in the skeletal muscle of the offspring.

The reproductive NOAEL was 2500 ppm (equal to 211 mg/kg bw per day), the highest dose tested (Gandalovicová, 1993).

(b) Developmental toxicity

Rat

In a developmental toxicity study conducted according to GLP and TG 414 (1981), Wistar rats (25 females/test group) were administered chlormequat chloride (purity 75.7% [757 g/L]) by gavage at doses of 0 (control treated with the vehicle bi-distilled water only), 25, 75 or 225 mg/kg bw per day with a constant dosing volume of 10 mL/kg bw from postcoitum day 6 to 15. The doses were selected on the basis of a range-finding study in the same strain of rats. The animals were observed for feed consumption and body weight gain regularly throughout the study period. On postcoitum day 21, all females were killed and assessed by gross pathology. The fetuses were dissected from the uterus, sexed, weighed and further investigated for any external, soft tissue and/or skeletal findings.

Of the rats, 25/25 were pregnant in all dose groups except the highest, where 23/25 females were pregnant. Clinical signs were only observed at the high dose level (225 mg/kg bw), and consisted of ruffled fur, ventral recumbency, tachypnoea/deep respiration, abnormal posture and isolated tremor. Feed consumption was distinctly reduced during the entire treatment period at the high dose of 225 mg/kg bw. A slight reduction was also noted at 75 mg/kg bw during the first half of treatment. Body weight gain and mean corrected body weight gain (corrected for uterus weight) were clearly reduced at 225 mg/kg bw but not at lower doses. No macroscopic changes were noted in dams at scheduled kill, which could be attributed to the test substance administration.

No effects on reproduction and fetal data were noted in any of the test groups. This includes external, visceral and skeletal investigations. There were no indications that chlormequat chloride causes malformations under the test conditions chosen.

The NOAEL for maternal toxicity was 75 mg/kg bw per day based on clinical signs and reduced body weight and feed consumption at 225 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 225 mg/kg bw per day, the highest dose tested (Becker & Biedermann, 1992a).

Rabbit

In a developmental toxicity study, Chinchilla rabbits (CHbb:CH hybrids, SPF quality; 16 females/test group) were administered chlormequat chloride (purity 75.7%; formulated in bi-distilled water) by gavage from postcoitum day 6 to 18 at 0 (control treated with bi-distilled water only), 10, 20 or 40 mg/kg bw per day, at a constant volume of 4 mL/kg bw with daily adjustment to the actual body weight. An additional dose group was added 7–11 days later as there were insufficient pregnancies at 20 mg/kg bw for the study to be in accordance with TG 414; the results of both groups were subsequently pooled.

Body weight and feed consumption were monitored throughout the study. The animals were examined twice daily for mortality and clinical signs. All surviving animals were killed on day 28 postcoitum and the fetuses were delivered by caesarean section. Reproduction and fetal data were compiled. Postmortem examinations of dams included gross macroscopic examinations of all internal organs. In addition, the uterus content, position of the fetuses and number of corpora lutea were examined. Fetuses were sexed, dissected and examined for external, internal and skeletal findings.

While there were no clinical findings or mortalities at 10 mg/kg bw per day, salivation was noted in two mid-dose females during the treatment period (see Table 4). The death of four females was not considered to be test substance-related: one animal died due to a gavage error; the others had only a slight impairment of body weight and no clinical signs (such as salivation), and died after the third, sixth and eighth administration of chlormequat chloride. Severe clinical signs such as salivation, ventral or lateral recumbency, tremor, tachypnoea and rhinorrhoea were noted in all animals at 40 mg/kg bw, from about 2.5 hours after dosing and lasting for two hours. The death of one animal showing these clinical signs was attributed to chlormequat chloride treatment.

Feed consumption was affected at the high dose level, with statistical significance in the middle of the treatment period ($P < 0.01$). After treatment, feed consumption, body weight and corrected body weight were found to be similar in all test groups, but at the high dose there was a statistically significant effect (postcoitum day 8–20) ($P < 0.01$). At this dose the corrected body weight (corrected for uterus weight) was also slightly lower when compared to the untreated control. There were no macroscopic findings related to chlormequat chloride in dams at any dose level.

The only treatment-related effect on reproductive parameters was a slight but statistically significant increase in mean postimplantation loss at 40 mg/kg bw per day, but the number of live pups per dam was greater at the high dose of 40 mg than in the controls. The increase of embryonic resorptions in the low-dose group was due to the very low control value and so was not considered to be a test substance-related effect.

Fetal body weights were affected only at the high dose (marginally reduced, without statistical significance), which is in accordance with maternal body weight development at this dose level. The sex ratio was normal in all groups. No treatment-related external, visceral or skeletal findings or malformations were noted.

Table 4. Summary of maternal data and reproductive/fetal parameters in a prenatal toxicity study with chlormequat chloride in rabbits

Parameter	Measure per dose level			
	0 mg/kg bw	**10 mg/kg bw**	**20 mg/kg bw**	**40 mg/kg bw**
No. of females mated	16	16	20	16
No. of pregnant dams	14	15	18	14
No. of dams used for calculation	14	15	14	13
No. of dams that died	0	0	4	1
Clinical signs (no. of dams affected)	–	–	Slight salivation (2)	Severe signs (14) [a]
Feed consumption on p.c. day 6–19, g/animal per day (% control)	186 (100%)	174 (93.5%)	187 (100.5%)	142 (76.3%) [b]

Parameter	Measure per dose level			
	0 mg/kg bw	10 mg/kg bw	20 mg/kg bw	40 mg/kg bw
Body weight gain on p.c. day 6–19, g/animal per day (% control)	119 (+3.3%)	74 (+2.1%)	103 (+2.9%)	−16 (−0.4%) [c]
Corrected body weight gain, net weight change on p.c. day 6–28 (% control)	−5.0%	−4.9%	−4.7%	−7.0%
No. of corpora lutea/dam (mean)	11.3	12.0	10.4	12.6
No. of implantations/dam (mean)	10.5	11.3	9.9	11.2
No. of total embryonic deaths (mean)	0.8	1.5	0.7	1.6*
Preimplantation loss (mean)	0.8	0.7	0.4	1.4
Postimplantation loss (mean)	0.8	1.5	1.1	1.6*
Embryonic resorptions/dam (mean)	0.1	0.5	0.1	0.2
Fetal resorptions/dam (mean)	0.7	0.9	0.6	1.5
No. of dead fetuses/dam	0	0	0	0
No. of live fetuses/dam (mean)	9.7	9.9	8.8	9.6
Fetal weights in g (mean)	33.3	33.7	33.4	32.2

bw: body weight; p.c.: postcoitum; *: $P < 0.05$ *Source*: Becker & Biedermann, 1992b

[a] One animal died [b] Statistically significant p.c. day 11–15 ($P < 0.01$).

[c] Statistically significant p.c. day 8–17 ($P < 0.01$) and 18–20 ($P < 0.05$).

The NOAEL for maternal toxicity was 10 mg/kg bw per day based on salivation seen at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day based on increased postimplantation loss and slightly reduced fetal weights (Becker & Biedermann, 1992b).

In a prenatal toxicity study dating from 1979, chlormequat chloride (purity 99%) was administered to female NN Russian/Himalayan CH/BB rabbits from post-insemination day 6 to 18 by gavage at doses of 0 (untreated control group), 1.5, 3, 6 or 12 mg/kg bw per day in a constant volume of 10 mL/kg bw per day. In all groups, including the control, temporary diarrhoea was observed. Severe agitation after gavage dosing was noted in two animals. No analytical determination was performed, and there was no vehicle (double distilled water) control group. Due to its hygroscopic nature, after the second treatment a 0.7631% stock solution was prepared and diluted for the various dosages.

Body weight and feed consumption were monitored throughout. The animals were examined daily for mortality and clinical signs. All surviving animals were killed on post-insemination day 28 and assessed by gross pathology. The fetuses were dissected from the uterus, sexed and weighed and their length determined. Internal organs were macroscopically examined, and skeletons were examined by X-ray. Transverse sections of the heads were prepared and assessed after fixing in Bouin's solution.

Maternal data and caesarean section/fetal results are presented in Table 5. Feed consumption was reduced during post-insemination days 6–12 at the low dose and post-insemination days 6–18 at all other dose levels when compared to the control ($P < 0.05$). Body weight remained unaffected while body weight gain was reduced at the high dose of 12 mg/kg bw per day during post-insemination days 6–12 ($P < 0.05$). One control animal and one at 6 mg/kg bw per day died; the causes of death were undetermined. Another two animals treated with 3 or 6 mg/kg bw per day died; severe agitation during gavage was noted as cause of death. Two dams treated with 6 mg/kg bw per day and one dam treated with 12 mg/kg bw per day spontaneously aborted. No pathological changes that could be attributed to the treatment were noted in the dams of all treated groups. The conception rate was unaffected. The average number of corpora lutea, viable and dead implants, resorptions (early, intermediate and late) and dead fetuses was unaffected by treatment. Placental weights, fetal weights and fetal length were comparable to the untreated control, and although there was a slightly statistically increased fetal weight at 12 mg/kg bw this was not considered test substance related. One fetus treated with 1.5 mg/kg bw had a cleft palate and pseudoankylosis. This isolated finding cannot be attributed to the test substance as it was not seen in any of the other studies. No effects were noted in the other treated groups and the

control. Examination of the internal organs and skeleton revealed no other anomalies. The number of variations and/or retardations in all treated groups was comparable to the untreated control (Merkle & Hofmann, 1979).

The Meeting concluded that this study was severely compromised by the temporary diarrhoea in all dose groups, including the control, and inadequate for the purposes of this risk assessment.

Table 5. Maternal and fetal data from prenatal toxicity study in Himalayan rabbits with chlormequat chloride

Parameter	Measure per dose level				
	0 mg/kg bw per day	**1.5 mg/kg bw per day**	**3 mg/kg bw per day**	**6 mg/kg bw per day**	**12 mg/kg bw per day**
Maternal data					
No. of inseminated does	15	15	21	21	14
No. of pregnant does (conception rate) [a]	12 (80%)	14 (93%)	15 (71%)	15 (71%)	12 (80%)
No. of does that aborted	0	0	0	2	1
No. of does that died	1	0	1	2	0
Clinical sign (no. of dams) [b]	Temporarily diarrhoea	Temporarily diarrhoea	Temporarily diarrhoea	Temporarily diarrhoea / salivation, accelerated respiration, apathia (1)	Temporarily diarrhoea / accelerated respiration, apathia (1)
Feed consumption	–	Reduced* (d 6–12 p.i.)	Reduced* (d 6–18 p.i.)	Reduced* (d 6–18 p.i.)	Reduced* (d 6–18 p.i.)
Body weight / body weight gain	–	No effect	No effect	No effect	Weight gain reduced** (d 6–12 p.i.)
Fetal data					
Corpora lutea (mean)	9.9	7.6	8.6	8.2	7.4
Total implantations/animal	5.3	4.1	5.5	4.3	5.0
Live fetuses/animal	4.9	3.5	4.9	3.9	4.3
Early resorptions (Salewski)	0	0	2	0	0
Early resorptions	3	1	4	3	8
Intermediate resorptions	1	6	0	3	0
Late resorptions	0	0	2	0	0
Dead fetuses	0	1	1	0	0
Placental weights; mean (g)	5.7	6.1	5.4	6.0	6.3
Fetal weights; mean (g)	37	37	36	36	40*

bw: body weight; p.i.: post insemination; *: $P < 0.05$; **: $P < 0.01$ *Source*: Merkle & Hofmann (1979).

[a] Results presented as the number and, in parentheses, the number of pregnant does as a percentage of the number of inseminated does.

[b] Results presented as the clinical sign and, in parentheses, the number of does with the described clinical sign.

In a study described in the 1972 monograph addendum (Annex 1, reference 82), groups of pregnant rabbits were fed diets containing chlormequat chloride at 0 or 1000 ppm (equivalent to 0 or 30 mg/kg bw per day) on days 1–28 of pregnancy and were killed two days before parturition. No evidence of teratogenicity was seen (Shaffer, 1970).

2.6 Special studies

(a) Neurotoxicity

Acute and subchronic neurotoxicity

No specific standard guideline-compliant neurotoxicity studies with chlormequat chloride have been performed. However, some neuropharmacological studies were conducted in order to determine the mechanism by which chlormequat chloride may have an effect on the nervous system (on muscarinic and/or nicotinic receptors). These general pharmacological tests were carried out to determine the physiological effects of chlormequat chloride injected intravenously. Oligopnoea, salivation and a tendency to inhibition of intestinal propulsion were observed in mice immediately after they were given chlormequat chloride at 7.4 mg/kg bw. In cats, there was mild inhibition of the vasopressor effect of noradrenaline 30 minutes after administration of 1 mg/kg bw chlormequat chloride. In rabbits, neuromuscular junctions were blocked by doses of greater than 1 mg/kg bw; this effect was counteracted by administration of 10 mg/kg bw D-tubocurarine and potentiated by administration of 1 mg/kg bw neostigmine. Coagulation of rat blood was unaffected by concentrations up to 3 mg/mL. In dogs, doses greater than 3 mg/kg bw caused a drop in blood pressure; at higher doses, increased respiratory and heart rates were observed. These effects were mitigated by prior intravenous administration of atropine at 1 mg/kg bw (Mutoh et al., 1987).

The action of chlormequat chloride was tested in vitro with the patch clamp technique for electrophysiological measurements described by Hamill et al. (1981). Muscles were excised from the feet of adult NMRI mice and dissociated enzymatically to obtain individual muscle cells. Chlormequat chloride (purity 95.6%) activated the nicotinic acetylcholine receptor channel at all concentrations between 10 and 100 mmol/L (Franke & Mellert, 1991).

The affinity of chlormequat chloride for subtypes of muscarinic acetylcholine receptors was investigated in vitro in membranes from bovine cerebral cortex, rat heart and rat submaxillary gland. The results were compared with those obtained for subtype-specific reference substances, and atropine was included as a high affinity reference compound with no subtype selectivity. Chlormequat chloride had low affinity for the muscarinic receptors in comparison with the reference substances (Weifenbach, 1991).

Delayed neurotoxicity

In an acute delayed neurotoxicity study conducted according to OECD TG 418 (1984), adult White Leghorn adult hens ($n = 20$; 12 months old; 1.10–1.84 kg) were administered chlormequat chloride (purity 63.5%) orally. Doses were applied at 2.0 mL/kg bw (2268 mg/kg bw) in the first treatment but, due to high mortality, this dose was reduced to 1.0 mL/kg bw (1134 mg/kg bw) and given to all remaining hens in the second treatment, 21 days after the first. Hens of the control and test group received no protection against potentially acute cholinergic effects prior to administration of the test material. The dose level in this study was selected on the basis of the results of a pre-study where hens ($n = 3$) each received single oral doses of 1.0, 2.0 or 3.0 mL/kg bw. All the hens at 3.0 mL/kg bw but none at the two lower dose levels died, and 2.0 mL/kg bw was selected as the maximum tolerated dose for the main study.

Six hens served as control and were orally treated with refined groundnut (peanut) oil at 10 mL/kg bw in the first and second treatments. Another six hens received undiluted tri-*o*-tolyl phosphate (TOTP) at a single dose of 0.65 mL/kg bw (750 mg/kg bw) as the positive control 1 hour after oral administration of 25 mg/kg bw atropine sulfate dissolved in physiological saline. All surviving hens of the test and the control group were killed 21 days after the second treatment. Positive control animals were killed on day 18 after the first treatment at the peak of locomotor ataxia. All animals were observed for signs of toxicity and mortality four times on day 1 and once daily thereafter until scheduled kill. Individual body weight and feed consumption was determined on a weekly basis. Assessment of locomotor ataxia was carried out in accordance with evaluation scores on days 1, 7, 14, 21, 28, 35 and 42, respectively. All surviving (including moribund) hens were necropsied at the end of the three-week observation period.

There were no deaths in the control and the TOTP-treated groups. After the first treatment with an oral dose of 2268 mg/kg bw, 7/20 hens were found dead on day 1 of treatment and another four died or were found dead on days 2–4. After the second treatment with an oral dose of 1134 mg/kg bw, 1/9 surviving hens died on day 2.

Control hens had mild diarrhoea on day 1 following each treatment with vehicle. Hens treated with the positive control TOTP had diarrhoea on days 1–3 post treatment and progressive ataxia from days 14 to 18. All surviving and/or moribund hens treated with chlormequat chloride at a single dose of 2268 or 1134 mg/kg bw had diarrhoea, tremor and salivation on day 1 of each treatment as well as diarrhoea and cyanotic comb and weakness in individual hens on day 2 of treatment (Table 6).

Table 6. Mortality during delayed neurotoxicity assessment

Group / treatment	Total no. of birds	No. of animals died / day of mortality	Terminated as scheduled on day 18/42*
G1 / Control (0 mg/kg bw)	6	0	6
G2 / TOTP(750 mg/kg bw)	6	0	6
G3 / Chlormequat chloride (2 268 mg/kg bw)	20	7 / day 1 4 / days 2–4	n.a.
G3 / Chlormequat chloride (1 134 mg/kg bw)	9	1 on day 2	8

bw: body weight; n.a.: not applicable; no.: number; TOTP: tri-*o*-tolyl phosphate *Source*: Suresh (1994b)

[a] Positive control group hens were sacrificed on day 18; control and chlormequat chloride–treated hens were killed on day 21 after the second treatment.

Body weight of surviving hens was comparable to or slightly higher than body weight of control hens, while body weight of TOTP-treated positive control hens was lower than the body weight of control hens during study weeks 1 and 2 but comparable to controls during study week 3. Feed intake of surviving treated hens treated with chlormequat chloride was comparable to or slightly higher than feed intake of control hens, while feed intake of TOTP-treated hens was reduced during weeks 1 through 3 compared to vehicle-treated controls.

There were no findings on necropsy of control and positive control group hens, and no gross lesions were detectable in chlormequat chloride–treated hens. In decedent hens, dehydration, watery stool, haemorrhage in the liver as well as petechiae of the lungs were noted. One hen died on day 3 in poor condition.

No clinically apparent neurological locomotor ataxia was observed in control group hens and in surviving hens treated with chlormequat chloride. Neurohistopathological examination revealed no lesions in the brain, spinal cord and sciatic nerve in surviving hens.

In conclusion, when two very high doses of chlormequat chloride producing acute systemic toxicity were administered at an interval of 21 days to White Leghorn hens, no neurological locomotor ataxia or neurohistopathological changes in the central nervous system and sciatic nerves were observed. Therefore, the test material is not considered to produce delayed neurotoxicity (Suresh, 1994b, 2002 amendment).

(b) Immunotoxicity

A 28-day dietary immunotoxicity study examined the effects of three plant growth regulators, including chlormequat chloride, on the immune response of young and aged male and female deer mice (*Peromyscus maniculatus*). Male and female mice over two years old (five males and four females) and female mice 6–8 months old (n = 10) were given chlormequat chloride at dietary concentrations of 40 mg/kg bw. The corresponding saline vehicle control groups also had 5, 4 and 10 mice, respectively.

Parameters relevant for assessment on the immune system were determined, including final body weight; relative weight of heart, kidney, thymus, spleen and liver; and clinical chemistry. In addition, both young and old mice received chlormequat chloride (40 mg/kg bw per day) over a period of 23 days before they were inoculated with the Venezuelan equine encephalitis virus (VEEV), the Modoc virus (MODV) and the Pichinde virus either separately or together. The vehicle control was saline. Mortality, viraemia and antibody titre were measured.

Although, in general, age and sex influenced many measured parameters in the untreated mice, the following findings were considered test substance related: decreased lymphocyte viability (22%)

in young females; decreased number of circulating white blood cells (61%) in all groups; decreased number of plaque-forming cells (73%) per gram of spleen in all groups.

Body weight, organ weights and all other parameters measured remained unaffected by chlormequat chloride treatment in all groups (young and old mice, males and females).

The study authors considered the increased mortality after VEEV infection in old and young females (30–33% when compared to saline control) to be a relevant finding at the dose level tested. The lack of statistical significance in young mice was attributed to the small sample size and the death of one control animal. Overall, a substance-related effect was assumed.

While infection with MODV was proven, no increased mortality was noted in any of the groups. The deaths after simultaneous inoculation of VEEV and MODV were probably due to VEEV, which was isolated from the brains of the dead mice. There was no effect on the magnitude or duration of VEEV MODV viraemias. Pichinde virus produced no measurable viraemia and the mean antibody titre was unaffected by treatment. However, early antibody response to MODV was significantly decreased and mean antibody titre did not decrease as rapidly as the saline control (Fairbrother, Yuill & Olson, 1986).

According to the authors, chlormequat chloride affected a few measured parameters of immune function and increased mortality due to infection with one of the viruses tested, but in comparison with other compounds tested, such as glyphosine, the effects of chlormequat chloride were evaluated as mild. However, the sample sizes were small, and aged mice are known to be more susceptible to infections, and so all data from this study cannot be considered robust. The mechanism of action of chlormequat chloride on cells of the mammalian immune system remains unknown.

(c) Pharmacological studies

In a series of in vitro and in vivo general pharmacology studies, ddY mice (3 males/dose group) received a single intravenous administration of chlormequat chloride at 0, 3.2, 4.9 or 7.4 mg/kg bw. The behaviour of the animals was observed before the administration and at 15, 30, 60 and 120 minutes post dosing according to the Irwin method.

In study 1, effects on locomotor coordination (rat-rod test) were examined after ddY mice (10 males/dose group) received a single intravenous administration of chlormequat chloride at 0, 3.2, 4.9 or 7.4 mg/kg bw. They were then placed on a treadmill (diameter 3 cm) rotating at 14 rpp at 15, 30, 60 and 120 minutes post dosing and their ability to stay on the wheel was monitored for one minute.

In study 2, effects on the autonomic nervous system were tested in cats (4–5/group). The effects on the nictitating membrane, blood pressure and heart rate were measured after intravenous administration of 0, 0.1, 0.3, 1 or 3 mg/kg bw. The test were performed with co-administration of norepinephrine, dimethylphenypiperazine or acetylcholine or with electrical stimulation of the cervical vagus or the preganglionic cervical sympathetic nerve. Observations were made at 30, 60 and 120 minutes post dosing.

In study 3, the effect on intestinal motility via charcoal propulsion were tested in NMRI mice (7–10 males/per dose group) after a single intravenous administration of chlormequat chloride at 0, 3.2, 4.9 or 7.4 mg/kg bw. At the same time, the mice were orally dosed with a charcoal suspension. Twenty minutes after dosing with the charcoal, the animals were killed and the transit distance of the charcoal was determined.

In study 4, the effects on neuromuscular junctions groups were assessed in rabbits (4/dose group) by electrical stimulation of the right peroneal nerve and the anterior tibial muscle. The groups were treated intravenously with chlormequat chloride at 0, 0.1, 0.3, 1 or 3 mg/kg bw. The contractions of the muscle were measured using a force displacement transductor. The observation period lasted for one hour after treatment.

In study 5, coagulation was determined in blood of Wistar rats that was mixed with 0.1 mL of a solution so that the final concentration of chlormequat chloride in the test-tube was either 0, 0.1 or 0.3 mg/L. Blood coagulation was determined at 37 °C.

In study 6, groups of four dogs were used for the determination of the following effects: respiration, electrocardiogram, blood pressure, heart rate and blood flow. The test substance (0, 1, 3, 10, 30, 100, 300, 1000 or 3000 µg/kg bw) was administered intravenously and observations were continued for two hours post dosing.

Intravenous administration of 7.4 mg/kg bw resulted in death of 2/9 mice. In addition to salivation and oligopnea (which also occurred at 4.9 mg/kg bw), slight mydriasis was observed. There were no effects at 3.2 mg/kg bw.

With respect to effects on locomotor coordination (rat-rod test), there were no test substance-related effects.

For the autonomous nervous system, an intravenous dose of 3 mg/kg bw resulted in death of all animals. At 1 mg/kg bw, a 15% inhibition of the vasopressor response to the injection of noradrenaline was noted 30 minutes after the administration of the test substance. This effect disappeared almost completely within 60 minutes after the administration. A transient nictitating membrane contraction and transient brachycardia were also observed at this dose level. Doses of 0.1 or 0.3 mg/kg bw were considered to be no-observed-effect levels (NOELs).

Intravenous administration of 7.4 mg/kg bw resulted in an inhibition of intestinal propulsion; and at this dose level, 3/10 mice died. There were no effects on intestinal movement at 3.2 and 4.9 mg/kg bw.

Intravenous administration of 1 and 3 mg/kg bw resulted in a transient inhibitory action on the neuromuscular junction as demonstrated by a reduction of muscular contraction after direct stimulation of 35% and 89%, respectively. There were no effects at a dose of 0.1 or 0.3 mg/kg bw.

There were no effects of the test substance on blood coagulation time.

After intravenous administration of 3 mg/kg bw, the respiration of the animals stopped after an initial increase immediately following the administration. All the waves in the ECG, except for the P wave, vanished immediately after the administration, but reappeared after artificial respiration was started. Heart rate and blood pressure dropped, and blood flow was also diminished.

Intravenous administration of 1 mg/kg bw resulted in increased respiration, a reduction in heart rate and blood pressure, and a decrease in blood flow. Increased respiration was observed at doses of 0.01 mg/kg bw and higher.

The effects on heart rate were different at low dose levels compared to high ones. In contrast to the effects at 1 and 3 mg/kg bw, at doses of 0.01 to 0.3 mg/kg bw heart rate increased transiently.

At 0.003 mg/kg bw, there was a tendency towards prolongation of the QRS interval in the ECG, a reduction of blood pressure and an initial decrease of blood flow, followed by a transient increase. Reversibility of all parameters was observed within three minutes after dosing.

The administration of 1 mg/kg bw of atropine prior to the administration of the test substance prevented or mitigated the vasodepressive reactions, the brachycardia, as well as the changes in blood flow.

In conclusion, the results of the different experiments indicate an inhibitory effect on the neuromuscular junctions as well as a muscarine-like action of the test substance on the parasympathetic nervous system. In addition, a stimulating effect on the whole autonomic nervous system was indicated (Mutoh et al., 1987).

(d) Neuropharmacological studies

In several of the studies described in this monograph, clinical signs such as lateral position, extension spasms and ataxia of hind limbs as well as salivation were observed at very high dose levels of chlormequat chloride. Furthermore, in acute toxicity studies, bradypnoea, decreased motor activity and motor incoordination as well as tremors, abnormal posture, salivation, loss of reflexes and muscle tone were noted.

These clinical signs, together with the observations made in the safety pharmacology study, indicate that chlormequat chloride affects the nervous system via interactions with muscarinic and nicotinic acetylcholine receptors. Neuropharmacologically, for muscarinic agonists, the following

effects have been noted: bronchospasms, intestinal spasms, diarrhoea, brachycardia, salivation and sweating. For nicotinic agonists the pharmacological effects are tremor, spasms and myoparesis of the skeletal muscle.

The following two studies were conducted to elucidate the mechanisms.

The potential affinity of chlormequat chloride (purity 95.6%) for muscarinic receptors was examined in vitro based on competition between chlormequat chloride and a ^{3}H-radiolabelled standard *N*-methylscopolamine. The test substance affinity to the receptor was expressed by replacement of radioactive *N*-methylscopolamine and the subsequent determination of the released radioligand. These results were then compared to the displacing potencies of reference substances for the specific muscarinic acetylcholine receptor subtypes: M1+2 (membranes of bovine cerebral cortex); M2 (membranes of rat heart); and M3 (membranes of rat submaxillary gland). The reference substances used were pirenzepine HCl salt for M1+2; methoctramine HCl salt for M2; and 4-diphenylacetoxy-*N*-methylpiperidine methiodide for M3. In addition, atropine sulfate was used as a high affinity reference compound without subtype selectivity.

The potency of chlormequat chloride was about five orders of magnitude lower than those of the reference standards (i.e. atropine). It can therefore be concluded that chlormequat chloride has a weak agonist activity on muscarinic receptors in vitro (Weifenbach, 1991).

Pharmaceutical doses of chlormequat chloride (purity 95.6%) were tested in vitro at the nicotinic acetylcholine receptors of adult mouse muscle using the patch clamp technique. Interosseal muscles from adult NMRI mice were excised and prepared with collagenase. The dissociated muscles were placed into culture dishes. Electrophysiological recordings of single channel currents were made using the clamp technique, with glass pipettes (tip diameter: 1–2 µm) on the muscle surface. These single channel openings represent the result of binding of a substance to the nicotinic receptor. Acetylcholine was used as the reference substance, and the experiments were conducted within six hours of preparing the muscle tissue, which is within the appropriate time range for maintenance of muscle cell properties after preparation.

Chlormequat chloride activated the nicotinic acetylcholine receptor in all experiments (19 different membrane patches) at concentrations of 10, 100 and 1000 µmol/L. The mean open time of the channels was about 0.35 ms, which is approximately three times lower than the value for acetylcholine. The single channel conductance was the same for chlormequat chloride and acetylcholine, but the frequency of channel openings elicited by 10 µmol/L at chlormequat chloride was 1/100 of the activity of the same concentration of acetylchloline, such that at a concentration of 1000 µmol/L, chlormequat chloride resulted in a response similar to 10 µmol/L of acetylcholine (Franke & Mellert, 1991).

(e) Developmental toxicity studies considered not to contribute to the quantitative risk assessment

Two recent experimental studies, from the same group, were available from the scientific literature and are described below. The Meeting noted that effects described were only seen at very high doses/concentrations, and therefore these studies do not contribute to the quantitative risk assessment.

Rat

Chlormequat chloride (purity 99%) was administered to Sprague Dawley rats (13/sex per dose group) daily by gavage on postnatal days 23–60 at doses of 0, 75, 150 and 300 mg/kg bw per day (close to the LD_{50}). There was no statement of GLP. Information on sex ratios was not provided.

Body weight and the length of the right femur were significantly decreased at 300 mg/kg bw per day. Histological analysis of proximal growth plates of the right femurs showed narrowed proliferative zones and hypertrophic zones in the treated groups. The messenger ribonucleic (mRNA) expression of growth hormone, growth hormone receptor and insulin-like growth factor 1 were decreased in the treatment groups. The results indicated that chlormequat chloride may affect the expression of growth hormone and insulin-like growth factor 1 and subsequently cause a decrease in body weight and bone length (Huang et al., 2016).

A subsequent study from the same group was specifically designed to evaluate the skeletal development toxicity of chlormequat chloride (purity 99%) in male pubertal Sprague Dawley rats (10/dose group) and to investigate whether chlormequat chloride impacts the development of chondrocyte, osteoblast and osteoclast through growth hormone and insulin-like growth factor 1. From postnatal days 23 to 70, rats were exposed daily by gavage at doses of 0, 75, 150 or 300 mg/kg bw per day.

The size of femurs and tibias, bone mineral density and biomechanical parameters were significantly decreased ($P < 0.01$) at 300 mg/kg bw per day compared with the control group. A significant effect for midshaft bone mineral density was also reported at 75 mg/kg bw per day ($P < 0.05$). The concentration of osteocalcin and C-terminal telopeptide of type I collagen (CTX-I) in blood at 150 mg/kg bw per day was also changed. The mRNA expression ratio of the receptor activator of NF-κB ligand (RANKL) and osteoprotegerin at 150 and 300 mg/kg bw per day was increased. Histological analysis of proximal and distal epiphyseal plates of the right femurs showed that both the proliferative zone and hypertrophic zone narrowed in treatment groups. The concentration of insulin-like growth factor 1 in blood was reduced with an increase in exposure doses of chlormequat chloride, and the mRNA expression of growth hormone receptor in the tibia was decreased.

The results suggest potential mechanisms by which chlormequat chloride might indirectly adversely impact upon the formation and activation of chondrocytes, osteoblasts and osteoclasts via the decline and inhibition of growth hormone receptor and insulin-like growth factor 1, leading to skeletal development damage (Huang et al., 2017).

Another published developmental toxicity study from the same group used the rat whole embryo culture model, limb bud micromass culture and 3T3 fibroblast cytotoxicity test to test chlormequat chloride (purity 99%) in embryos (12 per group): 0, 150, 500, 1000 µg/mL (equal to 0, 0.31, 0.93, 3.1 and 6.2 mmol/L). At doses of 150 µg/mL (0.93 mmol/L) embryo growth was retarded without causing significant morphological malformations; administration of 500 µg/mL (3.1 mmol/L) resulted in both retardation and morphological malformation of the embryos. However, the proliferation and differentiation of limb bud cells were not affected by chlormequat chloride up to 1000 µg/mL (6.2 mmol/L); nor did this concentration of chlormequat chloride affect cell viability as examined by 3T3 fibroblast cytotoxicity test either, suggesting that cellular toxicity may not play a role in chlormequat chloride-induced inhibition of rat embryo growth.

The authors considered that the results demonstrate that chlormequat chloride may affect embryo growth and development without inhibiting cell viability (Xiagedeer et al., 2016).

Hamster

Pregnant Syrian golden hamsters (8/group) were given chlormequat chloride (purity unspecified) by gavage at concentrations of 0, 25, 50, 100, 200, 300 or 400 mg/kg bw once on gestation day 8 or 100 mg/kg bw daily on gestation days 7, 8 and 9. The control group consisted of 15 animals. All animals were killed on gestation day 14.

Clinical signs of toxicity were seen in the groups receiving the higher doses of 300 and 400 mg/kg bw; 5/8 hamsters at 400 mg/kg bw died, leaving only three for evaluation. Animals at 100 mg/kg bw or higher had fewer fetuses than controls and more fetal resorptions. In animals at 200 mg/kg bw or higher, fetal size and weight were reduced. No abnormalities were observed in animals treated with single doses of 0, 25, 50 or 100 mg/kg bw. Malformations including anophthalmia, microphthalmia, cleft palate and polydactylism and evidence of developmental retardation were seen in the offspring of dams treated with three doses of 100 mg/kg bw or a single dose of 200, 300 or 400 mg/kg bw.

The limited details presented in the publication, including lack of information on feed consumption and body weight make it difficult to assess the maternal toxicity; the occurrence of malformations in historical controls was not presented (Juszkiewicz, Rakalska & Dzierzawski, 1970). This study was therefore considered to be of inadequate quality.

Skeletal developmental studies reported in the literature (Huang et al., 2016, 2017) suggest potential mechanisms of chlormequat chloride action in relation to retardation of embryo growth via disruption of the neurotransmitter acetylcholine. Using the rat whole embryo culture model, limb bud micromass culture and 3T3 fibroblast cytotoxicity test, chlormequat chloride at 150 µg/mL (0.93 mmol/L) retarded rat embryo growth without causing the significant morphological malformations observed at 500 µg/mL (3.1 mmol/L). At doses up to 1000 µg/mL (6.2 mmol/L), chlormequat chloride caused both retardation and morphological malformation of the embryos, but neither proliferation and differentiation of limb bud cells nor cell viability were affected, suggesting that cellular toxicity may not play a role in chlormequat chloride-induced inhibition of rat embryo growth.

The authors concluded that chlormequat chloride may affect embryo growth and development without inhibiting cell viability (Xiagedeer et al., 2016). Acetylcholine is related to the delay of embryo growth in the absence of malformations, and has been put forward as a potential biomarker (Teixidó et al., 2013).

(f) Endocrine activity and fertility

The submitted reproductive toxicity studies were all conducted according to old test guidelines, that is, without the additional endocrine-specific end-points, such as anogenital distance, that have been added to the test guidelines in the last five years. Several published studies explored endocrine-related end-points but did not provide any evidence of endocrine activity, although some observed effects on sperm function may be consistent with possible minor effects on fertility.

The Meeting considered that these studies did not provide information useful for this quantitative risk assessment.

Mouse

A study designed to investigate sensitive parameters of spermatogenesis impairment (not according to standard test guidelines) examined the reduced male fertility reported in the multigenerational dietary study in mice (Han: NMRI) (Hellwig et al., 1993; section 2.5). The number of testicular spermatozoa and the relative proportion of primary and secondary spermatocytes involved in spermatogenesis were measured, and the fertility of epididymal spermatozoa from tested male mice was investigated in an in vitro fertilization (IVF) system. The experimental feed was composed of chlormequat chloride–treated wheat in the first experiment and chlormequat chloride-free wheat and water mixed with pure chlormequat chloride in the second experiment.

In experiment 1, female F_0 mice were fed wheat containing chlormequat chloride at dietary level of 0 or 0.21 mg/kg wheat after mating. After birth of the F_1 litter, both generations were given feed containing chlormequat chloride at the same level. At day 64, the male F_1 mice were killed by cervical dislocation, and testes and epididymis were separated and used for fertility studies. In experiment 2, female F_0 mice were fed feed pellets containing chlormequat chloride (0 or 0.2 mg/kg pellet) and water containing chlormequat chloride (0 or 0.2 mg/L) after mating. After birth of the F_1 litter, both generations were given feed containing chlormequat chloride at the same level. On day 64, the male F_1 mice were treated as for experiment 1. For preparation of spermatozoa for IVF, 48 male mice from both feeding groups (experiment 1 and 2) were used as sperm donors. To investigate the number of spermatozoa and the alteration of testicular germ cell ratios, the testes of 128 male mice from both feeding groups (experiments 1 and 2) were used.

For the IVF, females were superovulated by intraperitoneal injection of 5 International Units (IU) of pregnant mare serum gonadotropin followed 48 hours later by 5 IU human choriogonadotropin (hCG). Thirteen hours after hCG injection, females were killed and 2–4 oviducts were placed in 1 mL TCM 199. Tubal cumulus–oocyte complexes (COCs) were recovered by oviduct flushing. All compact cumulus–oocyte complexes were pooled before being randomly assigned to treatment groups.

For preparation of spermatozoa for IVF, 48 male mice from different feeding groups (experiments 1 and 2) were used as sperm donors. Two hours before oocyte preparation, male mice were killed and spermatozoa for each treatment group/experiment were obtained from four caudal epididymis. The IVF was conducted within six hours and subsequent zygote cultivation were performed up to

24 hours; at the end of the culture period, eggs were evaluated for the proportions of cleaved embryos. The presence of two pronuclei in uncleaved eggs and the number of nuclei in each blastomere of cleaved eggs were determined. All eggs at mitotic stages with more than two pronuclei were considered to be fertilized. All embryos with more than two blastomeres and nucleus/blastomere were considered to be cleaved.

The chlormequat chloride content in the treated feed and water was 0.21 mg/kg and 0.2 mg/L, respectively. The fertilization and cleavage rates of oocytes incubated with spermatozoa from chlormequat chloride-fed mice (experiment 1) were reduced: the fertilization rate in chlormequat chloride-fed versus control mice was 65.1% versus 21.1% and the cleavage rate was 51.9% versus 20.3% ($P < 0.01$). Feeding of sperm donors with pure chlormequat chloride mixed with untreated wheat pellets or water (experiment 2) led to a reduction in fertilization and cleavage rates (control: 60.8%, 32.4%; chlormequat chloride in feed: 29.8%, 12.1%; chlormequat chloride in water: 30.1%, 10.2%; chlormequat chloride in feed and water: 36.6%, 12.5%; $P < 0.01$, respectively). The normal course of spermatogenesis was unchanged after the exposure to chlormequat chloride, and testicular weight, number of spermatozoa and the proportion of haploid, diploid and tetraploid testicular cells were not influenced. However, as noted above, the functional competence of epididymal spermatozoa (IVF with oocytes from untreated mice and their cleavage rate) from chlormequat chloride–fed donors was reduced, resulting in a significantly diminished fertilization and cleavage rate in vitro ($P < 0.01$) compared to the control data. The authors suggest that chlormequat chloride could interfere with epididymal protein secretion and the process of sperm maturation during passage through the epididymis, but that further investigations are needed (Torner et al., 1999).

In conclusion, while no effects on spermatogenesis in the testes were observed, sperm maturation during epididymal transit and resulting functional competence may be affected.

The same group also evaluated the effects of chlormequat chloride-treated wheat on the fertility of female mice in a two-generation study (Langhammer, 1999). Female F_0 mice were fed with chlormequat chloride-treated wheat (purity 460 g/L; residue level of 0.21 mg/kg wheat dry matter) or with the untreated control wheat (0.1 mg/kg upper limit of detection for chlormequat chloride) up to week 9 of the study, whereas males only received the treated wheat during the week of mating due to technical reasons (animals were kept in one cage). The pups were again treated until sexual maturation, mated and gave birth to the next generation. The procedure was repeated for a second cycle. Altogether, four sub-experiments were conducted:

- estrus cycle determination in F_1 females between weeks 3 and 9 postpartum;
- mortality of embryos from F_1 mothers up to gestation day 18;
- number, quality and development of embryos of F_1 mothers 48 hours postcoitum; and
- litter performance of F_1 and F_2 mothers.

The parameters measured included sexual development based on vaginal opening (indicating the day of sexual maturation and the respective body weight) and first estrus (indicating the possibility of first mating); prenatal fertility based on number of live versus dead fetuses on gestation day 18; postnatal fertility based on litter size and litter weight at birth and day 21 postpartum; and body weight development measured on postpartum days 21, 42 and 63.

After inducing superovulation in females, the in vitro evaluation of the oocytes and embryos was monitored and the progesterone and estradiol receptor content in the uterus and blood progesterone level were determined. The luteinizing hormone receptor content was measured in pooled ovaries. There were no effects on parameters measured with respect to general toxicity (body weight development) sexual development, or pre- and postnatal fertility.

Investigations on oocytes and embryos did not indicate a test substance-related effect. Progesterone and estradiol receptor content in the uterus were unaffected.

While differences noted included reduced luteinizing hormone receptor content (pooled ovaries, 35 versus 146 fmol/mg protein in the control) and a lower progesterone content in the blood of chlormequat chloride-treated females (22.8 versus 30.0 ng/mL in the control), the authors concluded that there was no essential change with respect to reproduction regulation and phenotypic fertility.

Subchronic chlormequat chloride study to determine the effects on fertility in farm animals

The fertility of five-month-old boars was investigated in a subchronic feeding study
Chlormequat chloride (purity 64.96%) was administered via the feed over a period of four months to four groups of male boars at dietary doses of 0, 1, 10 and 60/40 mg/kg bw per day. Due to marked systemic toxicity, the highest daily dose was reduced to 40 mg/kg bw after 28 days of exposure.

There were no effects on any of the sperm evaluation parameters as well as on the macroscopic and histopathological evaluations of the testes and epididymides (Sachsse et al., 1987).

A published in vivo study on the effect of chlormequat chloride in growing hens reported a significant increase ($P > 0.05$) in estrogen levels at a dose of 50 ppm. In this study, 115 three-week-old chickens were divided into four treatment groups of 39, 33, 31 and 12 chickens. They were placed on four dietary treatments consisting of 0 (control), 5 ppm chlormequat chloride from 14 weeks of age, 5 ppm chlormequat chloride from the start of the experiment at three weeks and 50 ppm from the start of the experiment. The basal diets were formulated according to the growing period and were iso-nitrogenous and iso-energetic. Feed intake, body weight changes and egg production were measured. Blood samples for estrogen determination were collected weekly from week 15 until the first egg was laid.

Depressive effects of 50 ppm chlormequat chloride on body weight gain during weeks 3–8 and feed efficiency over the whole period were observed. There were no treatment differences ($P > 0.05$) on feed intake, body weight gain during weeks 9–20 and age at the point of lay. Chlormequat chloride inclusion at 50 ppm significantly ($P < 0.05$) stimulated the estrogen concentration at 17 and 18 weeks of age, and was 65% higher than the control at 18 weeks (Gultom, Songsang & Ter Meulen, 2001).

The relevance of such a study to human endocrine disruption effects is not clear, particularly as all the other available evidence is negative for such adverse effects.

In vitro studies did not show chlormequat chloride at 50 µmol/L to have estrogen and androgen activity (Andersen et al., 2002), and there was no inference of aromatase activity (chlormequat chloride purity 99%) in human placental microsomes (Kjeldsen, Ghisari & Bonefeld-Jørgensen, 2013).

Recently the same group reported minimal in vitro activation (lowest observed effect concentration [LOEC] 10^{-4} mol/L) of the aryl hydrocarbon receptor (AhR), but this was not sufficient to yield a half maximal effective concentration (EC_{50}) or an AhR-relative potency (REP) compared to the positive control, 2,3,7,8-tetrachlorodibenzodioxin (TCDD) ($LOEC_{TCDD}/LOEC_{pesticide}$) (Ghisari et al., 2015).

(g) Microflora and human health risk assessment

No data were found in the open literature.

(h) Poisoning and antidotes

In vivo studies to determine the effectiveness of antidotes for chlormequat chloride poisoning showed that there was a slight but statistically nonsignificant increase in survival time after oral choline chloride treatment post oral chlormequat chloride poisoning in rats. Fischer (1988a) reported the effect of oral choline chloride therapy after oral chlormequat chloride (purity 72%) poisoning in rats. Choline is considered insufficiently effective as an antidote for chlormequat chloride poisoning and should therefore not be recommended.

Treatment with atropine by intraperitoneal injection (20 mg/kg bw) increases the toxicity after oral chlormequat chloride poisoning. Thus such a treatment is contraindicated for acute intoxication with chlormequat chloride (Fischer, 1988b).

3. Observations in humans

3.1 Medical data and information

(a) Medical surveillance on manufacturing plant personnel

A dossier provided by the sponsor, states that:

> …the manufacturing plant personnel is surveyed by regular medical examinations. This surveillance programme is not aimed to specifically detect chlormequat chloride-related symptoms or diseases. Thus, it does not indicate a causal association between the compound and any specific medical effect. Frequency and distribution of medical diagnoses in the manufacturing plant personnel did not reveal any peculiarities" (CCC Task force, 2004).

(b) Direct observation of incidental occupational exposure

The sponsor stated that:

> No case of incidental exposure to chlormequat chloride has been observed at BASF sites. Two fatal poisoning incidents have been reported after ingestion of CYCOCEL® which contains chlormequat chloride (460 g/l) and choline chloride (35 g/l). The amount of CYCOCEL® ingested was several hundreds to 4000 mL in one case (Freislederer et al. 1989) and "a mouthful" in the other (Winek et al. 1990). In the first case nausea, emesis, and coma, in the second sweating, increased salivation, visual disturbance, diarrhoea, seizures, cardiac dysrhythmia, and coma have been reported.
>
> After oral or inhalational exposure to CYCOCEL® or a similar product nausea, emesis, diarrhoea, salivation, irritation of mucous membranes, somnolence, and disturbance of equilibrium [have been described], after dermal exposure to CYCOCEL® irritation or allergic skin reactions have been described in a few cases. This information is based on personal communications and is not verified. (CCC Task force, 2004).

In a fatal occupational exposure in France, a 39-year-old farmer accidentally inhaled Cycocel C5™ (BASF Agro SAS, Ecully, France) containing 460 g/L of chlormequat chloride and 320 g/L of choline chloride. Twelve hours later an acute pulmonary oedema occurred, followed by respiratory and asystolic cardiac arrest that required external cardiac massage, with intubation. Atropine and adrenaline were administered but the patient subsequently died at home (Nisse et al., 2015).

(c) Pesticide poisoning centre reporting

There are several pesticide poisoning reports in the open literature, most of which were successful suicide attempts. Three fatal cases have been reported by Freislederer, Besserer & Mallach (1989); Winek, Wahba & Edelstein (1990); and Bardale, Sonar & Waghmare (2012). Seven fatal cases were reported by Nisse et al. (2015) and, most recently, one by Vijitharan et al. (2016), who cite further examples.

For six deaths by suicide reported from the Lille Poison Centre between April 2011 and January 2014 (Nisse et al., 2015), all had clinical features of acute poisoning, namely cholinergic crisis, cardiac arrest, acute pulmonary oedema, respiratory failure and death mostly within 1 hour of ingestion. Atropine had been used in many of the cases, and may have hastened death in some instances. This is especially likely where these patients were misdiagnosed with organophosphate or carbamate poisoning, as acute poisoning mimics anticholinesterase toxicity. As shown in in vivo studies, atropine does not act on nicotinic receptors of neuromuscular junctions, which are affected by chlormequat chloride too, and results in respiratory paralysis. Atropine affects absorption and excretion of chlormequat chloride and is associated with high mortality in these fatal cases where death occurred within a day of exposure.

The sponsor provided a statement evaluating case reports (mostly male) for the classification of acute toxicity of chlormequat chloride. These are listed in Table 7 (Frericks M, BASF SE, Ludwigshafen, Germany; personal communication 10 May 2017 [received 26 August 2017]).

The cases in the public literature and incidence reports from BASF are summarized in Table 7.

Table 7. Case reports for the classification of acute toxicity of chlormequat chloride

Source	Sex	Age (years)	Volume ingested	Estimated amount of chlormequat chloride (g) [a]	Calculated approximate concentration of chlormequat chloride (mg/kg bw) [b]	Outcome	Reference
Case report	M	33	30 mL	22.5	281.0	Lethal	Bardale, Sonar & Waghmare (2012)
Case report 1	F	34	Unknown	Unknown	Unknown	Survived	Yang et al. (2015)
Case report 2	M	39	Inhalation?	Unknown	Unknown	Lethal	Nisse et al. (2015)
Case report 3	M	46	Unknown	Unknown	Unknown	Lethal	Nisse et al. (2015)
Case report 4	M	59	Two mouthfuls	36.4	462.6	Lethal	Nisse et al. (2015)
Case report 5	M	46	150 mL	69	876.2	Lethal	Nisse et al. (2015)
Case report 6	M	37	Unknown	Unknown	Unknown	Lethal	Nisse et al. (2015)
Case report 7	M	15	Unknown	Unknown	Unknown	Lethal	Nisse et al. (2015)
Case report 8	M	27	120 mL	41.4	525.7	Lethal	Nisse et al. (2015)
Case report 9	M	20	300 mL	Unknown	Unknown	Lethal	Freislederer, Besserer & Mallach (1989)
Case report 10	M	45	20 (IP)	8	5.6	Lethal	Boumrah et al. (2016)
Case report 11	M	59	One mouthful	4.8	68	Lethal	Winek, Wahba & Edelstein (1990)
Case report 12	M	50	200 mL	150	1873	Lethal	Vijitharan et al. (2016)
Case report 13	M	Unknown	2–3 drops	Unknown	Unknown	No clinical signs	BASF reporting, no date
Case report 14	M	47	30–40 mL	22.5 [c]	281	Coma	BASF reporting, no date
Case report 15	M	Unknown	1 mouthful	0.34	58	No clinical signs	BASF reporting, no date
Case report 16	M	2	1 mL	Unknown	Unknown	Survived	BASF reporting, no date

bw: body weight; F: female; IP: intraperitoneal; M: male

[a] Takes into account the density as well as the active ingredient content of the respective formulation.

[b] Calculation to mg/kg bw uses the following parameters: body weight of male, 70 kg , and of female, 60 kg ; volume of one mouthful based on swallow volume according to Bennett et al. (2009): male, 39.4 mL; female, 24.5 mL.

[c] Worst case assumption, assuming that ingestion of 30 mL resulted in the toxic effect.

The case reports listed in Table 7 indicate that the lowest lethal dose was 68 mg/kg bw (Winek, Wahba & Edelstein, 1990). All other values were greater than 250 mg/kg bw. It should be noted that case report descriptions of amounts ingested are always subject to substantial subjective error due to self-reporting or estimations.

In one published case of acute dermatitis (Fischer, 1984), patch tests to chlormequat chloride gave negative reactions, while reactions to choline chloride have been reported to be positive.

3.2 Epidemiological studies

Neither data on exposure of the general public nor epidemiological studies specifically addressing exposure to chlormequat chloride are available.

(a) Exposure of adults and children living near agricultural lands

Urinary biomarker concentrations of chlormequat chloride in UK adults and children living near agricultural land were assessed. Adults and children residing 100 m or less from fields sprayed with captan, chlormequat chloride, chlorpyrifos or cypermethrin (based on spray event information provided by farmers) provided first-morning void urine samples during and outside of the spraying season. Selected samples (1–2 days after a spray event and at other times "background samples") were analysed and adjusted for creatinine levels. Generalized linear mixed models were used to investigate any elevations in urinary biomarker concentrations after spray events. The final dataset for statistical analysis contained 1518 urine samples from 140 participants, consisting of 523 spray events and 995 background samples. For chlormequat chloride, the geometric mean urinary biomarker concentrations following spray events were 15.4 µg/g creatinine, compared with 16.5 µg/g creatinine for background samples within the spraying season. Outside of the spraying season, lower concentrations for chlormequat chloride were observed (12.3 µg/g creatinine) (Galea et al., 2015).

Comments

Biochemical aspects

In rats, absorption of chlormequat chloride from the gastrointestinal tract was rapid. Elimination, mainly of nonmetabolized chlormequat, was almost entirely via the urine (approximately 90%) and was essentially complete within 24 hours (Blinn, 1967). Less than 1% of the administered dose remained in the tissues.

In a second metabolism and toxicokinetics study, rats were administered an intravenous dose of 0.1 mg/kg bw or a high (30 mg/kg bw) or repeated low (0.5 mg/kg bw) oral gavage dose. At 1.5 hours after administration, the highest amounts of radioactivity from the high dose were found in the gastrointestinal tract, followed by the kidneys, the liver and the heart. At termination 168 hours post dosing, very low levels of radioactivity were found in the kidneys, followed by the liver, the heart and the gastrointestinal tract.

For the repeated low-dose study, at two hours after the last of the seven daily doses of 0.5 mg/kg bw, the highest amounts of radioactivity were found in the kidneys, followed by the liver, the heart and the gastrointestinal tract, at levels much lower than in the high-dose study. At termination (168 hours post dosing), virtually no radioactivity remained. Faeces and bile do not play a major role in the elimination. There were no significant differences in absorption and excretion between the high- and low-dose levels. Excretion after a 14-day pretreatment was comparable to that after a single oral dose. The C_{max} increased less than proportionally with dose, whereas the AUC was linear over the tested dose range, indicating that chlormequat chloride has no accumulating potential (Giese & Hoffmann, 1989).

Other than chlormequat chloride, only trace metabolites were found in rat urine; these may have been salts of chlormequat and choline (Bronisz & Romanowski, 1968). An unidentified polar metabolite was also found in faeces (Giese & Kohl, 1989).

Toxicological data

The LD_{50} values of chlormequat chloride were 800–1000 mg/kg bw in rats, mice, hamsters, guinea-pigs, sheep and monkeys and 10–80 mg/kg bw in rabbits, cats and dogs.

The dermal LD_{50} was greater than 4000 mg/kg bw in rats and 440 mg/kg bw in rabbits. The rat inhalation LC_{50} was greater than 2.5 mg/L.

Chlormequat chloride is a partial agonist of the nicotinic acetylcholine receptor. The neurological signs of toxicity observed in pharmacological investigative studies may have been due to the pharmacological activity of chlormequat chloride. There were no consistent treatment-related findings at necropsy.

Chlormequat chloride is not irritating to skin and eyes of rabbits and does not induce skin sensitization in guinea-pigs.

In short-term studies of toxicity of chlormequat chloride in different species, the most important effects were clinical signs related to a generally reduced state of health and reduced feed consumption and body weights. In a 4-week mouse study at dietary concentrations of 0, 500, 1500 or 3000 ppm (equal to 0, 148, 439 and 885 mg/kg bw per day for males and 0, 223, 618 and 1190 mg/kg bw per day for females, respectively), there were no test substance-related effects. The NOAEL was 3000 ppm (equal to 885 mg/kg bw per day), the highest dose tested (Schilling, 1990).

In a four-week study of acute toxicity of chlormequat chloride in rats at dietary concentrations of 0, 500, 1500, 3000 or 4500 ppm (equal to 0, 47, 137, 258 and 367 mg/kg bw per day for males and 0, 51, 148, 291 and 418 mg/kg bw per day for females, respectively), the NOAEL was 1500 ppm (equal to 137 mg/kg bw per day) based on reduced body weight gain (Schilling et al., 1990).

In another four-week oral toxicity study, rats were administered dietary doses of chlormequat chloride of 0, 100, 1000 or 10 000 ppm (equal to 0, 8.5, 95, 1210 and 1110 [recovery group], all in mg/kg bw per day for males and 0, 9.8, 120, 1240 and 1140 [recovery group], all in mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 9.8 mg/kg bw per day) based upon emaciation and clinical signs of weakness observed in females at 1000 ppm (equal to 1240 mg/kg bw per day) (Suresh, 1991g, 2002 amendment).

In another four-week oral toxicity study, rats were administered chlormequat chloride at dietary concentrations of 0, 100, 500 or 2500 ppm (equal to 0, 8.3, 41 and 202 mg/kg bw per day for males and 0, 8.8, 45 and 211 mg/kg bw per day for females, respectively). No treatment-related effects were observed up to 2500 ppm (equal to 202 mg/kg bw per day), the highest dose tested (Nemec, 1991).

In a 90-day oral toxicity study, rats were given chlormequat chloride at dietary levels of 0, 300, 900 or 2700 ppm (equivalent to 0, 21, 61 and 189 mg/kg bw per day for males and 0, 24, 73 and 220 mg/kg bw per day for females, respectively). There were no mortalities or test substance-related clinical signs of toxicity. Reductions in feed consumption, body weight and body weight gain were observed in high-dose males (Tanabe & Nagao, 1981). The NOAEL was 900 ppm (equivalent to 61 mg/kg bw per day) based on reduced body weight gain and feed intake in males at 2700 ppm (equivalent 189 mg/kg bw per day).

In a second 90-day oral toxicity study, rats were given chlormequat chloride at dietary concentrations of 0, 100, 1000 or 5000 ppm (equal to 0, 6.0, 61 and estimated as 305 mg/kg bw per day for males and 0, 7.9, 89 and estimated as 445 mg/kg bw per day for females, respectively). Clinical signs of urinary incontinence were observed in one high-dose female and two high-dose males but not in the recovery group. In addition, three males were found to have prolapsed penis, but this finding was not clearly treatment related and not reported by other study. The NOAEL was 1000 ppm (equal to 61 mg/kg bw per day) based on decreased body weight gain in males at 5000 ppm (approximately 305 mg/kg bw per day) (Suresh, 1992b).

In a third 90-day oral toxicity study, rats were given dietary doses of chlormequat chloride at 0, 500, 1500 or 4500 ppm (equivalent to 0, 50, 150 and 450 mg/kg bw per day). The 4500 ppm group had their dose raised to 9000 ppm (equivalent to 900 mg/kg bw per day) in the eleventh week and additional groups of 10 males and 10 females were treated at 9000 ppm for 7 weeks, followed by a six-week recovery period. Body weight gain decreased in the two higher dose groups of 4500/9000 ppm and at 9000 ppm.

In the recovery phase, the females recovered completely, but the males did not within the study time frame. Feed consumption also decreased for these high-dose groups. The NOAEL was 1500 ppm (equivalent to 150 mg/kg bw per day) based upon decreased body weight gain and feed consumption at the highest dose (Sterner et al., 1977a).

In a 90-day oral toxicity study, dogs were administered chlormequat chloride in the diet at concentrations of 0, 100, 300 or 600 ppm, with the top dose level raised to 800 ppm in week eight, 1200 ppm in week 10, 2000 ppm in week 11 and 2500 ppm in week 12 (equivalent to 0, 2.5, 7.5 and 15/20/30/50/62.5 mg/kg bw per day). Body weight gain decreased in high-dose females and males from week 9 onwards. Salivation was a consistent treatment-related effect. At the higher doses, increased salivation was observed in two animals in the 300 ppm group 3–6 hours after feeding; this was pronounced in one animal in the 1200 ppm group and in all animals in the 2000 ppm and 2500 ppm groups from week 11. The NOAEL was 100 ppm (equivalent to 2.5 mg/kg bw per day) based on salivation at 300 ppm (equivalent to 7.5 mg/kg bw per day) (Sterner et al., 1977b).

In a one-year study, dogs were administered chlormequat chloride in the diet at concentrations of 0, 150, 300 or 1000 ppm (equal to 0, 4.7, 9.2 and 31 mg/kg bw per day for males and 0, 5.2, 10 and 32 mg/kg bw per day for females, respectively; corrected for purity). Diarrhoea was seen at 300 ppm in two males during the first and second weeks of the study. Salivation and vomiting was also seen at this dose, starting at week 1 and occurring intermittently thereafter. The NOAEL was 150 ppm (equal to 4.7 mg/kg bw per day), based on diarrhoea, vomiting and salivation at the LOAEL of 300 ppm (equal to 9.2 mg/kg bw per day) (Mellert et al., 1993).

The overall NOAEL in the 90-day and one-year dog studies was 4.7 mg/kg bw per day based on clinical signs, vomiting, salivation and decreased body weight gain at the LOAEL of 300 ppm (equal to 9.2 mg/kg bw per day).

In a 110-week mouse study of chlormequat chloride at dietary concentrations of 0, 150, 600 or 2400 ppm (equal to 0, 21, 84 and 336 mg/kg bw per day for males and 0, 23, 91 and 390 mg/kg bw per day for females, respectively), the NOAEL was 2400 ppm (equal to 336 mg/kg per day), the highest dose tested (Mellert et al., 1994).

In an 18-month study, mice were administered dietary dose levels of chlormequat chloride at 0, 250, 1000 or 4000 ppm (equal to 0, 20, 79 and 323 mg/kg bw per day for males and 0, 22, 91 and 352 mg/kg bw per day for females, respectively). Although there were no treatment-related increases in the incidence of neoplasms up to the highest dose tested, the limited end-points assessed prevent identification of a NOAEL for nonneoplastic effects (Suresh, 1998, 2002 amendment).

In a 102-week cancer bioassay study, dietary doses of chlormequat chloride at 0, 500 or 2000 ppm (equivalent to 0, 70 and 290 mg/kg bw per day) were given to mice for 102 weeks. No increases in tumour incidences were found (National Cancer Institute, 1979).

In a 78-week toxicity and carcinogenicity study, rats were given chlormequat chloride at dietary concentrations of 0, 281, 937 or 2810 ppm (equal to 0, 12, 43 and 136 mg/kg bw per day for males and 0, 15, 56 and 172 mg/kg bw per day for females, respectively). Tumour incidences were not increased. The NOAEL was 937 ppm (equal to 43 mg/kg bw per day) based on reduced body weight at 2810 ppm (equal to 136 mg/kg bw per day) (Schilling et al., 1992).

In a two-year toxicity and carcinogenicity study, rats were given chlormequat chloride at dietary concentrations of 0, 280, 940 or 2800 ppm (equal to 0, 13, 42 and 120 mg/kg bw per day for males and 0, 16, 55 and 170 mg/kg bw per day for females, respectively). No treatment-related neoplastic and nonneoplastic histopathological changes were seen. The NOAEL was 940 ppm (equal to 42 mg/kg bw per day), based on reduced weight gain and feed consumption at the highest dose (Mellert et al., 1992).

In a carcinogenicity study conducted by the National Cancer Institute (1979), rats were given chlormequat chloride at dietary concentrations of 0, 1500 or 3000 ppm (equivalent to 0, 75 and 150 mg/kg bw per day). There was no test substance-related increase in mortalities or signs of clinical toxicity in any of the treatment groups. In male rats, dose-related islet cell adenomas of the pancreas were observed. However, given the difficulties in diagnosis of these lesions (Nolte et al., 2016) and considering the available data quality, the Meeting concluded that under the conditions of this

bioassay, there was no convincing evidence that chlormequat chloride is carcinogenic in male and female Fischer F344 rats up to the highest dose tested (3000 ppm).

The Meeting concluded that chlormequat chloride is not carcinogenic in mice and rats.

Chlormequat chloride has been adequately tested in a wide range of in vitro and in vivo mutagenicity/genotoxicity assays. The Meeting concluded that the overall weight of evidence indicates that chlormequat is unlikely to be genotoxic.

In view of the lack of genotoxic potential and absence of carcinogenicity in mice and rats, the Meeting concluded that chlormequat is unlikely to pose a carcinogenic risk to humans.

In a multigeneration study of reproductive toxicity in rats at dietary concentrations of chlormequat chloride at 0, 300, 900 or 2700 ppm (equal to 0, 29, 86 and 250 mg/kg bw per day for males and 0, 23, 69 and 230 mg/kg bw per day for females, respectively). The NOAEL for parental toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced feed consumption and decrease in body weight and body weight gain in female and male parental animals of both generations at 2700 ppm (equal to 230 mg/kg bw per day). The NOAEL for offspring toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced pup weight gain and retarded development during lactation at 2700 ppm (equal to 230 mg/kg bw per day). The NOAEL for reproductive toxicity was 900 ppm (equal to 69 mg/kg bw per day) based on reduced numbers of pregnancies and delivered pups at 2700 ppm (equal to 230 mg/kg bw per day) (Hellwig et al., 1993). Reversible and transient tremor and hypersensitivity were also observed in F_0 and F_1 females at 2700 ppm (equal to 230 mg/kg bw per day), mainly during or after the lactation period. At this dose level there were some indications of an impairment of the reproductive function (a higher number of mating partners did not show signs of fertility within the scheduled mating period).

In another multigeneration study, rats were given dietary dosages of chlormequat chloride at 0, 10, 100 or 1000 ppm (equal to 0, 0.7, 6.8 and 69.3 mg/kg bw per day for males and 0, 1, 10.2 and 105 mg/kg bw for females, respectively). There were no parental mortalities in any group, no signs of clinical toxicity, no treatment-related effects on feed consumption at any time and no significant effects upon body weight. The parental NOAEL was 100 ppm (equal to 6.8 mg/kg bw per day), based on reduced body weight gain, clinical signs seen during lactation (females) and marginal anaemia at 1000 ppm (equal to 69.3 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 69.3 mg/kg bw per day), the highest dose tested. The NOAEL for effects on reproductive function was 1000 ppm (equal to 69 mg/kg bw per day) (Suresh, 1995).

In a third multigenerational study, rats were given dietary doses of chlormequat chloride at 0, 100, 500 or 2500 ppm (equal to 0, 8.4, 41.4 and 211 mg/kg bw per day for males and 0, 9.1, 46.3 and 241 mg/kg bw per day for females, respectively). The parental NOAEL was 500 ppm (equal to 41.4 mg/kg bw per day for males and 46.3 mg/kg bw per day for females) based on reduced body weight and body weight gain at 2500 ppm (equal to 211 mg/kg bw per day). The offspring NOAEL was 500 ppm (equal to 41.4 mg/kg bw per day) based on reduced pup weight and pup weight gain and dystrophic changes in the skeletal muscle of the offspring. The reproductive NOAEL was 2500 ppm (equal to 211 mg/kg bw per day), the highest dose tested (Gandalovicová, 1993).

In a developmental toxicity study, rats were administered gavage doses of 0, 25, 75 or 225 mg/kg bw per day. There were no indications of malformation. The NOAEL for maternal toxicity was 75 mg/kg bw per day based on clinical signs and reduced body weight and feed consumption at 225 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 225 mg/kg bw per day, the highest dose tested (Becker & Biedermann, 1992a).

In a developmental toxicity study, rabbits were administered gavage doses of chlormequat chloride at 0, 10, 20 or 40 mg/kg bw per day. There was a slight but statistically significant increase in mean postimplantation loss at 40 mg/kg bw per day, but the number of live pups per dam was greater at the high dose than in the controls. The NOAEL for maternal toxicity was 10 mg/kg bw per day based upon salivation seen at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day based on increased postimplantation loss and slightly reduced fetal weights (Becker & Biedermann, 1992b).

The Meeting concluded that chlormequat chloride is not teratogenic.

A number of studies retrieved from a search of the scientific literature investigated the potential effects of chlormequat on sperm quality and function and endocrine activity. These studies did not provide any evidence of endocrine activity of chlormequat. Some effects were observed on sperm function in certain in vitro and in vivo non-standard studies that may be consistent with possible minor effects on fertility observed in some standard multigeneration studies. The Meeting considered that these studies did not provide useful information for quantitative risk assessment.

Human data

No adverse effects on the health of workers involved in the normal manufacture of chlormequat were observed.

Lethal poisoning cases with commercial products containing chlormequat have been reported in the literature and in three case studies of oral intake from a manufacturing plant. From these studies the estimated lethal dose in humans appears to be consistent with the LD_{50} in dogs, which are more sensitive than rodents.

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

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.05 mg/kg bw established on the basis of the overall NOAEL of 4.7 mg/kg bw per day for diarrhoea, vomiting and salivation in the 1-year and 90-day studies of toxicity in dogs, and using a safety factor of 100.

The Meeting reaffirmed the ARfD of 0.05 mg/kg bw on the basis of a NOAEL of 4.7 mg/kg bw for clinical signs (diarrhoea, vomiting and salivation) observed in the one-year study in dogs. These effects were observed multiple times during the first week of treatment and were likely to be elicited after a single dose.

Levels relevant to risk assessment of chlormequat chloride

Species	Study	Effect	NOAEL	LOAEL
Mouse	One hundred and ten-week study of toxicity and carcinogenicity [a]	Toxicity	2400 ppm, equal to 336 mg/kg bw per day [b]	–
		Carcinogenicity	2400 ppm, equal to 336 mg/kg bw per day [b]	–
Rat	Two-year study of toxicity and carcinogenicity [a]	Toxicity	940 ppm, equal to 42 mg/kg bw per day	2800 ppm, equal to 120 mg/kg bw per day
		Carcinogenicity	2800 ppm, equal to 120 mg/kg bw per day [b]	–
	Two-generation reproductive toxicity study [a,d]	Reproductive toxicity	900 ppm, equal to 69 mg/kg bw per day	2700 ppm, equal to 230 mg/kg bw per day
		Parental toxicity	100 ppm, equal to 6.8 mg/kg bw per day	1000 ppm, equal to 69.3 mg/kg bw per day
		Offspring toxicity	500 ppm, equal to 41.4 mg/kg bw per day	2 500 ppm, equal to 211 mg/kg bw per day
	Prenatal/developmental toxicity study [c]	Maternal toxicity	75 mg/kg bw per day	225 mg/kg bw per day
		Embryo/fetal toxicity	225 mg/kg bw per day [b]	–
Rabbit	Prenatal developmental toxicity study [c]	Maternal toxicity	10 mg/kg bw per day	20 mg/kg bw per day
		Embryo/fetal toxicity	20 mg/kg bw per day	40 mg/kg bw per day
Dog	Ninety day- and 12-month toxicity studies [a,d]	Toxicity	150 ppm, equal to 4.7 mg/kg bw per day	300 ppm, equal to 9.2 mg/kg bw per day

[a] Dietary administration. [b] Highest dose tested. [c] Gavage administration. [d] Two or more studies combined.

Estimate of acceptable daily intake (ADI)

0–0.05 mg/kg bw

Estimate of acute reference dose (ARfD)

0.05 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 chlormequat

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly and almost completely absorbed
Dermal absorption	Less than 10%
Distribution	Widely distributed
Potential for accumulation	Low potential for bioaccumulation
Rate and extent of excretion	Rapid excretion mainly into urine (90%)
Metabolism in animals	Mostly excreted unchanged, little metabolism to choline or *N*-choline, salts of chlormequat
Toxicologically significant compounds in animals and plants	Chlormequat chloride
Acute toxicity	
Mouse, LD_{50}, oral	405 mg/kg bw
Rat, LD_{50}, oral	433 mg/kg bw
Rabbit, LD_{50}, oral	~75 mg/kg bw
Dog, LD_{50}, oral	37 mg/kg bw
Rat, LD_{50}, dermal	>4000 mg/kg bw
Rabbit, LD_{50}, dermal	>440 mg/kg bw
Rat, LC_{50}, inhalation	>2.51 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Guinea-pig, dermal sensitization	Non-sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Reduced body weight gain, neurological effects (salivation, tremors)
Lowest relevant oral NOAEL	4.7 mg/kg bw (dog)
Lowest relevant dermal NOAEL	150 mg/kg bw
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight gain, neurological effects (salivation, tremors)
Lowest relevant NOAEL	
Carcinogenicity	Not carcinogenic in mice and rats
Genotoxicity	No evidence of genotoxicity [a]

Reproductive toxicity	
Target/critical effect	Reduced fertility
Lowest relevant parental NOAEL	6.8 mg/kg bw per day
Lowest relevant offspring NOAEL	41.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	69 mg/kg per bw day
Developmental toxicity	
Target/Critical effect	Reduced body weight gain during lactation and focal dystrophy of muscles
Lowest relevant maternal NOAEL	10 mg/kg bw day (rabbit)
Lowest relevant embryo/fetal NOAEL	20 mg/kg bw day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No specific studies were conducted.
Subchronic neurotoxicity NOAEL	
Developmental neurotoxicity NOAEL	

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI [a]	0–0.05 mg/kg bw	90-day and one-year studies in dogs	100
ARfD [a]	0.05 mg/kg bw	One-year study in dogs	100

[a] Expressed as chlormequat chloride.

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Appendix 1: Literature search

The authors conducted literature searches in PubMed (March and June 2017). Articles appearing to be obviously not relevant for a toxicological or human health evaluation were excluded from the results list based on their titles and/or abstracts. Articles concerned with analytical techniques in different matrices, and non-English language abstracts were also excluded. Searches conducted for chlorocholine chloride substantially increased the number of non-relevant hits, and so are not included here. Articles describing research in plant metabolism were provided to the FAO experts.

For the remaining references, the full articles were retrieved and further assessed for relevance.

Table A1. Details on literature search and its results (date: June 2017)

Database		Number of hits
PubMed	Number of user queries: Chlormequat Chlormequat AND TOXICITY	134 26
	Number of hits for which the full articles were retrieved:	15
	Number of retrieved full articles excluded after detailed assessment:	1
	Number of references included in the evaluation:	14

See Also:

Toxicological Abbreviations

Chlormequat chloride (AGP:1970/M/12/1)

Chlormequat chloride (WHO Pesticide Residues Series 2)

Chlormequat chloride (Pesticide residues in food: 1976 evaluations)

Chlormequat chloride (Pesticide residues in food: 1994 evaluations Part II Toxicology)

Chlormequat chloride (JMPR Evaluations 1999 Part II Toxicological)

CYCLANILIPROLE

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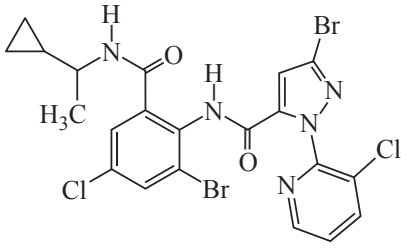
Explanation

Cyclaniliprole is the International Organization for Standardization-approved common name for 2′,3-dibromo-4′-chloro-1-(3-chloro-2-pyridyl)-6′{[(1RS)-1-cyclopropylethyl]carbamoyl}pyrazole-5-carboxanilide (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 1031756-98-5. Cyclaniliprole, an anthranilic diamide insecticide, is a ryanodine receptor modulator.

Figure 1. Structure of cyclaniliprole (IKI-3106)



Cyclaniliprole has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues.

All critical studies had statements of compliance with good laboratory practice and were conducted in accordance with relevant national or international test guidelines, unless otherwise indicated.

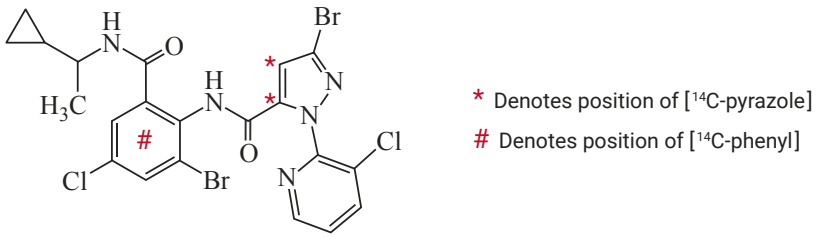
Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Penketh (2013a) undertook a series of absorption, distribution, metabolism and excretion studies in rats using [^{14}C-phenyl]- or [^{14}C-pyrazole]-labelled cyclaniliprole (> 97% radiochemical purity; see Fig. 2) in 0.5% aqueous carboxymethylcellulose (CMC). Radioactivity was analysed in excreta, blood and tissues by liquid scintillation counting.

Figure 2. Position of radiolabel in cyclaniliprole (IKI-3106) used in absorption, distribution, metabolism and excretion studies in rats



In a pilot excretion/mass balance experiment, groups of han Wistar [Crl:WI(Han)] rats (n = 1/sex) received a single gavage dose of [^{14}C-phenyl]- or [^{14}C-pyrazole]-labelled cyclaniliprole at 10 mg/kg body weight (bw). Urine was collected at 0–6 and 6–24 hours, and at 24-hourly intervals to 120 hours after dosing thereafter. Faeces were collected at 24-hourly intervals to 120 hours after dosing. Expired air was collected at 24-hourly intervals to 120 hours after dosing or until less than 1% of the administered dose was detected over a 24-hour period. Recovery of radioactivity was 98.32–100.56%, with the majority detected in faeces (94.4–97.11%) and the remainder in the carcass (1.27–3.95%), urine (0.39–0.74%) and cage wash (0.06–0.11%). No radioactivity was detected in expired air, and subsequent experiments did not collect expired air. Over 93% of radioactivity was eliminated within 48 hours. There was no difference in the mass balance of radioactivity between the two radiolabelled forms of cyclaniliprole or between male and female rats.

In an excretion/tissue distribution experiment, han Wistar [Crl:WI(Han)] rats (n = 4/sex per group) were administered a single gavage dose of [^{14}C-phenyl]cyclaniliprole at 10 or 400, or a single gavage dose of [^{14}C-pyrazole]-cyclaniliprole at 10 mg/kg bw. Urine was collected from each rat at 0–6 and 6–24 hours, and at 24-hourly intervals to 168 hours after dosing. Faeces were collected at 24-hourly intervals to 168 hours after dosing. Rats were killed at 120 or 168 hours after dosing. Blood was collected for the analysis of haematocrit and radioactivity, and tissues for the analysis of radioactivity.

The mass balance of radioactivity is summarized in Table 1 (see next page). Recovery of radioactivity was 88.55–103.58%, with the majority detected in faeces (87.33–102.96%). Relatively low levels of radioactivity were recovered from the carcass (0.22–1.95%), urine (0.29–0.63%) and cage wash (0.02–0.06%). The majority (> 85%) of radioactivity was eliminated within 48 hours. Tissue radioactivity comprised less than 2% of the administered dose, with most detected in plasma and whole blood (Table 2). Tissue to plasma ratios of radioactivity were less than 1.0 for all tissues.

Table 1. Mass balance of radioactivity in rats following a single oral dose of [^{14}C-phenyl]- or [^{14}C-pyrazole]cyclaniliprole

Sample	Mean % of the administered radioactive dose					
	[^{14}C-phenyl]cyclaniliprole				[^{14}C-pyrazole]cyclaniliprole	
	10 mg/kg bw		400 mg/kg bw		10 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Urine						
0–6 h	0.10	0.12	0.13	0.14	0.15	0.10
6–24 h	0.24	0.14	0.12	0.10	0.31	0.13
24–48 h	0.09	0.08	0.04	0.04	0.13	0.10
48–72 h	0.03	0.04	0.01	0.01	0.03	0.05
72–96 h	0.01	0.03	<0.01	0.01	0.01	0.03
96–120 h	<0.01	0.01	ND	ND	0.01	0.02
120–144 h	–	–	ND	ND	ND	0.01
144–168 h	–	–	ND	ND	ND	<0.01
Total	0.46	0.42	0.30	0.29	0.63	0.43
Cage wash	0.06	0.03	0.02	0.02	0.04	0.05
Faeces						
0–6 h	80.70	83.90	88.31	85.77	75.93	68.09
6–24 h	10.81	6.57	13.41	15.89	11.65	17.42
24–48 h	0.62	0.71	0.90	0.54	0.73	1.00
48–72 h	0.12	0.36	0.07	0.08	0.09	0.42
96–120 h	0.05	0.20	0.27	0.03	0.06	0.22
120–144 h	–	–	0.01	0.02	0.04	0.12
144–168 h	–	–	0.01	0.05	0.07	0.07
Total	92.31	91.74	102.96	102.38	88.56	87.33
Carcass	1.58	1.95	0.30	0.22	0.97	0.74
Balance	94.42	94.13	103.58	102.92	90.19	88.55

bw: body weight; ND: not detected

Source: Penketh (2013a)

Table 2. Proportion of tissue radioactivity following a single oral dose of [^{14}C-phenyl]- or [^{14}C-pyrazole]cyclaniliprole

Tissue	Mean % of the administered radioactive dose					
	[^{14}C-phenyl]cyclaniliprole				[^{14}C-pyrazole]cyclaniliprole	
	10 mg/kg bw [a]		400 mg/kg bw [b]		10 mg/kg bw [b]	
	Males	Females	Males	Females	Males	Females
Carcass	1.58	1.95	0.30	0.22	0.97	0.74
Plasma	0.72	0.59	0.14	0.12	0.92	0.64
Whole blood	0.69	0.58	0.15	0.12	0.94	0.63
Blood cells	ND	ND	ND	ND	ND	ND
Brain	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heart	0.01	0.01	<0.01	<0.01	0.01	0.01

(*continued on next page*)

Tissue	Mean % of the administered radioactive dose					
	[^{14}C-phenyl]cyclaniliprole				[^{14}C-pyrazole]cyclaniliprole	
	10 mg/kg bw [a]		400 mg/kg bw [b]		10 mg/kg bw [b]	
	Males	Females	Males	Females	Males	Females
Kidney	0.02	0.02	<0.01	<0.01	0.02	0.02
Liver	0.08	0.15	0.02	0.02	0.10	0.11
Lungs	0.02	0.02	<0.01	<0.01	0.03	0.02
Spleen	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Adrenals	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pituitary	<0.01	<0.01	ND	ND	<0.01	<0.01
Thyroid	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ovaries	–	<0.01	–	<0.01	–	<0.01
Uterus	–	–	–	–	–	<0.01
Epididymis	0.01	–	<0.01	–	0.01	–
Testes	0.03	–	0.01	–	0.03	–
Bone	0.03	0.03	ND	ND	0.04	0.02
Bone marrow	0.01	0.01	<0.01	<0.01	0.01	0.01
Fat (abdominal)	0.13	0.15	0.03	0.03	0.12	0.17
Muscle (skeletal)	0.45	0.32	0.10	0.08	0.55	0.33
GIT and contents	0.10	0.14	0.02	0.02	0.11	0.09
Total	1.58	1.43	0.32	0.26	1.96	1.41

bw: body weight; GIT: gastrointestinal tract *Source*: Penketh (2013a)

[a] 120 h after dosing. [b] 168 h after dosing.

Biliary excretion was analysed in groups of bile duct-cannulated Han Wistar [Crl:WI(Han)] rats (n = 5/sex per dose) administered a single gavage dose of [^{14}C-phenyl]cyclaniliprole at 10 or 400 mg/kg bw. Bile was collected from each rat at 0–3, 3–6, 6–9, 9–12, 12–24 and 24–48 hours after dosing. Urine and faeces were collected at 0–24 and 24–48 hours after dosing. Following scheduled kill, the gastrointestinal tract and its contents, the liver and carcass were retained. The mass balance of radioactivity is summarized in Table 3. Excretion of radioactivity in bile was 3.55% in males and 2.77% in females at the low dose, and 0.81% in males and 0.79% in females at the high dose. Similar to other experiments, the majority of radioactivity was eliminated in faeces within 48 hours, with relatively low levels excreted in urine and remaining in the carcass. Based on the level of radioactivity in bile, urine, liver and carcass, gastrointestinal absorption was approximately 10.67% in males and 8.96% in females at the low dose, and 2.32% in males and 4.77% in females at the high dose.

Table 3. Mass balance of radioactivity in bile duct–cannulated rats following a single oral dose of [^{14}C-phenyl]cyclaniliprole

Sample	Mean % of the administered radioactive dose			
	10 mg/kg bw		400 mg/kg bw	
	Males	Females	Males	Females
Bile				
0–3 h	0.09	0.08	0.07	0.06
3–6 h	0.23	0.24	0.09	0.11
6–9 h	0.48	0.32	0.11	0.10
9–12 h	0.44	0.11	0.13	0.08
12–24 h	1.55	0.73	0.25	0.22
24–48 h	0.75	1.29	0.17	0.22

Sample	Mean % of the administered radioactive dose			
	10 mg/kg bw		400 mg/kg bw	
	Males	Females	Males	Females
Total	3.55	2.77	0.81	0.79
Urine				
0–24 h	1.52	0.49	0.49	0.43
24–48 h	0.50	0.21	0.13	0.10
Total	2.02	0.69	0.62	0.52
Cage wash	0.06	0.03	0.07	0.02
Faeces				
0–24 h	75.23	72.39	67.50	54.86
24–48 h	16.35	15.94	33.94	9.88
Total	91.58	88.34	101.44	64.73
Liver	0.58	1.04	0.08	0.21
Gastrointestinal tract and contents	1.55	1.69	0.58	34.43
Carcass	4.52	4.46	0.82	3.25
Total recovery	103.86	99.01	104.41	103.95
Absorption[a]	10.67	8.96	2.32	4.77

bw: body weight *Source*: Penketh (2013a)

[a] Estimated from the sum of radioactivity in bile, urine, liver and carcass

In a plasma and whole-blood kinetic experiment, groups of han Wistar [Crl:WI(Han)] rats (n = 12/sex per dose) were administered a single gavage dose of [^{14}C-phenyl]cyclaniliprole at 10 or 400 mg/kg bw or a single gavage dose of [^{14}C-pyrazole]cyclaniliprole at 10 mg/kg bw. Blood was collected from subgroups (n = 4/sex per dose) at intervals from 0.25 to 168 hours after dosing.

Maximum concentrations of radioactivity (C_{max}) in plasma were reached at 24–72 hours after dosing, while C_{max} in blood was reached at 24–72 hours in males and 48–120 hours in females (Table 4). The amount of radioactivity in plasma and blood was lower in females than males as reflected in the C_{max} and the area under the concentration–time curve (AUC) values. As plasma concentrations of radioactivity did not decline over the experimental period, the AUC, elimination rate constant and plasma elimination half-life were not determined. C_{max} and AUC_{all} did not increase proportionally with dose; the study author suggested that this reflected dissolution rate-limited absorption at the high dose. Whole blood to plasma ratios of radioactivity of 0.5–0.6 in both sexes suggested little distribution of radioactivity to erythrocytes. The position of the radiolabel had no effect on pharmacokinetic parameters.

Table 4. Pharmacokinetic parameters in rats administered a single oral dose of [^{14}C-phenyl]- or [^{14}C-pyrazole]cyclaniliprole

Parameter	Measure per dose level and sex					
	[^{14}C-phenyl]cyclaniliprole				[^{14}C-pyrazole]cyclaniliprole	
	10 mg/kg bw		400 mg/kg bw		10 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Plasma						
C_{max} (µg equiv. × h/g)	2.47	1.82	19.1	13.6	2.70	1.51
T_{max} (h)	24	48	72	72	24	72
AUC_{24} (µg equiv. × h/g)	40.1	17.3	–	–	–	–
AUC_{120} (µg equiv. × h/g)	249	166	2 010	1 330	241	139
AUC_{all} (µg equiv. × h/g)	249	166	2 800	1 930	326	204

Parameter	Measure per dose level and sex					
	[^{14}C-phenyl]cyclaniliprole				[^{14}C-pyrazole]cyclaniliprole	
	10 mg/kg bw		400 mg/kg bw		10 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Whole blood						
C_{max} (µg equiv./g)	1.47	1.02	9.74	9.05	1.58	0.824
T_{max} (h)	24	48	120	72	24	120
AUC_{24} (µg equiv. × h/g)	24.2	10.3	–	–	–	–
AUC_{120} (µg equiv. × h/g)	145	96.5	958	729	140	81.4
AUC_{all} (µg equiv. × h/g)	145	96.5	1 380	1 070	189	119

AUC: area under the concentration–time curve;
AUC_{24}: area under the concentration–time curve 24 hours after administration of final dose;
AUC_{120}: area under the concentration–time curve at 120 hours;
AUC_{all}: overall area under the concentration–time curve;
C_{max}: maximum concentration; T_{max}: time to reach C_{max} bw: bodyweight

Source: Penketh (2013a)

In a repeated-dose excretion/tissue distribution experiment, han Wistar [Crl:WI(Han)] rats (n = 4/sex) were administered a single gavage dose of [^{14}Cphenyl]cyclaniliprole at 10 mg/kg bw per day for 14 days. Urine and faeces were collected separately from each rat for 24 hours after the first and seventh doses. Following the final dose, excreta was collected for up to 168 hours, with samples of tissues collected following scheduled kill. Consistent with single dose experiments, the highest concentrations of radioactivity were detected in faeces at 24 hours after the first (86.30% and 99.26% in males and females, respectively) and seventh doses (100.29% and 99.20% in males and females, respectively), and up to 168 hours after the final dose (116.40% and 119.76% in males and females, respectively). The corresponding urinary concentrations of radioactivity were less than 2% of the administered dose. The concentration of radioactivity in the carcass was 29.72% and 23.24% of the administered dose in males and females, respectively. Radioactivity distributed to most tissues; total tissue concentrations of radioactivity were 31.84% and 31.22% of the administered dose in males and females, respectively. The highest tissue concentrations of radioactivity were detected in plasma (~16%), whole blood (~16%), skeletal muscle (8–9%), abdominal fat (1.7–1.9%), liver (1.1%), bone (0.5–6%) and testes (0.55%), with all remaining tissues containing less than 0.5% each of the administered dose. Tissue to plasma ratios were less than 1.0 indicating no accumulation after repeat dosing (Penketh, 2013a).

In a repeated-dose plasma and whole-blood kinetic experiment, han Wistar [Crl:WI(Han)] rats (n = 12/sex per dose) were administered [^{14}C-phenyl]cyclaniliprole at 10 mg/kg bw per day by gavage for 14 days. Blood was collected from subgroups (n = 4/sex per dose) before dose 1, 3, 5, 10 and 14 and at various times to 168 hours after the final dose. Pharmacokinetic parameters determined following the final dose are shown in Table 5. Similar to the single dose experiment, plasma concentrations of radioactivity did not decline over the experimental period and therefore AUC, elimination rate constant and elimination rate half-life were not determined. Accumulation ratios (calculated from the AUC_{24} of the single dose experiment and the AUC_{24} from this experiment) were approximately 25 in males and 48 females, indicating extensive accumulation of radioactivity in plasma and whole blood. Whole blood to plasma ratios of radioactivity of approximately 0.6 in both sexes suggested little distribution of radioactivity to erythrocytes (Penketh, 2013a).

Table 5. Pharmacokinetic parameters in rats administered 14 repeated daily oral doses of [^{14}C-phenyl]cyclaniliprole at 10 mg/kg bw

Parameter	Measure per sex	
	Males	Females
Plasma		
C_{max} (µg equiv. × h/g)	54.3	39.6
T_{max} (h)	2	12
AUC_{24} (µg equiv. × h/g)	1140	855
AUC_{all} (µg equiv. × h/g)	7640	5450
Whole blood		
C_{max} (µg equiv./g)	28.7	24.3
T_{max} (h)	48	12
AUC_{24} (µg equiv. × h/g)	577	495
AUC_{all} (µg equiv. × h/g)	4190	3090

AUC: area under the concentration–time curve;
AUC_{24}: area under the concentration–time curve 24 hours after administration of final dose;
AUC_{all}: overall area under the concentration–time curve 168 hours after administration of final dose
C_{max}: maximum concentration; T_{max}: time to reach C_{max} bw: bodyweight

Source: Penketh (2013a)

In a tissue distribution experiment, Han Wistar [Crl:WI(Han)] rats (n = 9/sex per dose) were administered a single gavage dose of 10 or 400 [^{14}C-phenyl]cyclaniliprole. Subgroups (n = 3/sex) were killed at 24, 48 or 168 hours after the low dose and 48, 72 and 120 hours after the high dose, and blood and tissues sampled. The tissue distribution of radioactivity was generally consistent with that of the earlier experiment (see Table 2). At the low dose, total tissue radioactivity was 10.86% in males and 12.57% in females at 24 hours, decreasing to 0.92% and 2.57%, respectively, at 168 hours. At the high dose, total tissue radioactivity was 5.65% in males and 5.67% in females at 48 hours, decreasing to 0.32% and 0.26%, respectively, at 168 hours. At both doses, tissue concentrations of radioactivity were similar in males and females, with the highest concentrations of radioactivity detected in plasma and whole blood, followed by the liver, adrenal glands and fat. The majority of tissues showed a decline in radioactivity over time, with the exception of plasma and whole blood in females, where radioactivity increased from 24 to 120 or 168 hours. Tissue to plasma ratios of radioactivity were generally less than 1, with the exception of the gastrointestinal tract and its contents (4.67–10.8 at 24 hours at the low dose and 10.1–12.5 at 48 hours at the high dose) and, in low-dose females, in the liver (2.57 at 24 hours and 1.23 at 48 hours), adrenal glands (1.52 at 24 hours) and fat (1.25 at 24 hours).

Penketh (2013b) undertook an investigative absorption, distribution and excretion study in bile duct-cannulated beagle dogs that were administered a single gavage dose of [^{14}C-phenyl] or [^{14}C-pyrazole] labelled cyclaniliprole (98.2% radiochemical purity) in 0.5% aqueous CMC at 1 mg/kg bw. Four dogs were cannulated but one of these was excluded due to complications following surgery. One male and one female were administered [^{14}C-phenyl]cyclaniliprole, while one female was administered [^{14}C-pyrazole]cyclaniliprole. Blood, bile, urine and faeces were sampled at various times up to 48 hours after dosing. Dogs were killed after the 48-hour dose, and the liver, spleen, kidney, fat, muscle, gastrointestinal tract and contents, and carcass were collected. Radioactivity was analysed in excreta, blood and tissues by liquid scintillation counting.

The mean recovery of radioactivity in the two dogs administered [^{14}C-phenyl]cyclaniliprole was 54.8% (76.4% based on total body fluid). The highest radioactivity (expressed as per cent of the administered dose) was detected in faeces (30.2%), gastrointestinal tract (5.14%), bile (2.73%), liver (0.83%), urine (0.64%), kidney and spleen (0.26%), with 15% (35.55% based on total body fluid) in the carcass. Based on the level of radioactivity detected in bile, urine, liver and carcass, gastrointestinal absorption was estimated to be 40%. In the male dog, the highest tissue concentrations of radioactivity

were in plasma (1.36 µg equiv./g) and fat (1.21 µg equiv./g), with the concentrations in kidney, liver, spleen, muscle and whole blood 0.708 µg equiv./g or less. In the female dog, the highest tissue concentrations of radioactivity were in plasma (0.275 µg equiv./g) and whole blood (0.145 µg equiv./g), with the concentrations in kidney, liver, spleen, muscle and fat 0.111 µg equiv./g or less.

Recovery of radioactivity in the female administered [^{14}C-pyrazole]cyclaniliprole was 60.3% (87.2% based on total body fluid). The highest radioactivity (expressed as % of the administered dose) was detected in faeces (43.1%), gastrointestinal tract (3.51%), bile (2.90%), liver (0.94%), urine (0.91%), kidney and spleen (0.13%), with 8.85% (32.8% based on total body fluid) in the carcass. Based on the level of radioactivity detected in bile, urine, liver and carcass, gastrointestinal absorption was 37.5%. The highest tissue concentrations of radioactivity were detected in plasma (0.747 µg equivalents/g) and whole blood (0.441 µg equivalents/g), followed by fat (0.293 µg equivalents/g), liver (0.263 µg equivalents/g), spleen (0.091 µg equivalents/g) and muscle (0.058 µg equivalents/g).

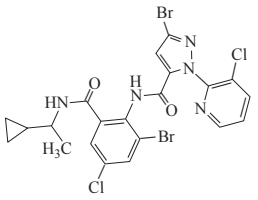
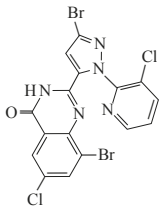
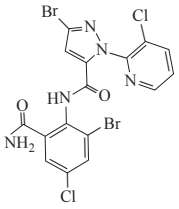
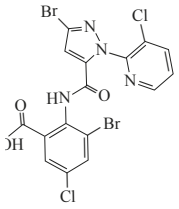
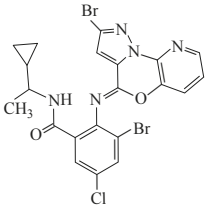
The C_{max} of radioactivity in plasma was 1.36 µg equivalents/g in the male and 0.399 and 0.903 µg equivalents/g in the two females. Time to reach C_{max} (T_{max}) was 48, 6 and 24 hours, respectively. The corresponding C_{max} and T_{max} values for whole blood were 0.708, 0.211 and 0.549 µg equivalents/g and 48, 12 and 24 hours, respectively. The AUC_{48} (µg equivalents × h/g) of radioactivity in plasma was 37.0 in the male and 17.3 and 31.2 in the two females, with the corresponding values in whole blood of 19.7, 8.18 and 18.8, respectively. As with to rats, plasma concentrations of radioactivity did not decline over the experimental period, and therefore AUC, elimination rate constant and elimination rate half-life were not determined. Whole blood to plasma ratios of radioactivity of 0.473–0.603 suggested little distribution of radioactivity to erythrocytes.

1.2 Biotransformation

In the study by Penketh (2013a) described in section 1.1, metabolites in excreta and tissues were analysed by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). There were no sex differences in metabolite profiles or the different radiolabelled forms of cyclaniliprole. Parent cyclaniliprole was the main metabolite identified in faeces and fat; it was also detected in plasma, liver and kidney, with none detected in bile or urine. The main plasma metabolite was 8-bromo-2-(3-bromo-1(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloroquinazolin-4(3*H*)-one (NSY-28), which was detected in the majority of samples but not faeces. Low levels of the metabolites 3-bromo-*N*-(2-bromo-6-carbamoyl-4-chlorophenyl)-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide (YT-1284) and 3-bromo-2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamido)-5-chlorobenzoic acid (NSY-27) were detected in excreta and liver, with YT-1284 detected only in kidney. The metabolite 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl)benzamide, designated NK-1375, was detected only in fat.

A summary of the metabolites identified in excreta, plasma and selected tissues is presented in Table 6, on the next page.

Table 6. Cyclaniliprole and cyclaniliprole metabolites identified in rat excreta and tissues

Sample	Radioactivity (%)				
	Cyclaniliprole (IKI-3106)	NSY-28[a]	YT-1284[b]	NSY-27[c]	NK-1375[d]
					
Faeces[e]					
Low dose	76.9–86.2	ND	≤ 0.3	≤ 0.6	ND
High dose	96.8–97.1	ND	ND	ND	ND
Repeated dose	95.3–97.0	ND	≤ 1.6	0.5–1.0	ND
Bile[e]					
Low dose	ND	0.2–0.3	0.6–0.7	0.2–0.3	ND
Urine[e]					
Low dose	ND	≤0.1	0.1–0.3	0.1–0.5	ND
Plasma[f]					
Low dose	1.5–4.5	91.3–96	ND	≤3.8	ND
High dose	4.7–5.0	91.1–91.3	ND	3.7–4.2	ND
Repeated dose	0.3	98.3–98.4	ND	0.8–1.1	ND
Liver[f]					
Low dose	61.2–85.4	4.4–10.8	1.9–6.3	3.7–5.3	ND
High dose	71.8–75.8	14.7–19.4	ND	≤3.9	ND
Kidney[f]					
Low dose	14.9–45.3	24.0–55.2	1.9–4.7	ND	ND
Fat[f]					
Low dose	46.4–56.8	3.1–3.4	ND	ND	4.2–5.9

ND: not detected

[a] 8-bromo-2-(3-bromo-1(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloroquinazolin-4(3*H*)-one.

[b] 3-bromo-*N*-(2-bromo-6-carbamoyl-4-chlorophenyl)-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide.

[c] 3-bromo-2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamido)-5-chlorobenzoic acid.

[d] 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl benzamide.

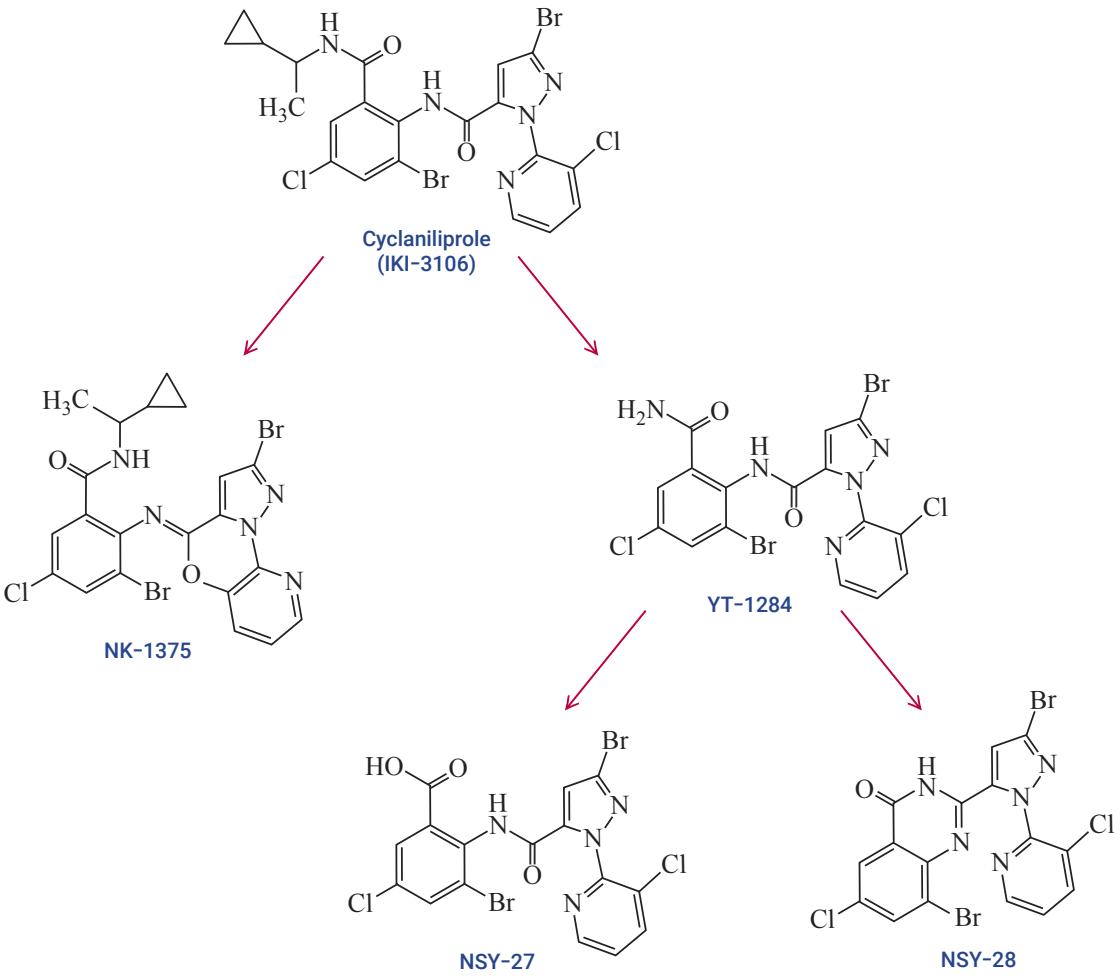
[e] Expressed as the % of the administered dose.

[f] Expressed as the % of tissue radioactivity.

Source: Penketh (2013a)

The proposed metabolic pathway for cyclaniliprole in rats is shown on the next page in Fig. 3.

Figure 3. Proposed metabolic pathway of cyclaniliprole (IKI-3106) in rats



IKI-3106: cyclaniliprole; NK-1375: 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl)benzamide; NSY-27: 3-bromo-2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamido)-5-chlorobenzoic acid;

NSY-28: 8-bromo-2-(3-bromo-1(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloroquinazolin-4(3*H*)-one; YT-1284: 3-bromo-*N*-(2-bromo-6-carbamoyl-4-chlorophenyl)-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide

Source: Redrawn from Penketh (2013a)

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity tests on cyclaniliprole, including skin and eye irritation and skin sensitization studies, are summarized in Table 7. No systemic toxicity was observed in any of these studies including deaths, clinical signs, body weight effects or pathology.

Table 7. Summary of acute toxicity studies with cyclaniliprole

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} or LC_{50}	Reference
Rat	Crl:CD(SD)	F	Oral	95.71	1% CMC	>2000 mg/kg bw	Moore (2011a)
Rat	Crl:CD(SD)	M + F	Dermal	95.71	1% CMC	>2000 mg/kg bw	Moore (2011b)
Rat	HsdHan™:WIST	M + F	Inhalation (nose only)	95.71	None	>4.62 mg/L	Leighton (2011)
Rabbit	Kb1:NZW	F	Skin irritation	95.71	None	Not irritating	Ueda (2011a)
Rabbit	Kb1:NZW	F	Eye irritation	95.71	None	Slightly irritating	Ueda (2011b)
Mouse	CBA/J	F	LLNA	95.71	DMF	Not sensitizing up to the maximum tested concentration of 50% w/v	Fukuyama (2011)
Guinea-pig	Kwl:Hartley	F	Maximization test	95.71	Olive oil (intradermal induction) acetone (topical inductions & challenge)	Not sensitizing up to the maximum tested concentration of 50% w/v	Nomura (2012)

bw: body weight; CMC: carboxymethylcellulose; DMF: *N*,*N*-dimethylformanide; F: female; M: male; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; LLNA: local lymph node assay; w/v: weight per volume

2.2 Short-term studies of toxicity

Mouse In a doserange-finding carcinogenicity study, cyclaniliprole (97.71% purity) admixed in the diet at concentrations of 0, 200, 1200 or 8000 parts per million (ppm) was fed ad libitum to Crl:CD1(ICR) mice (n = 12/sex per dose) for 13 weeks. The achieved doses were 0, 27, 159 and 1023 mg/kg bw per day for males and 0, 34, 179 and 1350 mg/kg bw per day for females, respectively. The mice were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly. Blood was sampled during week 13 for haematological and clinical chemistry analyses. After 13 weeks of treatment, the mice were killed and necropsied; organs were weighed and tissues histopathologically examined.

There were no deaths and no treatment-related clinical signs. Overall body weight gain in males at 1200 ppm was 19% higher than the controls and at 8000 ppm was 37% higher, with the latter statistically significant ($P < 0.05$); these increases were considered not toxicologically significant. There were no treatment-related effects on feed consumption or haematology parameters. Significantly reduced bilirubin ($P < 0.01$ or 0.05) was noted at every dose in males and at 1200 and 8000 ppm in

females; reduced bilirubin is of no toxicological relevance. There were treatment-related macroscopic or histopathological abnormalities. The reductions in relative kidney weight at 8000 ppm in males (−13%, $P < 0.05$) and in relative liver weight at 200 (−8%, $P < 0.05$), 1200 (−9%, $P < 0.05$) and 8000 ppm (−4%, $P < 0.05$) were small, did not follow a dose–response relationship and occurred in the absence of any other effects; on this basis they were considered to reflect normal biological variations.

The no-observed-adverse-effect level (NOAEL) was 8000 ppm (equal to 1023 mg/kg bw per day for males and 1350 mg/kg bw per day for females), the highest tested dietary concentration (O'Halloran, 2012).

Rat

In a 28-day dietary toxicity study by Ohnuma (2010), cyclaniliprole (97.15% purity) was admixed in the diet at concentrations of 0, 300, 1250, 5000 or 20 000 ppm and fed ad libitum to Wistar hannover GALAS (Brl:Han:WIST@Jcl) rats ($n = 6$/sex per dose). The achieved doses were 0, 26.4, 107, 426 and 1778 mg/kg bw per day for males and 0, 26.4, 113, 443 and 1800 mg/kg bw per day for females, respectively. The rats were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly. Blood and urine were collected after four weeks for haematological, clinical chemistry and urine analyses. After four weeks of treatment, the rats were killed and necropsied; organs were weighed and tissues sampled for potential histopathological examination. Only the liver was histopathologically examined due to increases in its weight at the highest dose.

There were no deaths or clinical signs and no treatment-related effects on body weight, feed consumption or haematological and urine analysis parameters. Total bilirubin was lower than the controls (−50%, $P < 0.01$) across all treatment groups of males and at 5000 and 20 000 ppm in females (−20%, $P < 0.01$); reduced bilirubin has no toxicological significance. There were no treatment-related macroscopic findings. At the highest dose, there were slight increases in absolute and relative liver weight in males (+14% and +12%, respectively), while absolute liver weight was increased at the highest dose in females (+13%, $P < 0.01$). In the absence of any histopathological changes in the liver or evidence of hepatotoxicity, these increases in liver weight were considered not toxicologically significant (Ohnuma, 2010).

In a 90-day dietary toxicity study by Ohnuma (2011), cyclaniliprole (97.15% purity) admixed in the diet at concentrations of 0, 600, 6000 or 20 000 ppm was fed to Wistar hannover GALAS (Brl:Han:WIST@Jcl) rats ($n = 10$/sex per dose). The achieved doses were 0, 39.9, 402 and 1331 mg/kg bw per day for males and 0, 43.3, 467 and 1594 mg/kg bw per day for females, respectively. The rats were observed daily for deaths and clinical signs. Body weight and feed consumption were recorded weekly. A functional observational battery (FOB) and motor activity assessment were performed during week 11. An ophthalmological examination was performed pretreatment and during week 13. Blood and urine were collected after 13 weeks of treatment for haematological, clinical chemistry and urine analyses. After 13 weeks of treatment, the rats were killed and necropsied; organs were weighed and tissues histopathologically examined.

There were no treatment-related deaths or clinical signs. The FOB and motor activity assessment were unremarkable, and there was no treatment-related effect on body weight or feed consumption. Activated partial thromboplastin time was significantly prolonged in females at every dose (18.3, 19.6 [$P < 0.05$], 19.3 [$P < 0.05$] and 20.1 [$P < 0.01$] seconds at 0, 600, 6000 or 20 000 ppm, respectively]. however, the prolongation at 600 and 6000 ppm was unlikely to be treatment-related because of the lack of a dose–response relationship. The increase at the highest dose was not considered treatment-related because the increase did not occur at 14, 26 or 52 weeks in the one-year rat study (Koyama, 2013) at the same dose; other parameters related to blood coagulation were unaffected by treatment; and the increase was only slight (< 10% of the concurrent control).

Total bilirubin was decreased by 60–70% ($P < 0.01$) in males and females at every dose, a finding that has no toxicological significance. Urine analysis parameters were unaffected by treatment, and there were no treatment-related macroscopic or histopathological findings. Treatment-related organ weight changes, occurring only in high-dose females, consisted of increased absolute and relative heart weights (+11% and +7%, respectively; $P < 0.05$ or 0.01), increased absolute and relative liver weights (+13% and +11%, respectively; $P < 0.01$) and increased absolute and relative ovary weights (+27% and

+24%, respectively; $P < 0.05$ or 0.01); none of these increases were accompanied with any pathology.

The NOAEL was 20 000 ppm (equal to 1331 mg/kg bw per day in males and 1594 mg/kg bw per day in females), the highest dietary concentration tested (Ohnuma, 2011).

In a one-year dietary toxicity study by Koyama (2013), cyclaniliprole (95.71% purity) admixed in the diet at concentrations of 0, 200, 2000, 6000 or 20 000 ppm was fed ad libitum to Wistar Hannover GALAS (Brl:Han:WIST@Jcl) rats ($n = 21$/sex per dose). The achieved doses were 0, 9.21, 89.6, 277 and 955 mg/kg bw per day for males and 0, 11.7, 117, 358 and 1213 mg/kg bw per day for females, respectively. The rats were observed daily for deaths and clinical signs. Body weight and feed consumption were recorded weekly from week 1 to 13 and once every four weeks thereafter. FOB and motor activity assessment were performed during week 49 on 10 rats/sex per dose. Ophthalmology was performed pretreatment and on all rats in the control and high-dose group during week 52. Blood was collected from 10 rats/sex per dose after 14, 26 and 52 weeks of treatment for the analysis of haematological and clinical chemistry parameters. Urine was collected from 10 rats/sex per dose during weeks 13, 25 and 51 for urine analysis. After 52 weeks of treatment, the rats were killed and necropsied; organs were weighed and tissues histopathologically examined.

One high-dose female was euthanized at 25 weeks due to human error. A second high-dose female was found dead during week 28; necropsy found bile calculi and luminal dilatation in the common bile duct. There was a significant ($P < 0.05$) increase in callus in the extremities in high-dose males (0, 2, 0, 3 and 5 rats per group of 21 at 0, 200, 2000, 6000 and 20 000 ppm, respectively). There were no treatment-related FOB, locomotor or ophthalmological findings and no effects on body weight, feed consumption, urine analysis or haematological parameters. Consistent with studies over shorter exposures, total bilirubin was significantly lower ($P < 0.01$ or 0.05) than the controls at every dose in both males and females (22–50% lower in males and 38–67% lower in females); this decrease has no toxicological significance. In females, calcium was significantly elevated ($P < 0.05$) at all doses during week 14 (4–5% higher than the control), but was comparable to the controls by the end of the study; in the absence of a dose–response relationship, this finding was not considered treatment related. There were no treatment-related macroscopic or histopathological findings or effects on organ weights.

The NOAEL was 20 000 ppm (equal to 955 mg/kg bw per day in males and 1213 mg/kg bw per day in females), the highest dietary concentration tested (Koyama, 2013).

Dog

In a 28-day dietary toxicity study by Yoshida (2013), cyclaniliprole (95.71% purity) was admixed in the diet at concentrations of 0, 100, 1000, 10 000 or 30 000 ppm and 300 g and fed to beagle dogs ($n = 1$/sex per dose) each day. The achieved doses were 0, 3.08, 27.3, 288 and 1035 mg/kg bw per day for males and 0, 3.20, 32.2, 309 and 1032 mg/kg bw per day for females, respectively. The dogs were observed daily for deaths and clinical signs. Body weight was recorded weekly and feed consumption daily. An ophthalmological examination was performed pretreatment and after four weeks of exposure. Blood and urine were collected pretreatment and after two and four weeks of exposure for haematological, clinical chemistry and urine analyses. After four weeks, the dogs were killed and necropsied, and organs were weighed. Only the liver was histopathologically examined.

There were no deaths and no treatment-related clinical signs or effects on body weight or feed consumption. Ophthalmology was unremarkable, and there were no treatment-related effects on haematological or urine analysis parameters. Alkaline phosphatase (ALP) was elevated in the 10 000 ppm female after two and four weeks of treatment (1.5- and 2-fold higher than pretreatment; 1.4- and 2-fold higher than the concurrent control), with marginally less of an effect in the female at 30 000 ppm (1.2- and 1.7-fold higher, respectively, than pretreatment); no increases occurred in males. There were no treatment-related macroscopic findings. Absolute and relative liver weights were elevated relative to the historical control maxima at 1000 (+20% and +13%, respectively) and 10 000 ppm (+21% and +19%, respectively) in males and at 10 000 ppm (+6% and +4%, respectively) in the female. Relative liver weight only was elevated at 30 000 ppm (+11% in males and +6% in females). histopathological examination found slight centrilobular hypertrophy in the liver of the male dog at 10 000 ppm (Yoshida, 2013).

In a 90-day dietary toxicity study by Ohtsuka (2013a), cyclaniliprole (95.71% purity) was admixed in the diet at concentrations of 0, 100, 1000 or 10 000 ppm, and 300 g was fed to beagle dogs (n = 4/sex per dose) each day. The achieved doses were 0, 2.68, 26.8 and 266 mg/kg bw per day for males and 0, 2.75, 26.9 and 270 mg/kg bw per day for females, respectively. The dogs were observed daily for deaths and clinical signs. Body weight was recorded weekly and feed consumption daily. An ophthalmological examination was performed pretreatment and after 13 weeks of treatment. Blood and urine were collected pretreatment and after 7 and 13 weeks of treatment for haematological, clinical chemistry and urine analyses. After 13 weeks of treatment, the dogs were killed and necropsied; the organs were weighed and tissues histopathologically examined.

There were no deaths or treatment-related clinical signs. There were no effects on body weight, feed consumption or ophthalmological findings. Neutrophil counts were significantly increased ($P < 0.05$) during week 13 in females at 1000 and 10 000 ppm (6.35, 8.68, 10.05 and 11.44 × 10^3/μL at 0, 100, 1000 and 10 000 ppm, respectively; historical control range 3.28–14.45 × 10^3/μL), but in the absence of other haematological or pathological changes, these increases were considered not treatment-related.

Selected clinical chemistry results are shown in Table 8. During weeks 7 and 13, ALP was significantly elevated in males at 1000 and 10 000 ppm (2–2.7-fold higher than the concurrent control; $P < 0.05$) and in females at 10 000 ppm (2.4–3.4-fold higher than the concurrent control; $P < 0.01$).

Table 8. Findings in dogs exposed orally to cyclaniliprole for 13 weeks

Parameter	Measure per sex and dietary concentration							
	Males				Females			
	0 ppm	100 ppm	1000 ppm	10 000 ppm	0 ppm	100 ppm	1000 ppm	10 000 ppm
ALP (U/L) [a]								
Pretreatment	389	418	352	375	325	331	309	291
Week 7	275	402	551*	597*	278	344	436	679**
Week 13	229	358	599*	637*	227	311	398	762**
Albumin (g/dL) [b]								
Pretreatment	3.04	3.08	3.11	3.09	3.14	3.30	3.26	3.21
Week 7	3.21	3.04	2.92*	2.93*	3.10	3.10	3.01	2.89
Week 13	3.25	2.91**	2.86**	2.82*	3.05	2.98	3.01	2.89
Calcium (mg/dL) [c]								
Pretreatment	10.5	10.5	10.6	10.0	10.5	10.6	10.6	10.8
Week 13	10.6	10.2*	10.3*	10.3*	10.2	10.3	10.4	10.0
Liver weight [d]								
Absolute (g)	265	345 (+30%)	339 (+28%)	365* (+38%)	267	331 (+24%)	344 (+29%)	322 (+21%)
Relative (%)	2.23	2.87* (+29%)	2.87* (+29%)	3.03** (+36%)	2.36	2.88 (+22%)	2.93 (+24%)	2.89 (+22%)
Histopathology – centrilobular hepatocellular hypertrophy [e]								
	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4

ALP: alkaline phosphatase; ppm: parts per million; U: enzyme unit; *: $P < 0.05$; **: $P < 0.01$

[a] Historical control range: 220–562 U/L (week 7) and 274–471 U/L (week 13) in males; 140–422 U/L (week 7) and 103–478 U/L (week 13) in females.

[b] Historical control range: 2.57–3.40 g/dL (week 7) and 2.61–3.49 g/dL (week 13) in males.

[c] Historical control range: 8.8–11.1 g/dL (week 13) in males.

[d] Expressed as the mean, with the % change compared to the concurrent control in parentheses.

[e] Presented as number of animals with the finding / number of animals examined.

Source: Ohtsuka (2013a)

Although serum albumin and calcium were significantly reduced ($P < 0.01$ or 0.05) in males at every dose, all values were within the historical control range and no dose–response relationship was evident. In high-dose males, serum calcium was lower than the controls prior to treatment initiation. In males, absolute and relative liver weight was increased at 10 000 ppm (+38% and +36%, respectively, relative to the concurrent control; $P < 0.05$). Relative liver weight was significantly higher ($P < 0.05$) than the controls in males at 100 and 1000 ppm (+29% at both doses), but occurred in the absence of any pathology and did not follow a dose–response relationship. No dose-related changes in liver weight occurred in females. On this basis, the increase in liver weight in males at 100 ppm was not considered adverse as it was not corroborated by other findings indicative of hepatotoxicity or reduced liver function, such as increased ALP or reduced albumin. histopathological examination found centrilobular hepatocellular hypertrophy in the liver of one high-dose male.

The NOAEL in males was 100 ppm (equal to 2.68 mg/kg bw per day) based on a consistent increase in ALP activity, a slight but consistent decrease in albumin and increased liver weight at and above 1000 ppm (equal to 26.8 mg/kg bw per day). The NOAEL in females was 1000 ppm (equal to 26.9 mg/kg bw per day) based on a consistent increase in ALP activity and a slight but consistent decrease in albumin at 10 000 ppm (equal to 270 mg/kg bw per day) (Ohtsuka, 2013a).

In a one-year dietary toxicity study by Ohtsuka (2013b), cyclaniliprole (95.71% purity) was admixed in the diet at concentrations of 0, 50, 150, 1000 or 10 000 ppm and 300 g and fed to beagle dogs ($n = 4$/sex per dose) each day. The achieved doses were 0, 1.29, 4.07, 27.2 and 259 mg/kg bw per day for males and 0, 1.47, 4.20, 27.6 and 288 mg/kg bw per day for females, respectively. The dogs were observed daily for mortality and clinical signs, with detailed examinations performed weekly. Body weight was recorded weekly from week 1 to 13 and every four weeks thereafter. Feed consumption was recorded daily. An ophthalmological examination was performed pretreatment and after 52 weeks of treatment. Blood and urine were collected pretreatment and after 13, 26 and 52 weeks of treatment for haematological, clinical chemistry and urine analyses. After 52 or 53 weeks of treatment, the dogs were killed and necropsied; the organs were weighed and tissues histopathologically examined.

There were no deaths or treatment-related clinical signs. There were no effects on body weight, feed consumption, ophthalmological findings or haematological and urine analysis parameters. Selected clinical chemistry findings are shown in Table 9. In both sexes, ALP was significantly elevated at and above 150 ppm (322–509% in males and 300–864% in females). During week 52, alanine aminotransferase (ALT) was lower than the controls in both sexes, reaching statistical significance in females at most doses. While this finding was treatment related, reduced ALT has no toxicological significance. During week 52, total protein was significantly lower than controls in females at 1000 and 10 000 ppm ($P < 0.05$), but in the absence of a dose–response relationship or effects at earlier time points, this difference is unlikely to be treatment related. Albumin was significantly lower ($P < 0.01$ or 0.05) than controls at 1000 and 10 000 ppm in both sexes.

There were no treatment-related macroscopic findings. Absolute and relative thyroid weights were elevated in high-dose females (+78% and +89%, respectively; $P < 0.01$), but in the absence of any pathological changes except for lymphocytic thyroiditis, a common, naturally occurring lesion in beagle dogs. Incidences of this finding were 1, 1, 1, 1 and 1 for males and 0, 1, 1, 1 and 1 for females at 0, 50, 150, 1000 and 10 000 ppm, respectively. After removing one high-dose female with this lesion (graded as severe), the thyroid weights in high-dose females still remained 35% (absolute) or 45% (relative) higher than controls. In males, absolute and relative liver weights were elevated by more than 20% at 1000 ($P < 0.05$) and 10 000 ppm ($P < 0.01$). In females, relative liver weight was significantly higher ($P < 0.01$ or 0.05) than the controls at every dose (+25, +27, +26 and +45%); the increased relative liver weights in females at 50 and 150 ppm were not considered adverse as they were not accompanied any other findings indicative of hepatotoxicity or impaired liver function. histopathological examination found treatment-related centrilobular hepatocellular hypertrophy in one male and one female at 10 000 ppm.

Table 9. Findings in dogs exposed orally to cyclaniliprole for 52 weeks

Parameter	Measure per dose level of cyclaniliprole									
	Males					Females				
	0 ppm	50 ppm	150 ppm	1000 ppm	10 000 ppm	0 ppm	50 ppm	150 ppm	1000 ppm	10 000 ppm
ALP (U/L)										
Pretreatment	426	395	426	375	331	362	353	411	409	378
Week 13	237	317	537	547	598* (252%)	207	372	610	664* (321%)	858** (414%)
Week 26	176	318	567* (322%)	619* (352%)	704** (400%)	202	411	605* (300%)	581	1037** (513%)
Week 52	154	290	583	728* (472%)	784** (509%)	140	431	681*	587	1210** (864%)
ALT (U/L)										
Week 52	40	28	37	32	28	42	26*	28*	34	25**
Total protein (g/dL)										
Week 52	6.44	6.25	6.45	5.94	6.04	6.84	6.32	6.56	6.12*	6.16*
Albumin (g/dL)										
Pretreatment	3.16	3.06	3.10	3.05	3.07	3.40	3.26	3.22	3.24	3.35
Week 13	3.07	2.94	2.88	2.70**	2.72**	3.29	3.08	3.01	2.91**	3.03
Week 26	3.22	2.98	2.88	2.76*	2.79*	3.22	2.87	3.04	2.88	2.95
Week 52	3.15	3.01	2.82	2.79	2.77	3.33	2.98	3.07	2.85*	2.79**
Liver weight [a]										
Absolute (g)	279	307	299	318	369** (+32%)	252	293	320	318	338*
Relative (%)	2.32	2.60	2.72	2.90* (+25%)	3.20** (+38%)	2.34	2.92* (+25%)	2.98* (+27%)	2.96* (+26%)	3.40** (+45%)
Histopathology – centrilobular hepatocellular hypertrophy [b]										
	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4

ALP: alkaline phosphatase; ALT: alanine aminotransaminase; U: enzyme unit; *: $P < 0.05$; **: $P < 0.01$

[a] Expressed as means, with the % change compared to the concurrent control in parentheses.

[b] Presented as number of animals with the finding / number of animals examined.

Source: Ohtsuka (2013b)

The NOAEL was 150 ppm (equal to 4.07 mg/kg bw per day in males and 4.20 mg/kg bw per day in females) based on a consistent increase in ALP, a slight but consistent decrease in albumin and increased liver weight at 1000 ppm (equal to 27.2 mg/kg bw per day in males and 27.6 mg/kg bw per day in females) (Ohtsuka, 2013b). These effects were consistent with those observed in the 90-day study of toxicity in dogs by Ohtsuka (2013a).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a chronic toxicity and carcinogenicity study by Chase (2013), cyclaniliprole (95.71% purity) admixed in the diet at concentrations of 0, 200, 1250 or 8000 ppm was fed ad libitum to CD-1 (Crl:CD1(ICR)) mice (n = 51/sex per dose) for 78 weeks. The achieved doses were 0, 22.7, 140 and 884 mg/kg bw per day for males and 0, 31.6, 186 and 1316 mg/kg bw per day for females, respectively. Mortality and clinical signs were assessed daily. Body weight and feed consumption were recorded weekly for 14 weeks and every four weeks thereafter. Blood smears from the control and high-dose groups were prepared during week 52 and 78 for the analysis of differential leukocyte counts and morphological abnormalities. After 78 weeks of treatment, the mice were killed and necropsied; organs were weighed and tissues histopathologically examined.

Survival was comparable across all groups, and there were no treatment-related clinical signs or effects on body weight or feed consumption. Analysis of blood smears from high-dose mice showed decreased neutrophils (−29% in males and −24% in females, $P < 0.01$) and increased lymphocytes (+32% in males and +21% in females, $P < 0.01$) during week 78 but not 52, and decreased monocytes in males during week 52 (−45%, $P < 0.05$) and 78 (−60%, $P < 0.05$). These changes were minor; in the absence of any pathology, they were considered not adverse. Relative liver weight was slightly elevated in high-dose males (+15%, $P < 0.05$), but in the absence of any corresponding pathology and given the relatively small magnitude of the increase, this increase was considered not toxicologically significant. Absolute brain weight was slightly increased in high-dose males (+3%, $P < 0.05$), a finding that was considered to be incidental.

There were no treatment-related macroscopic findings. The incidence of benign bronchioloalveolar adenomas was slightly increased in females at 1250 and 8000 ppm (14% at both doses versus 4% in the control) in the absence of accompanying alveolar epithelial hyperplasia. In addition, the increase was not statistically significant and the incidences were within the historical control range of 7.7–16%. Therefore, the increase in this lesion was not considered to be treatment related.

The NOAEL for chronic toxicity and carcinogenicity was 8000 ppm (equal to 884 mg/kg bw per day in males and 1316 mg/kg bw per day in females), the highest tested dietary concentration (Chase, 2013).

Rat

In a carcinogenicity study by Ohtsuka (2013c), cyclaniliprole (95.71% purity) was admixed in the diet at concentrations of 0, 200, 2000, 6000 or 20 000 ppm and fed ad libitum to Wistar Hannover [Brl:Han:WIST@Jcl(GALAS)] rats (n = 51/sex per dose) for 104 weeks. The achieved doses were 0, 7.93, 82.5, 249 and 834 mg/kg bw per day for males and 0, 10.3, 103, 306 and 1041 mg/kg bw per day for females, respectively. The rats were observed for mortality and clinical signs daily, with more extensive clinical examinations performed weekly. Body weight and feed consumption were recorded weekly for 13 weeks and every four weeks thereafter. Blood smears were prepared during weeks 54, 78 and 104 for the analysis of differential leukocyte counts and morphological abnormalities. After 104 weeks of treatment, the rats were killed and necropsied; the organs were weighed and tissues histopathologically examined.

Survival was comparable across all groups, and there were no treatment-related clinical signs. At 2000 and 20 000 ppm, body weights were significantly lower ($P < 0.05$) in males at weeks 20 and 24 (−5% and −6%), which is of marginal biological significance; no changes were evident in females or at other times. In high-dose females, feed consumption was significantly ($P < 0.01$ or 0.05) elevated at weeks 3, 4, 12, 44, 48 and 52, but these changes were considered not toxicologically significant. There were no treatment-related haematological findings. Macroscopic uterine masses were significantly increased ($P < 0.05$) in females at the highest dose (17% versus 2.3% in the control). The uterine masses corresponded microscopically to stromal endometrial stromal polyps in five females and a malignant schwannoma in one female. Their total incidences were 3, 6, 3, 6 or 6 for the stromal polyp and 0, 0, 0, 0 and 1 for the malignant schwannoma at 0, 200, 2000, 6000 and 20 000 ppm, respectively. When all the female rats were examined, the increases were not statistically significant, and therefore this macroscopic change was not considered treatment related. There were no treatment-related neoplastic lesions. The only treatment-related non-neoplastic lesion was follicular cell hypertrophy of the thyroid

gland in high-dose males (17% versus 2.7% in the controls, $P < 0.05$; 16% versus 2% in all high-dose male rats, $P < 0.05$).

The NOAEL for chronic toxicity was 6000 ppm in males (equal to 249 mg/kg bw per day) for follicular cell hypertrophy of the thyroid gland at 20 000 ppm (equal to 834 mg/kg bw per day), while the NOAEL in females was 20 000 ppm (equal to 1041 mg/kg bw per day), the highest tested dietary concentration.

The NOAEL for carcinogenicity was 20 000 ppm (equal to 834 mg/kg bw per day for males and 1041 mg/kg bw per day for females), the highest dose tested (Ohtsuka, 2013c).

2.4 Genotoxicity

The results of genotoxicity studies on cyclaniliprole are summarized in Table 10.

Table 10. Summary of genotoxicity studies with cyclaniliprole

End point	Test object	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 *uvrA*	61.7–5000 µg/plate (± S9) DMSO vehicle	95.71	Negative [a]	Matsumoto (2011)
Gene mutation	Mouse lymphoma L5178Y $TK^{+/-}$ cells 3- and 24-h treatments	20–320 µg/mL (± S9) DMSO vehicle	95.71	Negative [b]	Matsumoto (2013)
Cytogenetic test	Chinese hamster lung cells 24- or 48-h continuous treatment	15.4–625 µg/mL (± S9) DMSO vehicle	95.71	Negative [c]	Wada (2011a)
In vivo					
Micronucleus	Male ICR (Crlj:CD1) mice 24- or 48-hour sampling	500, 1000 or 2000 mg/kg bw, ip Methylcellulose vehicle	95.71	Negative [d]	Wada (2011b)

bw: body weight; DMSO: dimethyl sulfoxide; ip: intraperitoneal; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *TK*: thymidine kinase

[a] Precipitation at ≥ 313 µg/plate (−S9) and ≥ 1250 µg/plate (−S9).

[b] Cytotoxicity at > 106.7 µg/mL. Precipitation at ≥ 160 µg/mL.

[c] Precipitation at ≥ 156 µg/mL (± S9).

[d] No signs of toxicity.

2.5 Reproductive and developmental toxicity

(a) Single-generation or multigeneration studies

In a two-generation reproductive toxicity study by Fujii (2013), cyclaniliprole (95.71%) was admixed in the diet at 0, 500, 3000 or 20 000 ppm and fed ad libitum to two parental generations of Crl:CD(SD) rats (n = 24/sex per group) and their offspring through premating (15–16 weeks), mating, gestation and lactation. The rats were observed daily for mortality and clinical signs, with body weight and feed consumption recorded regularly for parental rats throughout the study. Standard reproduction parameters, including the analysis of oestrous cycling and sperm parameters, as well as litter and offspring parameters were recorded or calculated. All pups were also evaluated for physical development and subjected to a reflex response test (n = 1/litter). At scheduled kill, rats were necropsied and organs weighed. Reproductive tissues were histopathologically examined.

The achieved intakes of cyclaniliprole are summarized in Table 11. There were no treatment-related deaths, clinical signs or effects on body weight or feed consumption in parental rats. There was no treatment-related effect on reproduction, litter parameters or pups. There were no treatment-related macroscopic or histopathological findings. Organs weights were generally unaffected by treatment, with only a small increase in absolute liver weight (+11%, $P < 0.05$) occurring in F_0 parental females at 20 000 ppm, a finding that was considered not toxicologically significant.

Table 11. Mean intakes of cyclaniliprole in rats

Exposure period	Mean intakes (mg/kg bw per day) per dietary concentration					
	Males			Females		
	500 ppm	3000 ppm	20 000 ppm	500 ppm	3000 ppm	20 000 ppm
F_0 generation						
Premating	34.9	207	1406	39.2	228	1589
Gestation	–	–	–	31.6	186	1299
Lactation	–	–	–	60.3	361	2541
F_1 generation						
Premating	41.2	245	1683	45.6	274	1835
Gestation	–	–	–	34.4	200	1371
Lactation	–	–	–	61.3	356.3	2491

bw: body weight; F_0: parental generation; F_1: first filial generation *Source*: Fujii (2013)

The NOAEL for reproductive toxicity, parental toxicity and offspring toxicity was 20 000 ppm (equal to 1683 mg/kg bw per day for F_1 males and 1835 mg/kg bw per day for F_1 females), the highest dietary concentration.

(b) Developmental toxicity

Rat

In a developmental toxicity study by Takahashi (2012), cyclaniliprole (95.71% purity) in 1% weight per volumev(w/v)vCMC was administered by gavage to mated Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) female rats ($n = 24$/dose) at a dose of 0, 100, 300 or 1000 mg/kg bw per day from gestation day 6–19. The dams were observed daily for clinical signs, with body weight and feed consumption recorded throughout. On gestation day 20, surviving dams were killed and necropsied. Ovaries and uteri were macroscopically examined and the following recorded: gravid uterine weights, corpora lutea counts, total resorptions, implantations, live and dead fetuses and pup sex ratios. Fetuses were examined for external, visceral and skeletal abnormalities.

All dams survived to scheduled kill. There were no treatment-related clinical signs, effects on body weight or feed consumption or macroscopic findings. Litter and uterine findings were consistent across all groups. There were no effects on the nature, incidence or distribution of external, visceral or skeletal abnormalities.

The NOAEL for maternal and embryo and fetal toxicity was 1000 mg/kg bw per day, the highest tested dose (Takahashi, 2012).

Rabbit

In a developmental toxicity study by Takahashi (2013), cyclaniliprole (95.71% purity) in 1% w/v CMC was administered by gavage to inseminated female Japanese White Rabbits (Kbl:JW; $n = 25$/group) at 0, 100, 300 or 1000 mg/kg bw per day from gestation day 6 to 27. The dams were observed daily for clinical signs, with body weight and feed consumption recorded throughout. On gestation day 28, surviving dams were killed and necropsied. Ovaries and uteri were macroscopically examined and the following recorded: gravid uterine weights, corpora lutea counts, total resorptions, implantations, live and dead fetuses and pup sex ratios. Fetuses were examined for external, visceral and skeletal abnormalities.

All dams survived to scheduled kill. There were no treatment-related clinical signs, effects on body weight or feed consumption or macroscopic findings. Litter and uterine findings were consistent across all groups. There were no effects on the nature, incidence or distribution of external, visceral or skeletal abnormalities.

The NOAEL for maternal and embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Takahashi, 2013).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study by Chapman (2012), Crl:CD®(SD) rats (n = 10/sex per dose) were administered a single gavage dose of cyclaniliprole (95.71% purity) in 1% w/v CMC at 0, 500, 1000 or 2000 mg/kg bw. The rats were observed twice daily for mortality and clinical signs. Body weights were recorded on days 1, 8 and 15. Feed consumption was recorded prior to dosing and weekly following dosing. FOB and motor activity assessment were performed pretreatment, at 24 hours after dosing (i.e. the approximate time to peak effect) and at days 7 and 15. On day 15, survivors were killed and necropsied. Brains were measured and weighed. Neurological tissue from five rats/sex from the control and 2000 mg/kg bw group was histopathologically examined.

There were no treatment-related findings, including any evidence of neurotoxicity.

The NOAEL was 1000 mg/kg bw, the highest tested dose (Chapman, 2012).

In a subchronic neurotoxicity study by Allen (2012), cyclaniliprole (95.71% purity) was admixed in the diet at concentrations of 0, 600, 3100 or 16 000 ppm and fed ad libitum to Crl:CD®(SD) rats (n = 10/sex per dose) for 13 weeks. The achieved doses were 0, 40, 204 and 1085 mg/kg bw per day for males and 0, 49, 240 and 1279 mg/kg bw per day for females, respectively. The rats were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly. FOB and motor activity assessment were performed pretreatment and during weeks 2, 4, 8 and 13. Rats were killed after 13 weeks of treatment and necropsied; brain measurements and weights were recorded. Neurological tissue from 5 rats/sex from the control and high-dose groups were histopathologically examined.

There were no deaths or treatment-related clinical signs. At the highest dose, body weight gain was approximately 8% and 12% lower than the controls in males and females, respectively. In the absence of any statistical differences or effects on absolute body weight or feed consumption, and given the transient nature of this finding, it was not considered treatment-related. The FOB and motor activity assessment were unremarkable. There was no treatment-related effect on brain morphometry or weight, or on the incidence or nature of histopathological changes in the nervous system.

The NOAEL was 16 000 ppm (equal to 1085 mg/kg bw per day), the highest dietary concentration.

(b) Immunotoxicity

In a 28-day systemic toxicity and immunotoxicity study by Coleman (2013), cyclaniliprole (95.71% purity) was admixed in the diet at a concentration of 0, 200, 1250 or 8000 ppm and fed ad libitum to female Crl:CD1(ICR) mice (n = 10/group). The achieved doses were 0, 34, 209 and 1352 mg/kg bw per day, respectively. A positive control group (n = 8) received cyclophosphamide (20 mg/kg bw per day) by gavage on days 22–26. All the mice were immunized with sheep red blood cell (sRBC) antigen on day 25. The mice were observed daily for mortality and clinical signs, with a more detailed physical examination performed weekly. Body weight, feed consumption and water consumption were recorded weekly. The mice were killed on day 28 and necropsied; spleen and thymus weights were recorded. Splenocyte preparations were prepared to analyse adaptive or acquired immune response according to the Jerne plaque-forming cell assay.

No treatment-related effects including any effects on immune function were identified in this study.

The NOAEL for systemic and immunotoxicity toxicity was 8000 ppm (equal to 1352 mg/kg bw per day), the highest dose tested (Coleman, 2013).

(c) Studies on metabolites

All cyclaniliprole metabolites detected in plants or livestock relevant to human dietary exposures were also detected in rats.

The main residue in all commodities tested was parent cyclaniliprole, with the metabolite NK-1375 also detected in some plant commodities at 10–30% of total residues. While NK-1375 is formed in the rat (Penketh, 2013a), it was detectable only in fat at relatively low levels (4.2–5.9% of total tissue radioactivity). An acute oral toxicity test (Table 12) and bacterial reverse mutation assay (Table 13) were conducted with NK-1375.

Table 12. Results of an acute toxicity study with NK-1375

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} (mg/kg bw)	Reference
Rat	Crl:CD(SD)	F	Oral	98.60	1% CMC	> 2000	Bull (2012)

CMC: carboxymethylcellulose; LD_{50}: median lethal dose;
NK-1375: 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl)benzamide

Table 13. Results of a genotoxicity study with NK-1375

End point	Test object	Concentration or dose	Purity (%)	Result	Reference
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 *uvrA*	5–5000 µg/plate (± S9) DMSO vehicle	98.60	Negative[1a]	May (2012)

S9: 9000 × *g* supernatant fraction from rat liver homogenate; DMSO: dimethyl sulfoxide;
NK-1375: 3-bromo-2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*-(1-cyclopropylethyl)benzamide

[a] Precipitation ≥ 500 µg/plate.

Structurally, NK-1375 is very similar to the parent, and is formed in plants following cyclization of the amide linkage with the pyridine ring in cyclaniliprole with dehydrochlorination. A structural comparison of NK-1375 with cyclaniliprole using Toxtree (version 2.6.13) identified no unique structural alerts that would not have been covered by the comprehensive toxicity tests on the parent.

On this basis, it was concluded that NK-1375 is likely to be of no greater toxicity than cyclaniliprole.

3. Observations in humans

The sponsor reported that annual medical examinations have been performed on manufacturing plant personnel since the commencement of manufacture of cyclaniliprole. No adverse events, including poisonings, have been reported.

Comments

Biochemical aspects

In studies conducted using [^{14}C]cyclaniliprole, C_{max} of radioactivity in plasma were reached at 24–120 hours after a single oral dose of 10 or 400 mg/kg bw in rats (Penketh, 2013a) and 6–48 hours after a single oral dose of 1 mg/kg bw in dogs (Penketh, 2013b). Based on the level of radioactivity in bile, urine, liver and carcass, gastrointestinal absorption in rats was estimated to be 11% in males and 9% in females at 10 mg/kg bw, and 2% in males and 5% in females at 400 mg/kg bw (Penketh, 2013a). In dogs, gastrointestinal absorption was approximately 40% following a single oral dose of 1 mg/kg bw (Penketh, 2013b). In rats, the majority of radioactivity was eliminated in faeces (>85%) within 48 hours, with relatively low levels excreted in urine (<1%). Following a single oral dose of 10 mg/kg bw to rats, approximately 2% of radioactivity remained in tissues at 168 hours, with 31% remaining following repeated dosing (Penketh, 2013a). In both rats and dogs, the highest tissue concentrations of radioactivity were generally detected in whole blood and plasma (Penketh, 2013a,b). It was not possible to determine plasma elimination half-lives as plasma radioactivity did not decrease over the experimental period (up to 168 hours) (Penketh, 2013a,b). Radioactivity in all other tissues decreased over time.

In rats, parent cyclaniliprole was the main compound identified in faeces and fat. Cyclaniliprole was also detected in plasma, liver and kidney; none was detected in bile or urine. The main plasma metabolite was NSY-28 (up to 98% of total tissue radioactivity) (Penketh, 2013a). Low levels of the metabolites YT-1284 and NSY-27 were detected in excreta and liver, with YT-1284 also detected in kidney and NSY-27 in plasma. The metabolite NK-1375 was detected only in fat (Penketh, 2013a).

Toxicological data

In rats, the oral and dermal median lethal dose (LD_{50}) values were greater than 2000 mg/kg bw (Moore, 2011a,b) and the inhalation median lethal concentration (LC_{50}) was greater than 4.62 mg/L (Leighton, 2011). Cyclaniliprole was not irritating to the skin of rabbits (Ueda, 2011a) but was slightly irritating to rabbit eyes (Ueda, 2011b). Cyclaniliprole was not a skin sensitizer in mice (Fukuyama, 2011) or guinea pigs (Nomura, 2012).

Evidence of limited toxicity was seen in repeated-dose studies. In mice, rats and dogs, the liver is the target organ, with dogs the most sensitive species.

In a 13-week study in mice, which tested dietary concentrations of 0, 200, 1200 or 8000 ppm cyclaniliprole (equal to 0, 27, 159 and 1023 mg/kg bw per day in males and 0, 34, 179 and 1350 mg/kg bw per day in females, respectively), the NOAEL was 8000 ppm (equal to 1023 mg/kg bw per day), the highest dose tested (O'Halloran, 2012).

In a 28-day range-finding study in rats, which tested dietary concentrations of 0, 300, 1250, 5000 or 20 000 ppm cyclaniliprole (equal to 0, 26.4, 107, 426 and 1778 mg/kg bw per day in males and 0, 26.4, 113, 443 and 1800 mg/kg bw per day in females, respectively), the slight increase in liver weight observed at the highest dose was not considered adverse (Ohnuma, 2010).

In a 90-day study of toxicity in rats, which tested dietary concentrations of 0, 600, 6000 or 20 000 ppm cyclaniliprole (equal to 0, 39.9, 402 and 1331 mg/kg bw per day in males and 0, 43.3, 467 and 1594 mg/kg bw per day in females, respectively), the NOAEL was 20 000 ppm (equal to 1331 mg/kg bw per day), the highest dose tested (Ohnuma, 2011).

In a 90-day study of toxicity in dogs, which tested dietary concentrations of 0, 100, 1000 or 10 000 ppm cyclaniliprole (equal to 0, 2.68, 26.8 and 266 mg/kg bw per day in males and 0, 2.75, 26.9 and 270 mg/kg bw per day in females, respectively), the NOAEL was 100 ppm (equal to 2.68 mg/kg bw per day) based on a consistent increase in ALP activity, a slight but consistent decrease in albumin and increased liver weight at and above 1000 ppm (equal to 26.8 mg/kg bw per day) (Ohtsuka, 2013a).

In a one-year study of toxicity in dogs, which tested dietary concentrations of 0, 50, 150, 1000 or 10 000 ppm cyclaniliprole (equal to 0, 1.29, 4.07, 27.2 and 259 mg/kg bw per day in males and 0, 1.47, 4.20, 27.6 and 288 mg/kg bw per day in females, respectively), the NOAEL was 150 ppm (equal to 4.07 mg/kg bw per day) based on a consistent increase in ALP, a slight but consistent decrease in albumin and increased liver weight at 1000 ppm (equal to 27.2 mg/kg bw per day) (Ohtsuka, 2013b).

The overall NOAEL for the 90-day and one-year studies of toxicity in dogs was 150 ppm (equal to 4.07 mg/kg bw per day) based on increased ALP, reduced albumin and increased liver weight at 1000 ppm (equal to 26.8 mg/kg bw per day).

In a 78-week study of chronic toxicity and carcinogenicity in mice, which tested dietary concentrations of 0, 200, 1250 or 8000 ppm cyclaniliprole (equal to 0, 22.7, 140 and 884 mg/kg bw per day in males and 0, 31.6, 186 and 1316 mg/kg bw per day in females, respectively), no treatment-related increase in tumour incidences was observed. The NOAEL for chronic toxicity and carcinogenicity was 8000 ppm (equal to 884 mg/kg bw per day), the highest dose tested (Chase, 2013).

In a one-year study of toxicity in rats, which tested dietary concentrations of 0, 200, 2000, 6000 or 20 000 ppm cyclaniliprole (equal to 0, 9.21, 89.6, 277 and 955 mg/kg bw per day in males and 0, 11.7, 117, 358 and 1213 mg/kg bw per day in females, respectively), the NOAEL was 20 000 ppm (equal to 955 mg/kg bw per day), the highest dose tested (Koyama, 2013).

In a 104-week study of carcinogenicity in rats, which tested dietary concentrations of 0, 200, 2000, 6000 or 20 000 ppm cyclaniliprole (equal to 0, 7.93, 82.5, 249 and 834 mg/kg bw per day in males and 0, 10.3, 103, 306 and 1041 mg/kg bw per day in females, respectively), no treatment-related increase in tumour incidences was observed. The NOAEL for chronic toxicity was 6000 ppm (equal to 249 mg/kg bw per day) for follicular cell hypertrophy of the thyroid gland at 20 000 ppm (equal to 834 mg/kg bw per day). The NOAEL for carcinogenicity was 20 000 ppm (equal to 834 mg/kg bw per day), the highest dose tested (Ohtsuka, 2013c).

The Meeting concluded that cyclaniliprole is not carcinogenic in mice or rats.

Cyclaniliprole was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found (Matsumoto, 2011, 2013; Wada, 2011a,b).

The Meeting concluded that cyclaniliprole 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 cyclaniliprole is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats, which tested dietary concentrations of 0, 500, 3000 or 20 000 ppm cyclaniliprole (equal to 0, 41.2, 245 and 1683 mg/kg bw per day in males and 0, 45.6, 274 and 1835 mg/kg bw per day in females, respectively), the NOAELs for reproductive toxicity, parental toxicity and offspring toxicity were 20 000 ppm (equal to 1683 mg/kg bw per day), the highest dose tested (Fujii, 2013).

In a developmental toxicity study in rats, which tested cyclaniliprole at gavage doses of 0, 100, 300 or 1000 mg/kg bw per day from gestation day 6 to 19, the NOAELs for maternal and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Takahashi, 2012). In a developmental toxicity study in rabbits, which tested cyclaniliprole at gavage doses of 0, 100, 300 or 1000 mg/kg bw per day from gestation day 6 to 27, the NOAELs for maternal toxicity and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Takahashi, 2013).

The Meeting concluded that cyclaniliprole is not teratogenic.

In an acute neurotoxicity study in rats, which tested cyclaniliprole at a single gavage dose of 0, 500, 1000 or 2000 mg/kg bw, the NOAELs for acute systemic toxicity and neurotoxicity were 2000 mg/kg bw, the highest dose tested (Chapman, 2012).

In a 13-week subchronic neurotoxicity study in rats, which tested dietary concentrations of 0, 600, 3100 or 16 000 ppm cyclaniliprole (equal to 0, 40, 204 and 1085 mg/kg bw per day in males and 0, 49, 240 and 1279 mg/kg bw per day in females, respectively), the NOAELs for systemic toxicity and neurotoxicity were 16 000 ppm (equal to 1085 mg/kg bw per day), the highest dose tested (Allen, 2012).

The Meeting concluded that cyclaniliprole is not neurotoxic.

In a 28-day immunotoxicity study in female mice, which tested dietary concentrations of 0, 200, 1250 or 8000 ppm cyclaniliprole (equal to 0, 34, 209 and 1352 mg/kg bw per day, respectively), the NOAELs for systemic toxicity and immunotoxicity toxicity were 8000 ppm (equal to 1352 mg/kg bw per day), the highest dose tested (Coleman, 2013).

The Meeting concluded that cyclaniliprole is not immunotoxic.

Toxicological data on metabolites and/or degradates

All relevant metabolites of cyclaniliprole detected in plants or livestock were also detected in rats.

The main residue in all commodities tested was parent cyclaniliprole, with the metabolite NK-1375 also detected in some plant commodities at 10–30% of total residues. While NK-1375 is formed in the rat (Penketh, 2013a), it was detectable only in fat at relatively low levels (4.2–5.9% of total tissue radioactivity). In an acute toxicity study in rats, the LD_{50} for NK-1375 was greater than 2000 mg/kg bw (Bull, 2012), while an Ames test indicated no evidence of in vitro mutagenicity (May, 2012). Structurally, NK-1375 is very similar to the parent; a structural comparison of NK-1375 with cyclaniliprole using Toxtree (version 2.6.13) identified no unique structural alerts that would not already be covered by the comprehensive toxicity tests on the parent.

On this basis, the Meeting concluded that NK-1375 is likely to be of no greater toxicity than cyclaniliprole, and therefore the acceptable daily intake (ADI) established for cyclaniliprole would cover potential dietary exposure.

Human data

No adverse events, including poisonings, have been reported in personnel involved in the manufacture of cyclaniliprole.

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

Toxicological evaluation

The Meeting established an ADI of 0.04 mg/kg bw based on the overall NOAEL of 4.07 mg/kg bw per day in 13-week and 1-year studies of toxicity in dogs for elevated ALP activity, reduced albumin and increased liver weight at 26.8 mg/kg bw per day, and using a 100-fold safety factor.

The ADI was established for the sum of cyclaniliprole and the metabolite, NK-1375, expressed as cyclaniliprole.

The Meeting concluded that it is not necessary to establish an acute reference dose (ARfD) for cyclaniliprole in view of its low acute toxicity and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of cyclaniliprole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Thirteen-week study of toxicity [a]	Toxicity	8000 ppm, equal to 1023 mg/kg bw per day [b]	–
	Twenty-eight day study of toxicity and immunotoxicity [a]	Toxicity, immunotoxicity	8000 ppm, equal to 1352 mg/kg bw per day [b]	–
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	8000 ppm, equal to 884 mg/kg bw per day [b]	–
		Carcinogenicity	8000 ppm, equal to 884 mg/kg bw per day [b]	–

(*Continued on next page*)

Species	Study	Effect	NOAEL	LOAEL
Rat	Acute neurotoxicity study [c]	Toxicity	2000 mg/kg bw [b]	–
	Thirteen-week study of neurotoxicity [a,d]	Toxicity, neurotoxicity	16 000 ppm, equal to 1085 mg/kg bw per day	–
	One-year study of toxicity [a]	Toxicity	20 000 ppm, equal to 955 mg/kg bw per day [b]	–
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	6000 ppm, equal to 249 mg/kg bw per day	20 000 ppm, equal to 834 mg/kg bw per day
		Carcinogenicity	20 000 ppm, equal to 834 mg/kg bw per day [b]	–
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	20 000 ppm, equal to 1683 mg/kg bw per day [b]	–
		Parental toxicity	20 000 ppm, equal to 1683 mg/kg bw per day [b]	–
		Offspring toxicity	20 000 ppm, equal to 1683 mg/kg bw per day [b]	–
	Developmental toxicity study [c]	Maternal toxicity	1000 mg/kg bw per day [b]	–
		Embryo/fetal toxicity	1000 mg/kg bw per day [b]	–
Rabbit	Developmental toxicity study [c]	Maternal toxicity	1000 mg/kg bw per day [b]	–
		Embryo/fetal toxicity	1000 mg/kg bw per day [b]	–
Dog	Thirteen-week and 1-year studies of toxicity [a,d]	Toxicity	150 ppm, equal to 4.07 mg/kg bw per day	1000 ppm, equal to 26.8 mg/kg bw per day

[a] Dietary administration.

[b] Highest dose tested.

[c] Gavage administration.

[d] Two or more studies combined.

Estimate of acceptable daily intake (ADI applies to the sum of cyclaniliprole and NK-1375, expressed as cyclaniliprole)

0–0.04 mg/kg bw

Estimate of acute reference dose (ARfD)

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. Results of mechanistic studies on the human relevance of increased ALP activity in dogs.

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	T_{max}: 24–120 h (rats); 6–48 h (dogs) Gastrointestinal absorption: < 10% in rats (10 mg/kg bw dose); 40% in dogs (1 mg/kg bw dose)
Dermal absorption	No data
Distribution	Widespread tissue distribution; highest levels in blood
Potential for accumulation	Unlikely to accumulate
Rate and extent of excretion	Slow elimination from plasma
Metabolism in animals	Limited
Toxicologically significant compounds in animals and plants	Cyclaniliprole, NK-1375
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.62 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Slightly irritating
Guinea pig, dermal sensitization	Not sensitizing
Mouse, dermal sensitization	Not sensitizing
Short-term studies of toxicity	
Target/critical effect	Effects on liver, elevated alkaline phosphatase
Lowest relevant oral NOAEL	4.07 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	Follicular cell hypertrophy of the thyroid gland
Lowest relevant NOAEL	249 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice or rats [a]
Genotoxicity	No evidence of genotoxicity [a]
Reproductive toxicity	
Reproduction target/critical effect	No effect on reproduction
Lowest relevant parental NOAEL	1683 mg/kg bw per day (highest dose tested, rat)
Lowest relevant offspring NOAEL	1683 mg/kg bw per day (highest dose tested, rat)
Lowest relevant reproduction NOAEL	1683 mg/kg bw per day (highest dose tested, rat)
Developmental toxicity	
Developmental target/critical effect	No evidence of developmental toxicity
Lowest maternal NOAEL	1000 mg/kg bw per day (highest dose tested – rat, rabbit)
Lowest embryo/fetal NOAEL	1000 mg/kg bw per day (highest dose tested – rat, rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2000 mg/kg bw (highest dose tested, rat)
Subchronic neurotoxicity NOAEL	1085 mg/kg bw per day (highest dose tested, rat) Not neurotoxic

Other toxicological studies	
Immunotoxicity NOAEL	1352 mg/kg bw per day (highest dose tested, mice) Not immunotoxic
Toxicological studies on NK1375	
Rat, LD_{50}, oral	> 2000 mg/kg bw per day
Genotoxicity	Not mutagenic in vitro
Human data	No adverse effects in manufacturing personnel

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Studies	Safety factor
ADI [a]	0–0.04 mg/kg bw	Ninety-day and one-year studies in dogs	100
ARfD	Unnecessary		

[a] Applies to cyclaniliprole + NK-1375, expressed as cyclaniliprole.

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FENAZAQUIN

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Explanation

Fenazaquin is the International Organization for Standardization (ISO)–approved common name for 4-tert-butylphenethyl-quinazolin-4-yl-ether (International Union of Pure and Applied Chemistry [IUPAC] name), with the Chemical Abstracts Service number 120928-09-8. It is an acaricide and has a quinazoline structure.

Fenazaquin has not been evaluated previously 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).

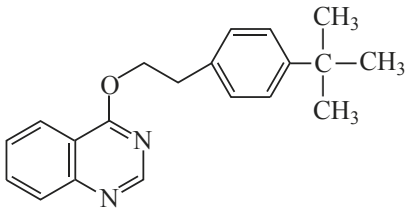
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise indicated.

Evaluation for acceptable daily intake

1. Biochemical aspects

Fig. 1 shows the structure of fenazaquin.

Figure 1. Structure of fenazaquin



1.1 Absorption, distribution and excretion

(a) Oral route

To compare the toxicokinetics of fenazaquin in several animal species, radiolabelled fenazaquin (lot number and specific radioactivity or radiochemical purity not indicated) in a 10% aqueous (weight per volume [w/v]) acacia suspension vehicle was administered by gavage to Fischer 344 rats, CD-1 mice or Syrian golden hamsters at 1, 10 or 30 mg/kg bw for rats (n = 3/sex per dose); 30, 300 or 750 mg/kg bw for mice (n = 3/sex per dose); and 5, 25 or 125 mg/kg bw for hamsters (n = 3/sex per dose). The total radiocarbon concentrations were measured in plasma samples taken at 0.25 (rat), 0.5, 1, 2, 4, 8, 14 (mouse), 24 (rat and hamster), 27 (mouse), and 48, 96 and 168 (rat and hamster) hours (Billington, 1997).

In rats, fenazaquin was readily absorbed by males and females. The time to reach peak plasma concentration (T_{max}) was eight hours in males and females at all doses. The area under concentration–time curve (AUC) and peak plasma concentration (C_{max}) showed a good correlation with the administered doses, with absorption and elimination kinetics comparable across doses and sexes. Elimination was complete after 168 hours, with a half-life of approximately 20.5–34.7 hours, without large differences between sexes and dose groups. The pharmacokinetic profile was similar in males and females.

In mice, T_{max} was 0.5–4 hours at 30 and 300 mg/kg bw in males and females, indicating that absorption occurred readily. The AUC was comparable in both sexes at these doses. A second peak, observed after 48 hours in females at 750 mg/kg bw was considered to be due to the unusually high dose administered. Half-lives at 30 and 300 mg/kg bw, respectively, were 2.9 and 27.5 hours in males and 2.8 and 9.1 hours in females; half-lives at 750 mg/kg bw could not be determined. Elimination was complete after 48 hours in mice at 30 mg/kg bw and after 72 hours in mice at 300 and 750 mg/kg bw. Elimination was dose related and became substantially slower at doses above 300 mg/kg bw.

T_{max} values in hamsters treated with fenazaquin were 1 or 2 hours in both sexes at 5 and 25 mg/kg bw. At 125 mg/kg bw, T_{max} was 4 hours in males and 8 hours in females. C_{max} and AUC, as well as half-lives, were comparable in males and females. Half-life times were almost the same in males and females for all doses.

The pharmacokinetics of fenazaquin in rat, mouse and hamster are summarized in Table 1.

Table 1. Plasma kinetic data in rats, mice and hamsters following a single oral dose of [^{14}C]fenazaquin

Animal – dose administered / pharmacokinetic parameter	Measure per oral dose of [^{14}C]fenazaquin					
	Male	Female	Male	Female	Male	Female
Rat – dose	1 mg/kg bw		10 mg/kg bw		30 mg/kg bw	
$C_{max.}$ (µg/mL)	0.202	0.255	2.52	3.99	4.82	8.47
$T_{max.}$ (h)	8	8	8	8	24	8
$t_{½}$ (h)	29.0	34.7	21.2	23.3	23.8	20.5
AUC (hr/µg per mL)	7.55	6.26	78.7	78.5	227	249
Mouse – dose	30 mg/kg bw		300 mg/kg bw		750 mg/kg bw	
C_{max} (µg/mL)	8.0	6.4	39.0	17.3	34.5/21.1	28.5/64.7
T_{max} (h)	0.5	1	4	1	4/48	2/48
$t_{½}$ (h)	2.9	2.8	27.5	9.1	136	ND [a]
AUC (h/µg per mL)	42.5	34.9	380	302	1 170	1 964
Hamster – dose	5 mg/kg bw		25 mg/kg bw		125 mg/kg bw	
C_{max} (µg/mL)	0.66	0.79	2.39	2.82	7.30	10.5
T_{max} (h)	2	1	2	2	4	8
$t_{½}$ (h)	75.1	88.9	90.4	56.3	50.7	65.6
AUC (h/µg per mL)	6.59	8.00	37.0	43.5	248	293

AUC: area under the concentration–time curve; bw: body weight; C_{max}: peak plasma concentration; ND: not determined; $t_{½}$: half-life; T_{max}: time to reach peak plasma concentration

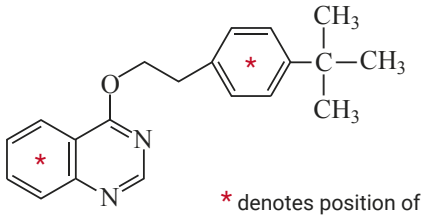
[a] Not determined due to the two peak concentrations.

Source: Billington (1997)

Fenazaquin at 30 or 25 mg/kg bw was more rapidly absorbed in mice and hamsters than in rats. The elimination half-lives were 20.5–23.8 hours in rats, 2.8–2.9 hours in mice and 50.7–90.4 hours in hamsters, indicating that fenazaquin was eliminated more quickly in mice than in rats and hamsters.

A study conducted in accordance with GLP and United States Environmental Protection Agency (USEPA) and Organisation for Economic Co-operation and Development (OECD) test guidelines investigating the distribution and excretion of fenazaquin in Fischer 344 rats in three separate experiments (Althaus & Magnussen, 1992). The *t*-butyl-phenyl-labelled (radiochemical purity >99.0%, lot no. 657-4BE-5; 97.33–97.72%, lot no. 553-VJ7–187) and quinazoline-phenyl-labelled (radiochemical purity 98.8–99.2%; lot no. 553-VE9-091; specific radioactivity: 73.26×10^{10} µBq/mg) fenazaquin used in this study is shown in Fig. 2. Lot no. and purity of unlabelled fenazaquin were ACD13041 and 97.36–98.80%, respectively.

Figure 2. Positions of radiolabels in distribution and excretion study with fenazaquin in rats



In the first experiment, rats (n = 3/sex) were administered a single oral dose of radiolabelled fenazaquin (specific radioactivity 31.64×10^{10} µBq/mg) at 1 mg/kg bw to determine the extent to which the radiocarbon was expired as $^{14}CO_2$ by rats. Sampling time intervals were 6, 14 and 48 hours after dosing.

In the second experiment, differences in absorption, excretion (via urine and faeces) and tissue distribution were compared in rats (n = 3/sex) after oral administration of a single low dose of fenazaquin at 1 mg/kg bw (specific radioactivity: 25.97×10^{10} µBq/mg) and a single high dose of fenazaquin at 30 mg/kg bw (specific radioactivity: 0.89×10^{10} µBq/mg). Urine and faeces were collected separately at 24-hour intervals for seven consecutive days after dose administration. The remaining untreated urine and faeces samples were stored for determination and characterization of metabolites. Seven days after dosing, all the rats were killed and the tissues were assayed for radiocarbon content.

In the third experiment, rats (n = 8/sex) were administered unlabelled fenazaquin (1 mg/kg bw) by gavage for 14 consecutive days. On day 15, five males and five females were dosed with a single dose (1 mg/kg bw) of radiolabelled fenazaquin (specific radioactivity: 25.89×10^{10} µBq/mg).

The radioactivity in urine in the second and third experiments showed that absorption within seven days after dosing was at least 18% of the low dose and 16% of the high dose. However, following oral administration of fenazaquin to bile duct–cannulated male F344 rats at a target dose of 1 mg/kg bw, absorption was rapid and high, representing approximately 65.1% of the administered dose (see Table 4).

In terms of distribution of fenazaquin in tissues/organs, levels of radioactivity were very low in all organs and tissues seven days after dosing. Concentrations of radioactivity were highest in the carcass, accounting for between 0.47% and 1.6% of total radioactivity administered. In liver, the radioactivity ranged from 0.026% to 0.036% of total radioactivity administered after seven days. Concentrations in the other tissues and organs investigated were reported to be lower than 0.04% of the total radioactivity administered. Modestly increased levels of fenazaquin were seen in the fat of rats receiving the high oral dose (30 mg/kg bw) compared to those receiving the low dose (1 mg/kg bw) multiple times. Fenazaquin distribution in tissues did not correlate with organ-specific toxicity and was not indicative of a potential to accumulate (Table 2).

Table 2. Distribution of radiolabel in tissues after single- or repeated-dose administration of fenazaquin

Tissue	Sex	Measure per dose of fenazaquin					
		Single dose at 1 mg/kg bw		Single dose at 30 mg/kg bw		Repeated doses at 1 mg/kg bw	
		µg equiv./g	% of dose	µg equiv./g	% of dose	µg equiv./g	% of dose
Blood	Male	0.004	ND	0.115	ND	0.005	ND
	Female	0.004	ND	0.073	ND	0.003	ND
Bone	Male	0.004	ND	0.178	ND	0.006	ND
	Female	0.007	ND	0.191	ND	0.005	ND
Brain	Male	0.000	0.000 8	0.002	0.000	0.000	0.001
	Female	0.000	0.001 4	0.005	0.000	0.000	0.001
Carcass	Male	0.002	0.470 1	0.061	0.467	0.005	1.06
	Female	0.005	1.575 4	0.101	0.942	0.004	1.141
Fat	Male	0.054	ND	2.18	ND	0.079	ND
	Female	0.131	ND	2.67	ND	0.091	ND
Heart	Male	0.000	0.000 1	0.013	0.000	0.002	0.001
	Female	0.000	0.000 2	0.008	0.000	0.002	0.001
Kidney	Male	0.001	0.002 2	0.044	0.003	0.002	0.004
	Female	0.002	0.004 4	0.043	0.003	0.001	0.003
Liver	Male	0.004	0.032 2	0.122	0.036	0.003	0.036
	Female	0.003	0.028 7	0.098	0.026	0.003	0.027
Lung	Male	0.003	0.001 8	0.105	0.002	0.006	0.004
	Female	0.005	0.004 1	0.091	0.002	0.004	0.003

Plasma	Male	0.001	ND	0.051	ND	0.005	ND
	Female	0.001	ND	0.046	ND	0.003	ND
Muscle	Male	0.000	ND	0.015	ND	0.002	ND
	Female	0.001	ND	0.024	ND	0.002	ND
Spleen	Male	0.003	0.000 7	0.138	0.001	0.001	0.000
	Female	0.002	0.000 8	0.171	0.002	0.003	0.001
Uterus	Male	–	–	–	–	–	–
	Female	0.008	0.002 2	0.098	0.001	0.002	0.000
Ovaries	Male	–	–	–	–	–	–
	Female	0.023	0.001 8	0.582	0.001	0.015	0.001
Testes	Male	0.000	0.000 5	0.037	0.002	0.002	0.002
	Female	–	–	–	–	–	–

bw: body weight; eq: equivalent; ND: not determined *Source*: Althaus & Magnussen (1992)

Rats treated with fenazaquin at 1 mg/kg bw as a single or a repeated dose had comparable amounts of radioactivity in urine, faeces and tissues; the mean percentage of the dose recovered was 100.6–108.8%. Radioactivity in urine of rats at 30 mg/kg bw was also comparable to the radioactivity in urine of rats treated with a single or repeated dose at 1 mg/kg bw. A slightly lower trend of faecal radioactivity excretion was observed at 30 mg/kg bw. Total recovery at 30 mg/kg bw was lower (90.4–92.0%) due to the slightly lower radioactivity in faeces, although radioactivity in urine was comparable in all dose groups. No sex-related differences in radioactivity were found in the overall urinary and faecal excretion. Multiple dosing with fenazaquin did not induce or repress excretion. $^{14}CO_2$ in expired air from rats dosed with 1 mg/kg bw fenazaquin was nondetectable or below the limit of quantification (approximately 0.02–0.04% of the dose) in almost all collected samples at the various time points.

Radioactivity was readily excreted in urine and faeces by both sexes in all dose groups (Table 3). More than 75% of the mean recovered radioactivity for each dose group was accounted for in excreta within the first 48 hours after dosing and more than 84% within 72 hours after dosing.

Table 3. Excretion in urine and faeces after single- or repeated-dose administration of fenazaquin in male and female rats

Dose	Excreta	Sex	% of total administered radioactivity per days after dosing							Total administered radioactivity	
			1 d	2 d	3 d	4 d	5 d	6 d	7 d	Mean [a]	SEM
Single dose											
1 mg/kg bw	Urine	M	15.87	3.76	0.80	0.25	0.10	0.05	0.05	20.86	2.63
		F	16.75	1.59	0.44	0.20	0.18	0.11	0.07	19.35	1.55
	Faeces	M	48.95	23.17	9.34	2.78	1.03	0.36	0.17	85.79	3.33
		F	67.54	9.70	2.74	0.84	0.23	0.11	0.06	81.23	2.46
30 mg/kg bw	Urine	M	10.04	7.90	0.88	0.28	0.18	0.13	0.13	19.55	3.08
		F	9.20	5.32	1.16	0.37	0.17	0.11	0.12	16.44	2.22
	Faeces	M	18.56	39.43	9.75	2.44	0.99	0.50	0.28	71.93	3.05
		F	29.46	32.52	7.22	2.50	0.86	0.29	0.15	73.01	1.55
Repeated dose											
1 mg/kg bw	Urine	M	16.36	1.57	0.44	0.17	0.12	0.08	0.06	18.80	0.79
		F	15.22	1.74	0.77	0.24	0.13	0.10	0.05	18.25	1.20
	Faeces	M	63.47	17.12	5.96	1.31	0.58	0.26	0.17	88.85	1.39
		F	63.86	12.80	4.20	1.31	0.33	0.16	0.09	82.74	1.90

bw: body weight; F: female; M: male; SEM: standard error of the mean

[a] Mean of 3/sex. *Source*: Althaus & Magnussen (1992); Billington (1997)

The major route of excretion was via faeces, accounting for 71.9–88.9% (range for both sexes of all three dose groups after 7 days) of the administered doses while 16.4–20.9% was recovered in urine (Althaus & Magnussen, 1992).

In a study conducted in accordance with GLP and USEPA test guidelines, the absorption and excretion of [phenyl-U-^{14}C]fenazaquin (radiochemical purity >98%; lot no. EPPS-05-067-86-21) was assessed in bile duct-cannulated male F344.IcoCrj rats (n = 6) after oral administration at 1 mg/kg bw. The bile ducts of the animals were cannulated to continuously collect bile. A second cannula was inserted into the duodenum for infusion of artificial bile. Both cannulas were connected by a loop in order to support bile infusion during the animals' recovery, transportation and acclimatization. After a postsurgery recovery period of 11 or more days, the animals were shipped to the test facility.

Absorption was rapid and high, representing approximately 65.1% of the administered dose (Table 4**)**. Bile secretion represented 60.5% of the administered dose. Radiolabel excreted into the urine and faeces within 48 hours after administration comprised 3.8% and 32.6% of the administered dose, respectively. Low amounts of radioactivity (≤0.7% of the administered dose) were recovered from the gastrointestinal tract and contents and the remaining carcass 48 hours after dosing. Blood and plasma concentrations of radioactivity were also low 48 hours after dosing, representing 0.001 and 0.002 µg equivalents/g in blood and plasma, respectively (Bernard, 2012).

Table 4. Summary of absorption and excretion of radiolabelled fenazaquin in bile, urine and faeces in bile duct–cannulated rats

Tissue or excreta / time	% administered dose							
	Rat no. 1	**Rat no. 2**	**Rat no. 3**	**Rat no. 4**	**Rat no. 5**	**Rat no. 6**	**Mean [a]**	**SD**
Bile								
0–1 h	0.43	0.31	0.05	1.60	0.25	0.14	0.46	0.58
1–2 h	5.71	5.68	5.72	6.97	6.01	5.06	5.86	0.63
2–4 h	16.38	15.96	23.55	15.25	19.18	17.58	17.98	3.06
4–8 h	23.18	22.62	21.06	17.67	25.02	19.67	21.54	2.63
8–12 h	7.11	7.77	9.24	10.18	7.36	8.80	8.41	1.20
12–24 h	5.22	5.09	4.58	5.41	5.52	4.88	5.11	0.35
24–32 h	0.63	0.71	0.55	0.51	0.57	0.53	0.58	0.08
32–48 h	0.66	0.73	0.66	0.19	0.60	0.52	0.56	0.19
Subtotal	59.32	58.87	65.40	57.79	64.51	57.18	60.51	3.54
Urine								
0–24 h	3.43	3.44	2.91	3.45	3.43	3.49	3.36	0.22
24–48 h	0.40	0.47	0.37	0.44	0.31	0.33	0.39	0.06
Subtotal	3.84	3.91	3.28	3.89	3.74	3.83	3.75	0.24
Faeces								
0–24 h	28.89	27.45	28.73	32.94	31.27	29.92	29.87	1.97
24–48 h	3.01	3.82	1.57	3.32	1.89	3.03	2.77	0.87
Subtotal	31.89	31.27	30.29	36.26	33.16	32.94	32.64	2.07
Cage wash	0.05	0.11	0.23	0.07	0.10	0.10	0.11	0.06
Total excretion	95.10	94.17	99.21	98.01	101.51	94.05	97.01	3.05
Residues								
Whole blood	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Plasma	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Gastrointestinal tract	0.14	0.17	0.13	0.21	0.14	0.10	0.15	0.04

(*continued on next page*)

Tissue or excreta / time	% administered dose							
	Rat no. 1	Rat no. 2	Rat no. 3	Rat no. 4	Rat no. 5	Rat no. 6	Mean [a]	SD
Carcass	0.60	0.61	0.69	0.44	0.57	0.50	0.57	0.09
Subtotal	0.74	0.78	0.82	0.66	0.71	0.61	0.72	0.08
Systemic absorption [b]	63.95	63.68	69.74	62.41	69.06	61.71	65.09	3.44
Total recovery	95.85	94.95	100.03	98.67	102.22	94.65	97.73	3.07

no.: number; SD: standard deviation *Source*: Bernard (2012)

[a] Mean of six rats.

[b] Based on radioactivity recovered in urine, bile, cage wash, whole blood, gastrointestinal tract (including contents) and carcass.

(b) Dermal route

In vivo dermal penetration studies of fenazaquin were not conducted.

An in vitro guideline-compliant rat and human dermal penetration study was conducted using radiolabelled fenazaquin (radiochemical purity >97%; batch no. EPPS-06-120-72-27) and non-radiolabelled fenazaquin (purity 99%, batch no. 371-66A) at two doses. The high dose level (200 g equiv./L) was equivalent to the commercially supplied concentrate, and the low dose level (0.05 g equiv./L) represents a typical in-use concentration of the product. Total absorption through human skin samples corresponded to 0.02% and 0.48% of the applied high and low dose, respectively. In the excised rat skin, the total absorption was 0.54% and 7.33% of the high and low dose level, respectively (Shaw, 2007).

1.2 Biotransformation

Althaus & Magnussen (1992) analysed fenazaquin metabolism using samples collected as previously described (section 1.1a). The metabolites were identified by comparing the *R*f values of the radioactive zones with the appropriate reference compounds. The metabolites present in sufficient quantity were additionally purified and identified by high-performance liquid chromatography (HPLC). Metabolites isolated by thin-layer chromatography (TLC) and/or HPLC in sufficient quantities underwent structural determination by nuclear magnetic resonance (NMR) and/or mass spectrometry.

Metabolites in urine and faeces are summarized in Table 5.

Urinary excretion accounted for 16.4–20.9% of total radioactivity by seven days after dosing. No great differences in radioactivity among the designated extraction fractions were observed between male and female rats within the same dose groups or between different dose groups. Fenazaquin was not a significant component in urine (<0.5% of urinary radioactivity). In all cases, 59.4–66.3% of the urinary radioactivity was extractable as nonconjugates, 23.3–29.0% as conjugates (aglycones following enzymatic hydrolysis) and 8.4–11.9% of the remainder was in the spent urine. There were two major metabolites: an acidic nonconjugate (AN)-1 and a neutral aglycone (NA)-1 (24.1–29.0% and 6.3–17.1% of urinary radioactivity, respectively). The percentage of AN-1 and NA-1 was comparable in all dose groups. No differences were observed between sexes. One minor metabolite, neutral nonconjugate (NN)-1, accounted for 2–3% in females in all dose groups and less than 1% in males. Several metabolites that could not be separated but were grouped as NN-2 and NN-3 complexes, accounted for 7.3–13.9% and 7.3–11.4% in all dose groups of both sexes, respectively. Each complex consisting of three to five compounds, indicating that there were 10 or more metabolites in total, each accounted for less than 5% of urinary radioactivity.

After seven days, 71.9–88.9% of total radioactivity was excreted via faeces by both sexes in all dose groups. There were no differences in the distribution of radioactivity in urine between male and female rats within the same dose groups or different dose groups. For all samples, 70.6–79.9% of faecal radioactivity was extracted by the subsequent methanol and acetonitrile/water procedure. Only 8.3–10.4% of the faecal radioactivity was not extracted with this procedure. Fenazaquin represented 1.2–4.2% of the faecal radioactivity in the single and repeated low doses and 11.5–20.6% of the single high dose. The most abundant metabolite in faeces was faecal (F)-2, representing 16.3–22.8% of faecal radioactivity, followed by F-3 at 6.5–12.6% and F-1 at 4.6–9.4%. Metabolite F-1A represented less than 3% of the radioactivity and 4-hydroxyquinazoline (4-OH) was minor (<1.0% faecal radioactivity). There were no major differences in the faecal metabolite pattern between sexes and dose groups.

Table 5. Metabolites in rat urine and faeces in a toxicokinetic study of orally administered radiolabelled fenazaquin

Excreta / Compound	% radioactivity administered per dose level of fenazaquin					
	Single dose				Repeated dose	
	1 mg/kg bw		30 mg/kg bw		1 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Urine						
Fenazaquin	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Nonconjugates	66.3	65.7	61.1	59.4	62.1	66.3
AN-1	27.9	24.1	29.0	25.3	25.6	26.9
NN-1	0.5	2.3	0.7	2.0	0.4	2.9
NN-2 complex [a]	13.2	13.9	7.3	7.3	10.4	12.1
NN-3 complex [a]	8.1	11.4	7.3	8.2	8.1	7.3
Aglycones	25.3	23.6	29.0	28.7	28.8	23.3
NA-1	15.1	13.7	17.1	13.1	10.0	6.3
Total	91.6	89.3	90.1	88.1	90.9	89.6
Faeces						
Fenazaquin	1.2	2.2	11.5	20.6	2.1	4.2
F-1	8.0	6.5	6.0	4.8	9.4	4.6
F-1A	2.6	0.8	1.9	0.6	1.7	0.6
F-2	20.2	16.9	22.8	16.3	22.4	17.2
F-3	12.2	11.4	8.5	6.5	11.0	12.6
4-OH	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

AN: acidic nonconjugate; bw: body weight; F: faecal metabolite;

F-2: (2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)ethyl) phenyl)acetic acid), or fenazaquin acid;

F-3: 2,2-dimethyl-2-(4-(2-((6-hydroxy-4-quinazolinyl)oxy)ethyl)phenyl)acetic acid, or 2-hydroxy-fenazaquin acid;

NN: neutral nonconjugate; **NA**: neutral aglycone; **NN-2** complex: neutral nonconjugate-2 complex;

NN-3 complex: neutral nonconjugate 3 complex; **4-OH**: 4-hydroxyquinazoline

[a] At least 3–5 components that could not be separated; it was estimated that each component accounted for less than 5% of urinary radioactivity).

Source: Althaus & Magnussen (1992)

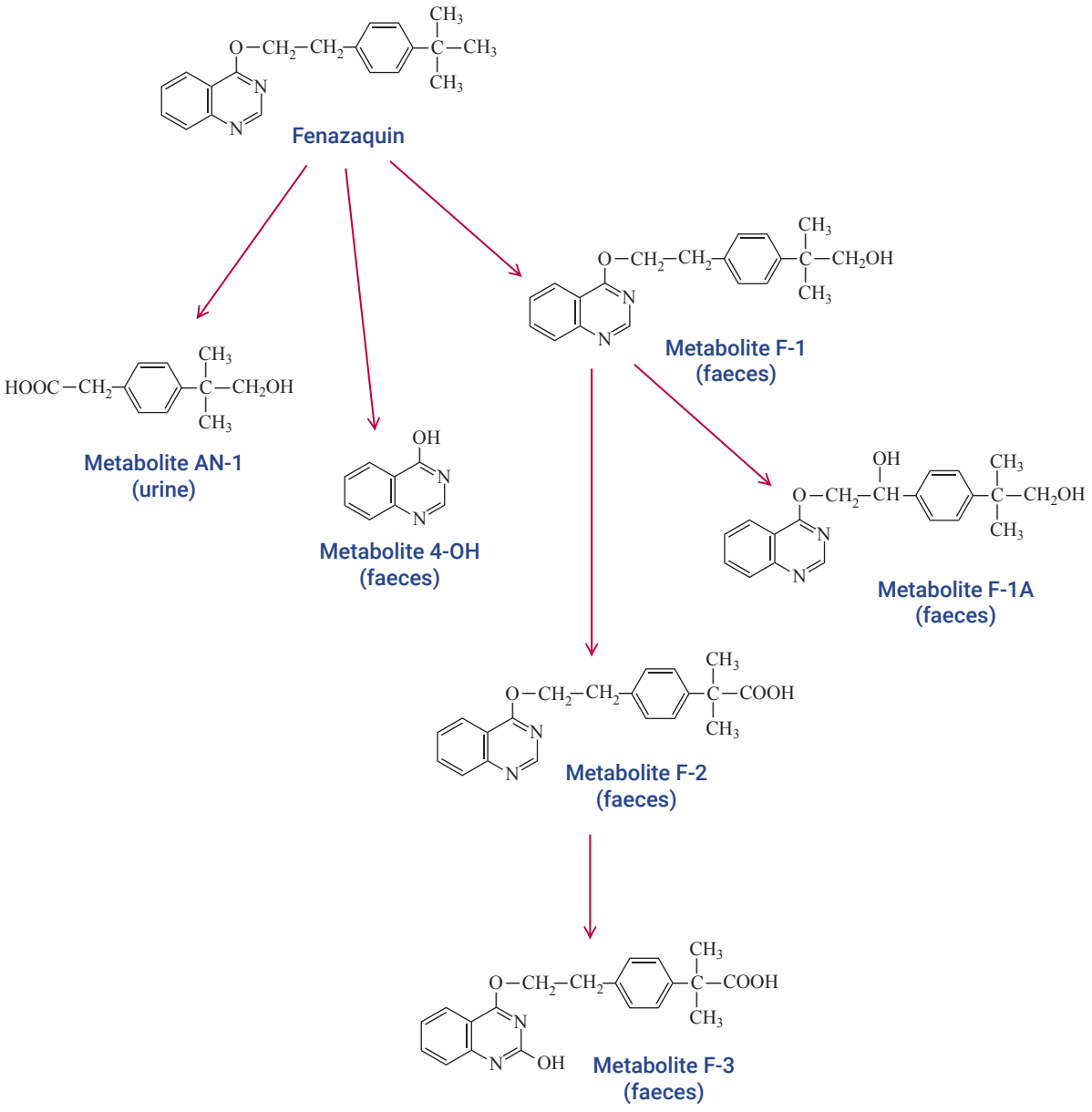
Faecal and urine metabolites were analysed using TLC and/or HPLC. Metabolite F-1 was identified as 2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)ethyl)phenyl)ethanol, the oxidized form of fenazaquin at the alkyl side chain. Another primary faecal metabolite, F-2 was a further oxidized form of metabolite F-1 with completely oxidized side chain, that is, 2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)ethyl) phenyl)acetic acid (or fenazaquin acid). Metabolite F-3, a livestock metabolite, was proposed to be metabolite F-2 with an oxidized quinazoline ring in position 2, that is, 2,2-dimethyl-2-(4-(2-((6-hydroxy-4-quinazolinyl)oxy) ethyl)phenyl)acetic acid (or 2-hydroxy-fenazaquin acid). Metabolite F-1A was identified as 2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)-1-hydroxy-ethyl)phenyl)ethanol. The minor faecal metabolite 4-OH resulted from hydrolysis of the ether bridge of fenazaquin. The urinary metabolite AN-1 was identified as 4-(2-hydroxy-1,1-dimethylethyl)phenylacetic acid. Metabolite NA-1 could not be identified due to poor recoveries during the TLC isolation steps. Minor metabolites could also not be identified.

To summarize, following oral administration in rats, fenazaquin was rapidly absorbed. The bile duct–cannulated rat studies showed that oral absorption of fenazaquin was high, totalling about 60% of the administered dose. Fenazaquin and/or metabolites did not accumulate in tissues. The major route of excretion was via the faeces (71.9–88.9% after 7 days). Excretion in the urine ranged from 16.4% to 20.9%. Most of the fenazaquin dose was readily excreted in rats, with more than 75% of the administered radiocarbon recovered in excreta within 48 hours after dosing and more than 84% within 72 hours.

There was no radiolabel in the expired air. Major differences between sexes or dose levels in the rate and route of excretion of radioactivity were not observed. Metabolic pathways of fenazaquin involved cleavage of the ether bond, resulting in the formation of the respective alcohol (4-OH quinazoline metabolite) and carboxyl acid (AN-1) derivatives. Other biotransformation reactions included oxidation of one of the methyl groups on the alkyl side chain to produce either alcohol (F-1) or carboxylic acid (F-2) metabolites. Finally, hydroxylation at the *O*-ether alkyl moiety of F-1 or F-2 resulted in F-1A and F-3 metabolites, respectively.

The proposed metabolic pathway and structures of metabolites in rats are shown in Fig. 3.

Figure 3. Proposed metabolic pathway and structures of metabolites in rats



AN-1: 4-(2-hydroxy-1,1-dimethylethyl)phenylacetic acid;

F-1: 2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)ethyl)phenyl)ethanol;

F-1A: 2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)-1-hydroxy-ethyl)phenyl)ethanol;

F-2: (2,2-dimethyl-2-(4-(2-((4-quinazolinyl)oxy)ethyl) phenyl)acetic acid), or fenazaquin acid;

F-3: 2,2-dimethyl-2-(4-(2-((6-hydroxy-4-quinazolinyl)oxy)ethyl)phenyl)acetic acid, or 2-hydroxy-fenazaquin acid;
4-OH: 4-hydroxyquinazoline

2. Toxicological studies

2.1 Acute toxicity

Studies on the acute toxicity, skin or eye irritation and the skin sensitization potential of fenazaquin are summarized in Table 6.

Table 6. Summary of the acute toxicity, skin and eye irritation and skin sensitization potential of fenazaquin

Species	Strain	Route (method)	Purity / batch no.	Result	Reference
Rat	Fischer 344	Oral (gavage)	98% ACD13041	LD_{50}: > 50 mg/kg but < 500 mg/kg in both sexes	Francis et al. (1989)
Rat	Fischer 344	Oral (gavage)	97.28% ACD13041	LD_{50}: 134 mg/kg bw for males, 138 mg/kg bw for females	Wright, Davis & Francis (1992a)
Mouse	CD-1	Oral (gavage)	97.9% ACD13041	LD_{50}: 2449 mg/kg bw for males, 1480 mg/kg bw for females	Wright, Davis & Francis (1992b)
Hamster	Syrian golden hamster	Oral (gavage)	97.28% ACD13041	LD_{50}: 812 mg/kg bw for males, 933 mg/kg bw for females	Wright, Davis & Francis (1992c)
Rabbit	New Zealand White	Dermal	98% ACD13041	LD_{50}: > 5000 mg/kg bw in both sexes	Francis, Rock & Clair (1989a)
Rat	Fischer 344	Inhalation (nose only)	98% ACD13041	LC_{50}: > 1.9 mg/L in both sexes	Francis, Herman & Wolff (1990)
Rabbit	New Zealand White	Skin irritation	98% ACD13041	Not irritant	Francis, Rock & Clair (1989a)
Rabbit	New Zealand White	Eye irritation	98% ACD13041	Not irritant	Francis, Rock & Clair (1989b)
Guinea-pig	Hartley Albino	Dermal sensitization (modified Buehler method)	98% ACD13041	Not sensitizing	Francis, Rock & Clair (1989c)
Guinea-pig	Dunkin Hartley	Dermal sensitization (Magnusson–Kligman maximization test)	99.2% RMM 1836	Not sensitizing	Jones (1994)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NZW: New Zealand White

(a) Lethal doses

In an acute oral toxicity study in rats, fenazaquin (purity 98%; batch no. ACD13041) was administered by gavage to young adult Fischer 344 rats (n = 5/sex per group) at 0, 50, 500 or 2000 mg/kg bw. The test material was suspended in 10% (w/v) aqueous acacia solution (10 mL/kg, maximum of test suspension administered). All survivors were killed on day 15 after treatment.

All males and females at 500 and 2000 mg/kg bw died. The majority of deaths occurred within five hours after dosing and all deaths occurred by six days after dosing. Clinical signs of toxicity observed in treated animals included hypoactivity, lethargy, ataxia, diarrhoea, gasping, posterior soiling and hunched posture. Males also exhibited coma, poor grooming, chromorhinorrhoea, clear ocular discharge, piloerection and an absence of faeces and urine in the cage tray. None of the rats at 50 mg/kg bw showed signs of toxicity by day 5, and their body weight gains were normal compared to controls at days 8 and 15. At necropsy, no abnormalities were observed in the animals that died or that survived to scheduled kill.

The acute oral toxicity of fenazaquin was greater than 50 mg/kg bw but less than 500 mg/kg bw for both sexes in Fischer 344 rats (Francis et al., 1989).

To determine the median lethal dose (LD_{50}) in rats, fenazaquin (purity 97.28%; batch no. ACD13041) was administered by gavage to young adult Fischer 344 rats (n = 5/sex per group) at 0, 100, 180 or 300 mg/kg bw in one experiment and at 0, 50, 100 or 250 mg/kg bw in another. The test material was suspended in 10% (w/v) aqueous acacia solution (10 mL/kg, maximum of test suspension administered). All survivors were killed on day 15 after treatment.

All the males at 180 and 300 mg/kg bw and 4/10, 3/5, 3/5 and 5/5 females at 100, 180, 250 and 300 mg/kg bw, respectively, died by day 2, with the majority dying within five hours following treatment. Treatment-related clinical signs included hypoactivity, hunched posture, straub tail, low carriage, soft stool, diarrhoea, perineal/posterior soiling, piloerection, clear ocular discharge, generalized leg weakness, ataxia, immobilization and coma. On the first day, hypoactivity and hunched posture were prevalent in all survivors. Male or female survivors at 100 mg/kg bw showed no signs of toxicity by days 4 or 6, respectively. At necropsy, fluid and materials were found in stomach or caecum but no other specific treatment-related findings were observed.

The oral LD_{50} in rats was calculated using the trimmed Spearman-Karber method to be 134 mg/kg bw (no confidence limits available) for males and 138 mg/kg bw (95% confidence interval [CI]: 102–187) for females (Wright, Davis & Francis, 1992a).

In an acute oral toxicity study in mice, fenazaquin (purity 97.9%; batch no. ACD13041) was administered by gavage to young adult CD-1 mice (n = 5/sex per group) at 0, 1500, 2000 or 3000 mg/kg bw in one experiment and 0, 1000, 1600 or 2500 mg/kg bw in another. The test material was suspended in 10% (w/v) aqueous acacia solution (25 mL/kg). All survivors were killed on day 15 after treatment.

Deaths occurred in 1/5, 2/5 and 3/5 males at 1500, 2000 and 3000 mg/kg bw and in 2/5, 3/5, 2/5, 3/5, 4/5 and 5/5 females at 1000, 1500, 1600, 2000, 2500 and 3000 mg/kg bw, respectively. Most of the deaths occurred by day 2 and all deaths had occurred by dayv5. Mean body weight gains were depressed in females at 1000, 1500 and 1600 mg/kg bw and slightly depressed in males at 2000 mg/kg bw by days 8 and 15. Common clinical signs were hypoactivity, hunched posture, low carriage, ataxia, generalized leg weakness, ptosis, piloerection, tremors and coma. None of the survivors showed signs of toxicity by day 7 after piloerection and hunched posture abated. At necropsy, the animals that died were observed to have abundant fluid in the stomach and/or jejunum.

The oral LD_{50} in mice was calculated using the trimmed Spearman-Karber method to be 2449 mg/kg bw (95% CI: 1307–4591) in males and 1480 mg/kg bw (95% CI: 666–3287) in females (Wright, Davis & Francis, 1992b).

In an acute oral toxicity study in hamsters, fenazaquin (purity 97.28%; batch no. ACD13041) was administered by gavage to young adult Syrian golden hamsters (n = 5/sex per group) at doses of 0, 500, 1000 or 2000 mg/kg bw. The test material was suspended in 10% (w/v) aqueous acacia solution (20 mg/kg bw mL/kg). All survivors were killed on day 15 after treatment.

Four males and four females at 1000 mg/kg bw and five males and five females at 2000 mg/kg died by day 3. Hypoactivity was observed in all treated animals at various times on the first day. Most animals at 1000 or 2000 mg/kg bw exhibited one or more of the following treatment-related signs of toxicity: ataxia, lethargy, clear ocular discharge, ptosis, hyperaemia and coma. By day 2, none of the surviving animals showed signs of toxicity except one female at 1000 mg/kg bw, which had recovered by day 3. By day 8, mean body weights were slightly depressed in animals at 500 mg/kg bw.

The oral LD_{50} in hamsters was calculated using the trimmed Spearman-Karber method to be 812 mg/kg bw (95% CI: 634–1041) in males and 933 mg/kg bw (95% CI: 689–1264) in females (Wright, Davis & Francis, 1992c).

In an acute dermal toxicity and dermal irritation study, fenazaquin (purity 98%; batch no. ACD13041) was applied directly to a clipped area (approximately 10% of the body surface) on the back

of New Zealand White rabbits (n = 5/sex per group) at 5000 mg/kg bw for 24 hours as a limit test. The animals were observed for 14 days. There were no unscheduled deaths during the course of the study. No treatment-related behavioural changes and no clinical signs were noted. No abnormalities were noted at necropsy. Dermal irritation was not observed.

The dermal LD_{50} in rabbits was greater than 5000 mg/kg bw (Francis, Rock & Clair, 1989a).

In an acute inhalation toxicity study, Fischer 344 rats (n = 10/sex per group) were exposed to fenazaquin (purity 98%; batch no. ACD13041) via a nose cone at 0.06, 0.8 or 4.6 mg/L for four hours. The median equivalent aerodynamic diameters were 9.23, 3.74 and 6.67 µm on a mass basis and 2.99, 3.79 and 6.45 µm on an activity basis in the 0.06, 0.8 and 4.6 mg/L groups, respectively.

One female at 0.8 mg/L and 9/10 males and 10/10 females at 4.6 mg/L died. Signs of toxicity at 0.8 mg/L were hypoactivity, dyspnoea, ataxia, poor grooming and nasal discharge and at 4.6 mg/L were hypoactivity, moribundity, dyspnoea, lethargy, poor grooming and rales. No signs of toxicity were observed in surviving animals on day 4. At necropsy, the lungs of animals were red and wet.

Under the test conditions, the four-hour median lethal concentration (LC_{50}) of fenazaquin was 1.9 (95% CI: 1.7–2.2) mg/L of air (Hamilton, Russo & Thurston, 1977, 1978; Francis, Herman & Wolff, 1990).

(b) Dermal irritation

In an acute dermal toxicity and dermal irritation study (see section 2.1a) by Francis, Rock & Clairv(1989a), no dermal irritation was observed following treatment.

(c) Ocular irritation

In an eye irritation study, fenazaquin (purity 98%; batch no. ACD13041) was instilled into the conjunctival sac of one eye of each New Zealand White rabbit (n = 3/sex) at 35 mg (equal to a volume of 0.1161 mL). The eyes were examined and ocular reactions were graded using the Draize method. Treated eyes were evaluated at 1, 24, 48, 72 hours and seven days after dosing. The eyes were not washed.

Slight corneal dullness, slight iritis and slight conjunctivitis developed within one hour of treatment. All treated eyes were normal within two days of treatment. Treated eyes in all animals gave a negative response to sodium fluorescein dye instilled 24 hours after treatment. Based on reactions that resolved 24–72 hours after the instillation of the test article, fenazaquin was considered to not be an eye irritant (Francis, Rock & Clair, 1989b).

(d) Dermal sensitization

A dermal sensitization study of fenazaquin (purity 98%; batch no. ACD13041) was evaluated using the modified Buehler method in albino Hartley guinea-pigs (n = 12) For each induction treatment, the test article was applied to a 1.5 cm^2 patch that was attached to the nuchal area of each animal and held in place under occlusion for six hours. On day 22, 10 days after the last induction application, 50 mg of the neat test substance was applied to the shaved and previously untreated area in the centre of the back of each animal. Control animals (n = 6) remained untreated during the induction and were treated only at the application stage. Dinitrochlorobenzene was used as a positive control sensitizer (n = 6 animals). The skin reactions were evaluated for erythema and oedema using an eight-point scoring system 24 hours after each induction exposure as well as 24, 48 and 72 hours after application, respectively.

No deaths occurred during the study. Treatment sites of all guinea pigs treated with fenazaquin were normal throughout the induction phase of the study. Twenty-four hours after challenge, there was no dermal irritation in any of the animals challenged with fenazaquin. Fenazaquin was not sensitizing in guinea pigs (Francis, Rock & Clair, 1989c).

Another sensitizing study of fenazaquin (purity 98%; batch no. ACD13041) was conducted in female albino Dunkin Hartley guinea-pigs (n = 10/test group; n = 5/control group) using the Magnusson–Kligman maximization test. Concentration levels of fenazaquin were 25% and 50%. No skin reactions were noted at the test material or vehicle challenge sites or in control group animals 24 and 48 hours after treatment.

Fenazaquin was not sensitizing in guinea pigs (Jones, 1994).

2.2 Short-term studies of toxicity

(a) Oral administration

In the Billington (1997) pharmacokinetic study in hamsters, mice and rats, T_{max} values were lower in hamsters and mice than in rats (Table 1). Acute toxicity tests determined the LD_{50} values to be 134 and 138 mg/kg bw for male and female rats, 2449 and 1480 mg/kg bw for male and female mice and 812 and 933 mg/kg bw for male and female hamsters, respectively (see Table 6). All three species also had decreased body weights. The effects were most severe in rats, in which body weight gains were reduced in a dose-related manner in both sexes. There was a small but significant decrease in body weight in male mice at the highest dose and a nonsignificant decrease in body weight in female hamsters but not in male hamsters (see Table 7).

Table 7. Body weight changes in mice, rats and hamsters treated with fenazaquin for two weeks

Estimated daily dose (mg/kg bw)	Dietary concentration (%)	Body weight change (g)	
		Males	Females
Mouse study (Study M21989)			
0	0	3.2	0.9
225	0.15	4.0	2.4
450	0.3	4.2	2.5
900	0.6	0.5*	−0.8
Rat study (Study R04790)			
0	0	65.0	32.0
47	0.05	42.4*	19.0*
85	0.10	21.0*	11.0*
175	0.25	−14.4*	−12.6*
Hamster study (Study H00489)			
0	–	9.0	17.6
5	–	11.0	19.6
25	–	13.0	20.0
75	–	11.4	12.5
150	–	9.2	5.6

bw: body weight; *: $P < 0.05$ *Source:* Francis (1990)

Hamsters

Based on results from pilot studies and a comparative toxicokinetics study, plasma levels in mice dropped very rapidly while plasma elimination in hamsters and rats was slower, indicating that systemic exposure to higher levels of fenazaquin in hamsters and rats was longer than in mice. This difference in kinetics correlated well with the toxicity profiles in these three species. In two-week pilot studies in CD-1 mice, Syrian golden hamsters and Fischer 344 rats, the hamsters and rats were substantially more sensitive than mice to decrease in body weight gain, the major manifestation of fenazaquin toxicity. In these studies, mice showed no toxicity at 450 mg/kg bw, but hamsters and rats significantly decreased their body weight gain at 25 and 47 mg/kg bw per day, respectively. As a result, the hamster was selected as the appropriate species for short- and long-term studies of fenazaquin.

In a 90-day gavage study, fenazaquin (purity 97.62%; batch no. ACD 13041) suspended in 10% (w/v) aqueous acacia solution was administered by gavage to Syrian golden hamsters (Lak:LVG(SYR); n = 15/sex per group) at the following doses: 0, 5, 25, 50 (females only), 75 (males only), 100 (females only) or 150 (males only) mg/kg bw per day. Observations were made daily for clinical signs. Body weight and feed consumption were measured weekly. At study end, the animals were killed and examined for macroscopic and microscopic pathology. Haematology, blood biochemistry, urine analysis and microsomal enzyme activity in the liver were measured at terminal kill. Due to excessive spillage of feed by animals during the study, feed consumption was not measured after 11 weeks.

All animals survived to scheduled kill except for three males that died as a result of an accident during gavage administration (one at 5 mg/kg bw per day and two at 150 mg/kg bw per day); one female at 100 mg/kg bw per day that died from acute haemorrhagic typhlitis; and one male at 25 mg/kg bw per day whose cause of death in week 13 was not determined. Mortality was not affected by treatment, and no treatment-related clinical signs were observed during the study.

Major treatment-related changes are summarized in Table 8. Statistically significant depressions of body weights started at weeks 3 or 4 at the two highest doses in both sexes, respectively, and were consistent during the study. Body weight gains at the two highest doses were 46% and 26% in males and 61% and 39% in females compared to control values, respectively. Red blood cell (RBC) counts and packed cell volume (PCV) were statistically and significantly decreased; however, given the small magnitude of their decreases (< 6%), these were not considered adverse. Other treatment-related changes in haematological or urine analysis parameters were not observed. In blood chemistry, serum cholesterol levels were slightly (about 10%) decreased at 75 mg/kg bw per day and above in males, and slightly decreased at 25 mg/kg bw per day and above in females, although the dose relationship was not clear in females; the slight decreases at the two highest doses in both sexes were considered treatment related. Lower serum cholesterol levels were also detected in other treated species that had reduced body weight and/or body weight gains. The treatment-related lower body weights might have been the reason for the decreases. In addition, the lower levels of serum triglyceride seen in females at 50 mg/kg bw per day and above and in total protein at the two highest doses in both sexes might also be a secondary effect resulting from lower body weights observed at those doses.

Table 8. Treatment-related observations in a 90-day oral toxicity study in hamsters

Parameters	**Measure per dose level of fenazaquin**				
	0 mg/kg bw per day	**5 mg/kg bw per day**	**25 mg/kg bw per day**	**M: 75 mg/kg bw per day / F: 50 mg/kg bw per day**	**M: 150 mg/kg bw per day / F: 100 mg/kg bw per day**
Males					
Body weight on day 88 (g)	137.4	147.1	127.7	114.8**	105.8**
Body weight gain on days 1–88 (% control)	100	121.7	84.0	45.8**	26.4**
Serum cholesterol (mg/dL)	117.4	112.6	107.5	99.6*	95.4*
Total protein (g/dL)	6.343	6.314	6.243	5.927*	5.946*
Hepatic PNA activity [a]	38.0	39.8	46.5	58.2*	64.6*
Liver					
Absolute weight (g)	4.29	4.57	4.20	4.50	4.55
Relative weight (g/100 g bw)	3.12	3.13	3.29	3.91*	4.30*
Testes					
Absolute weight (g)	3.68	3.57	3.29	2.15*	1.99*
Relative weight (g/100 g bw)	2.69	2.48	2.61	1.97	1.90
Prostate					
Absolute weight (g)	0.53	0.47	0.42	0.27*	0.27*
Relative weight (g/100 g bw)	0.38	0.32	0.33	0.24*	0.25*
Hypospermatogenesis					
Incidence [b]	0/15	1/15	2/15	5/15	8/15
Severity (slight/ moderate/severe)	–	(0/1/0)	(1/0/1)	(0/3/2)	(1/2/5)

Parameters	Measure per dose level of fenazaquin				
	0 mg/kg bw per day	5 mg/kg bw per day	25 mg/kg bw per day	M: 75 mg/kg bw per day / F: 50 mg/kg bw per day	M: 150 mg/kg bw per day / F: 100 mg/kg bw per day
Females					
Body weight on day 88 (g)	173.9	185.1	168.7	141.7**	125.1**
Body weight gain on days 1–88 (% control)	100	116.0	97.6	61.0**	39.0**
Serum cholesterol (mg/dL)	114.6	108.7	93.7*	90.5*	97.6*
Serum triglyceride (mg/dL)	180.8	212.7	162.7	138.9*	138.9*
Total protein (g/dL)	6.660	6.793	6.587	6.247*	6.029*
Hepatic PNA activity [a]	31.9	30.8	41.0*	46.6*	51.2*
Liver					
Absolute weight (g)	5.72	6.32	6.32	5.57	5.21
Relative weight (g/100 g bw)	3.27	3.41	3.57	3.92*	4.15*

bw: body weight; no.: number; PNA: p-nitroanisole-O-demethylase; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett t-test)

[a] Measured as amount of p-nitrophenol produced in nmol/mg protein per hour.

[b] Expressed as the no. of animals with the finding / no. of animals examined.

Source: Francis, Boss & Gries (1992a)

Statistically significant changes were observed in various organ weights at the two highest doses: increases in relative liver weights (about 20%) at the two highest doses in both sexes; relative kidney weights at the highest dose in males and the two highest doses in females; decreases in absolute and relative reproductive organ weights at the two highest dose in males. The increases in liver weight without supporting hepatotoxicity parameters were not considered adverse. The slight increase in *p*-nitroanisole-*O*-demethylase (PNA) activity may have been as a result of induction of the hepatic enzyme by fenazaquin. No corresponding renal toxicity was manifested in histopathology or clinical chemistry, indicating that the increases in kidney was manifested in histopathology or clinical chemistry, indicating that the increases in kidney weights were not adverse. The lower reproductive weights in testes and prostate reflecting hypospermatogenesis were considered treatment-related adverse effects. The lower weights in several organs, such as the female reproductive organs, heart or spleen, without corresponding histopathological findings were considered secondary effects of lower body weights in the two highest doses in both sexes.

The no-observed-adverse-effect level (NOAEL) was 25 mg/kg bw per day based on lower body weights and reduced body weight gains at 50 mg/kg bw per day (Francis, Boss & Gries, 1992a).

Rat

In a 90-day feeding study in accordance with OECD Test Guideline 408, fenazaquin (purity > 99%; batch no. N85-JX1–70) in a 10% (w/v) aqueous acacia solution vehicle was administered by gavage to Fischer 344 rats (n = 15/sex per group) at concentrations of 0, 1, 3, 10 or 30 mg/kg bw per day. Subgroups (n = 10/sex per group) at 0 and 30 mg/kg bw per day were withdrawn from treatment for a four-week recovery study after the 90-day treatment. Activity of PNA, a hepatic enzyme, was measured.

All male animals survived to study end; 3/25 females at 30 mg/kg bw per day died due to gavage accidents. There were no treatment-related clinical signs. The results are summarized in Table 9.

Statistically significant depressions in body weight and reduced body weight gain were found in both sexes at 30 mg/kg bw per day throughout the treatment period. The reduced body weight gain was about 18% in males and 14% in females, compared to their respective controls. The reduced gains

in both sexes largely recovered during the four-week recovery period, although statistically significant depressions remained. Feed consumption was slightly but consistently reduced in males (8%) and females (4%) at 30 mg/kg bw per day. No treatment-related changes were observed in haematology, urine analysis or ophthalmology examinations.

In blood biochemistry, a statistically significant decrease in serum cholesterol at 30 mg/kg bw per day was considered a secondary effect related to lower body weight. The slight decrease in cholesterol levels at 10 mg/kg bw per day was not considered toxicologically significant as body weights and other parameters indicating hepatotoxicity were absent (Table 9); levels returned to normal four weeks after treatment was concluded. Several blood biochemical parameters exhibited minor changes at higher doses; however, slight changes (within 10%) were not considered toxicologically significant. Slight but statistically significant increases in PNA activity in all male treated groups and in females at 30 mg/kg bw per day, indicated an induction of PNA activity. After the one-month recovery period, the PNA activity continued to be elevated in females but was comparable to the control value in males.

At study end, treatment-related changes in organ weights were found in the liver, kidneys and adrenals in males and females at 10 and 30 mg/kg bw per day (Table 9). Corresponding morphological changes were not found in these organs/tissues. Liver weights were increased at 30 mg/kg bw per day for males and at 10 mg/kg bw per day and above for females. The increases were within 20% of the control. Because no concomitant hepatotoxicity was observed, these weight increases were not considered to be adverse. Hepatic metabolic enzyme induction might be the reason for the observed increases.

Absolute and/or relative weights of adrenals were increased in both sexes at 30 mg/kg bw per day (20% higher than their control values) and at 10 mg/kg bw per day (10% higher than their control values). However. the increases were not concomitant with any parameters, including histopathological or blood biochemistry changes, indicative of adverse effects, and the adrenal weights recovered four weeks after treatment withdrawal. The increases at 30 mg/kg bw per day might be stress-related reactive changes due to consistently lower body weights at this dose. The slight and reversible increases (10% and lower) at 10 mg/kg bw per day were not considered to be adverse because there were no corresponding parameters indicating adverse effects. The slight increases in kidney weights (within 10%) at 10 mg/kg per day and above for males and 30 mg/kg bw per day for females were not considered to be adverse because of the absence of concomitant findings indicating renal toxicity. No treatment-related histopathological findings were found.

Table 9. Treatment-related observations in a 90-day feeding study in rats

Parameter	Measures per dose level of fenazaquin						
	Treatment period					Recovery	
	0 mg/kg bw per day	1 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	0 mg/kg bw per day	30 mg/kg bw per day
Males							
Body weight (g)	306.2	307.0	306.4	299.5	268.7**	335.4	308.5**
Body weight gain (g)	188.7	190.1	187.3	184.6	153.9**	27.3	40.7**
Feed consumption (g/rat)	16.0	15.9	16.0	15.8	14.8**	16.3	16.2
Serum cholesterol (mg/dL) [a]	45.80	45.67	39.67	33.60*	31.80*	46.10	42.60
PNA activity [b]	21.67	23.70*	23.65*	24.60*	26.57*	35.19	33.84
Liver							
Absolute weight (g)	7.22	7.51	7.39	7.34	7.37	7.95	7.36*
Relative weight (g/100g bw)	2.37	2.44	2.41	2.45	2.73*	2.37	2.39

Parameter	Measures per dose level of fenazaquin						
	Treatment period					Recovery	
	0 mg/kg bw per day	1 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	0 mg/kg bw per day	30 mg/kg bw per day
Kidneys							
Absolute weight (g)	1.76	1.84	1.83	1.80	1.71	1.91	1.80*
Relative weight (mg/100 g bw)	577.5	595.9	596.0	601.0*	634.9*	569.4	584.8
Adrenals							
Absolute weight (mg)	41.4	44.1	44.7	45.7*	51.7*	43.6	42.2
Relative weights (mg/100 g bw)	13.57	14.35	14.60	15.32*	19.23*	13.02	13.68
Females							
Body weight (g)	182.4	178.7	179.0	181.1	170.5**	193.1	184.7*
Body weight gain (g)	89.8	87.2	85.5	88.4	77.7**	9.0	12.9*
Feed consumption (g/rat)	12.1	11.8	11.8	12.0	11.6**	12.4	12.5
Serum cholesterol (mg/dL) [a]	84.86	81.27	79.93	78.65	75.33*	82.90	73.40*
PNA activity [b]	18.34	17.26	19.95	20.52	22.24*	23.87	26.64*
Liver							
Absolute weight (g)	4.22	4.19	4.20	4.48*	4.72*	4.52	4.27
Relative weight (g/100 g bw)	2.33	2.35	2.35	2.48*	2.79*	2.34	2.31
Kidneys							
Absolute weight (g)	1.12	1.10	1.12	1.15	1.12	1.17	1.11
Relative weight (mg/100 g bw)	617.1	617.1	628.4	636.3	664.2*	607.9	601.8
Adrenals							
Absolute weight (mg)	49.5	50.8	52.0	55.8*	59.1*	52.3	50.8
Relative weight (mg/100 g bw)	27.27	28.50	29.08	30.85*	34.91*	27.12	27.47

bw: body weight; PNA: p-nitroanisole-O-demethylase; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett t-test)

[a] Historical control data for Fischer 344 rat (> 13 weeks of age) range from 37 to 91 mg/dL for males (n = 208) and 62 to 121 mg/dL for females (n = 208).

[b] Measured as *p*-nitrophenol produced (in nmol/mg protein per hour).

Source: Francis, Ward & Gries (1992)

The NOAEL was 10 mg/kg bw per day based on lower body weights, decreased body weight gains and reduced feed consumption at 30 mg/kg bw per day (Francis, Ward & Gries, 1992).

In a 90-day OECD test guideline–compliant feeding study, fenazaquin (purity 98.4%; batch no. 271MH8) was administered at dietary concentrations of 0, 15, 45, 150 or 450 ppm (equal to 0, 1.0, 3.0, 9.6 and 28.7 mg/kg bw per day for males and 0, 1.2, 3.5, 11.5 and 33.0 mg/kg bw per day for females, respectively) to Fischer 344 rats (n = 10/sex per dose level). Activities of liver metabolism enzymes, that is, PNA, 7-ethoxyresorufin-*O*-deethylase (7-ER), benzphetamine-*N*-demethylase (BNZ), and microsomal cytochrome P450 content were measured in males and females.

Treatment-related changes are summarized in Table 10. All animals survived until scheduled kill. There were no treatment-related clinical signs. Mean body weights, mean body weight gains and feed consumption in males and females at 450 ppm were statistically significantly lower than the control values throughout the study. Females at 150 ppm showed a significant decrease in feed consumption during the first month of the study.

There were no treatment-related changes in haematological, urine analysis or ophthalmological parameters. In blood biochemistry, alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), blood urea nitrogen and creatinine were decreased in males or females at 450 ppm; however, the small decreases in these parameters were considered to not be toxicologically significant. A lower (22% less than control) serum cholesterol level in males at 450 ppm was considered to be secondary to the lower body weights. Other slight changes in several blood biochemical parameters in the 450 ppm group were also considered not toxicologically significant. PNA, 7-ER and BNZ activities were approximately 21–43% higher than control values at the 150 and 450 ppm treatments in both sexes. Hepatic microsomal cytochrome P450 monooxygenase content showed a slight and nonstatistically significant increase in males at 450 ppm.

An 11% increase in relative liver weights in males at 450 ppm, with no other changes in parameters, indicated treatment-related hepatotoxicity that was not particularly adverse. The increase in adrenal weight (approximately 20%) concomitant with histopathological findings in males at 450 ppm might be a secondary effect of continuous stress, such as lower body weight, at this dose. A slight increase in adrenal weight (< 10%) in males at 150 ppm was considered not toxicologically significant because the changes were minor and there were no corresponding findings for the increase. Changes in other organ weights without histopathological or blood biochemical abnormalities at 450 ppm were either considered not toxicologically significant or were secondary effects related to lower body weights at the high dose.

Table 10. Treatment-related observations in the second 90-day feeding study in rats

Parameters	Measure per concentration of fenazaquin				
	0 ppm	15 ppm	45 ppm	150 ppm	450 ppm
Males					
Body weight on day 83 (g)	309.3	295.1	296.9	298.5	265.4**
Body weight gain (% control)	100	95.2	94.5	94.8	79.5**
Feed consumption on days 0–90 (% control)	100	96.2	96.8	94.9	86.1**
Serum cholesterol (mg/dL)	41.80	45.40	39.90	34.70	32.80*
Serum total protein	7.420	7.370	7.250	7.170	6.940*
Liver					
PNA activity [a]	30.81	34.52	38.97*	39.01*	41.45*
7-ER activity [b]	2.89	2.67	2.74	3.06	3.82*
BNZ activity [c]	132.19	141.19	150.61	162.53*	161.75*
Relative weight (g/100 g bw)	2.381	2.365	2.379	2.399	2.636*
Adrenals – relative weight (mg/100 g bw)	13.97	14.12	14.06	15.01	16.77*
Kidneys – absolute weight (mg/100 g bw)	596.1	569.8	591.8	589.8	640.4*

Parameters	Measure per concentration of fenazaquin				
	0 ppm	15 ppm	45 ppm	150 ppm	450 ppm
Females					
Body weight on day 83 (g)	178.3	178.3	177.0	174.6	165.3**
Body weight gain (% control)	100	100.0	97.2	95.0	85.7**
Feed consumption on days 0–90 (% control)	100	100.0	99.1	96.5	88.7**
Serum cholesterol (mg/dL)	65.00	65.40	66.30	64.80	59.40
Serum total protein	6.960	7.020	7.150	6.970	6.450*
Liver					
PNA activity [a]	24.54	26.80	27.23	29.65*	33.40*
7-ER activity [b]	3.41	3.92	3.82	4.19*	4.89*
BNZ [c]	40.00	44.34	43.82	53.56*	72.09*
Absolute weight (g)	4.093	4.055	4.200	4.225	4.448*
Relative weight (g/100 g bw)	2.295	2.277	2.375	2.419*	2.690*
Kidney – relative weight (mg/100 g bw)	605.1	618.0	627.3	634.6*	666.7*

bw: body weight; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett t-test)

BNZ: benzphetamine-*N*-demethylase;

PNA: *p*-nitroanisole-*O*-demethylase;

7-ER: 7-ethoxyresorufin-*O*-deethylase;

[a] Activity measured as amount of *p*-nitrophenol produced (in nmol/mg protein per hour).

[b] Activity measured as amount of resorufin produced (in mg protein/hour).

[c] Activity measured as amount of formaldehyde (HCHO) produced (in mg protein/hour).

Source: Cocke, Francis & Gries (1992)

The NOAEL was 150 ppm (equal to 9.6 mg/kg bw per day) based on body weight depression, reduced body weight gain and reduced feed consumptions at 450 ppm (equal to 28.7 mg/kg bw per day) (Cocke, Francis & Gries, 1992).

Dog

In a 90-day GLP-compliant oral toxicity study, fenazaquin (purity 98.1%; batch no. 435MH8) was administered to beagle dogs (n = 4/sex per dose) in the diet at dose levels of 0, 1, 5 or 15 mg/kg bw per day for 13 weeks. The animals were examined for death and moribundity twice daily. Body weights and feed consumption were determined weekly. Ophthalmoscopy was performed before treatment initiation and scheduled kill. Haematology and blood biochemistry were analysed before treatment initiation and in weeks 6 and 13. Urine analysis was performed in week 13. At study end, all surviving animals were killed and macroscopically and microscopically examined. Data on feed consumption were not statistically analysed.

Major treatment-related changes are summarized in Table 11. There were no unscheduled deaths during the study. The only treatment-related clinical sign observed was the thinner appearance of both sexes at 15 mg/kg bw per day. During ophthalmoscopy, no consistent or significant findings were observed. For males and females at 15 mg/kg bw per day, body weights started to decrease in week 1 and continued to do so until study end, with body weights at about 90% that of concurrent controls. Feed consumption was lower in both sexes at 15 mg/kg bw per day (79% and 10% of control values for males and females from day 86 to 92, respectively). The decreases in body weights and feed consumption in both sexes at 15 mg/kg bw per day were considered to be treatment related. In blood biochemistry, slight decreases in serum cholesterol (within 20%) and increases in potassium (within 20%) were found in both sexes at 15 mg/kg bw per day at week 6 and 13. The reduced cholesterol levels were considered to be an effect secondary to the continuous depression in body weight at this dose.

Table 11. Treatment-related observations in a 90-day feeding study in dogs

Parameters	Measures per dose level of fenazaquin							
	Males				Females			
	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	15 mg/kg bw per day	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	15 mg/kg bw per day
Body weights on day 7 (kg)	9.4	9.3	9.3	9.3	8.0	8.3	8.4	8.2
Day 1	9.9	9.6	9.8	9.3	8.4	8.2	8.8	8.6
Day 8	9.8	9.8	9.9	9.3*	8.6	8.6	8.8	8.3*
Day 15	10.2	10.2	10.0	9.1*	8.6	8.7	9.0	8.1*
Day29	10.4	10.3	10.4	9.1*	9.1	9.0	9.3	8.4*
Day 57	10.9	11.0	10.5	9.7*	9.5	9.3	9.8	8.6*
Day 92	10.8	10.9	10.6	9.6*	10.0	9.6	10.2	9.0*
Feed consumption (g)								
Day −6 to 1[a]	418.5	352.4	397.5	425.7	343.0	363.5	336.6	364.0
Day 1 to 8	373.9	410.4	364.1	319.3	363.1	397.5	338.5	296.8
Day 8 to 16	418.1	383.3	377.0	276.3	352.1	388.2	353.7	273.6
Day 23 to 30	415.3	395.9	389.6	315.8	394.4	414.3	351.9	293.6
Day 51 to 58	341.9	342.6	279.4	328.3	389.0	386.9	334.7	283.1
Day 86 to 92	356.1	331.3	289.9	282.0	309.9	362.7	313.0	277.9
Serum cholesterol (mg/dL)								
Pretest 1	184	181	223	178	168	176	156	181
Pretest 2	194	200	225	188	168	186	162	197
Week 6	146	162	168	127*	168	162	139	139*
Week 13	154	171	170	140*	187	188	154	154*
Serum potassium (mmol/L)								
Pretest 1	4.7	4.7	5.1	4.8	5.1	4.8	4.8	4.8
Pretest 2	4.6	5.0	4.9	4.9	4.8	4.7	4.7	4.8
Week 6	4.5	4.6	4.7	4.8*	4.7	4.5	4.9	4.8
Week 13	4.5	4.6	4.7	4.9*	4.5	4.4	4.7	5.1*
Histopathology								
Decreased hepatocellular vacuolation	0/4	1/4	0/4	3/4	0/4	0/4	1/4	2/4

bw: body weight; *: $P \leq 0.05$ (three-tailed ANOVA with Bonferroni adjustment)

[a] Statistical analysis was not conducted.

Source: Cosse et al. (1992)

The increases in potassium in females at week 13 were considered to not be toxicologically significant as there were no changes in other electrolyte levels or in related findings. Microscopically decreased vacuolation of hepatocytes was noted in males and females at 15 mg/kg bw per day. This finding was related to body weight depression. Other treatment-related morphological changes were not found in pathology or urine analysis.

The NOAEL was 5 mg/kg bw per day based on decreases in body weight and feed consumption at 15 mg/kg bw (Cosse et al., 1992).

In a 12-month oral toxicity study in dogs, fenazaquin (purity 98.1%; batch no. 435MH8) was administered to beagle dogs (n = 4/sex per group) at dietary concentrations of 0, 1, 5 or 12 mg/kg bw per day. The highest dose level was reduced to 10 mg/kg bw per day on day 95 because of the poor palatability of the diet (87.2% of mean feed consumption from day 1 to 94 in males and 78.1% in females compared to controls). The time-weighted average dose consumed by this dose group was calculated to be 12 mg/kg bw per day. The test article was distributed homogeneously and was stable for the duration of use in diet. All animals were checked for clinical signs, body weights, feed consumption and deaths throughout the study. Ophthalmological examinations of all animals were performed prior to treatment initiation and one week prior to necropsy. Haematology and blood biochemistry were assessed before treatment initiation, after approximately 3 and 6 months, and in the week prior to necropsy. After six months of dosing and during the scheduled necropsy, urine was obtained for analysis. At scheduled kill, the animals were necropsied and organs were weighed. Organs and tissues were fixed for histopathological evaluation.

Treatment-related findings are summarized in Table 12. Male and female dogs at 12 mg/kg bw per day appeared thinner throughout the study, and body weights and body weight gains were statistically significantly decreased. In males, the mean body weight of the control group was higher than that in both the 1 and 5 mg/kg bw per day groups because the control group included one large dog; the weights and weight gains of the three remaining control male dogs were comparable to those at 1 and 5 mg/kg bw. As a result, treatment-related changes in body weights were observed at 12 mg/kg bw in males. In females, a statistically significant decrease in body weight was also observed at 5 mg/kg bw, with the decrease starting on day 176; however, the decrease was slight (within 10%). In females at 1 mg/kg bw, body weights were approximately 1 kg (10%) higher than the control value, differences that reflect normal variability. Therefore the slight decrease in body weight in females was not considered treatment related. The lower feed consumption at 12 mg/kg bw was attributed to the poor palatability of the feed. Ophthalmology, urine analysis and haematology found no treatment-related changes during the study.

Statistically significant findings in blood biochemistry were an increase in ALT in males at 12 mg/kg bw (200%, 147% and 115% of age-matched controls at 3, 6 and 12 months) and a decrease in cholesterol in males at 12 mg/kg bw (75%, 69% and 71% of age-matched controls at 3, 6 and 12 months). Since the ALT values were within the laboratory's historical control values (average = 25 mU/mL; range = 20–31 mU/mL; N = 28), the increases were considered to not be treatment related. The decreased cholesterol levels were considered to be an effect secondary to the decreased body weights at this dose. There were no treatment-related changes in organ weights or macroscopic and microscopic findings.

Table 12. Treatment-related observations in a 12-month feeding study in dogs

Parameter	Measure per dose level of fenazaquin							
	Males				Females			
	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	12 mg/kg bw per day	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	12 mg/kg bw per day
Body weight (kg)		*	*			*	*	
Day 92	12.0	11.6	11.4	10.6	9.7	10.0	9.3	8.6
Day 100	12.1	11.6	11.8	10.4	9.6	10.1	9.1	8.6
Day 106	12.1	11.6	11.8	10.7	9.6	10.2	9.4	8.8
Day 113	12.5	11.7	11.7	10.8	9.5	10.2	9.3	8.7
Day 120	12.1	11.7	11.6	10.7	9.5	10.2	9.3	8.4
Day 148	12.7	11.8	11.8	11.2	9.6	10.4	9.5	8.8
Day 176	12.7	11.7	12.0	11.3	9.9	10.5	9.6	9.0
Day 232	13.1	12.3	12.2	10.7	10.5	11.0	9.7	9.1
Day 288	13.5	12.7	12.8	11.3	10.8	11.7	10.1	9.1
Day 344	13.8	12.7	12.6	11.2	10.2	11.2 [b]	9.5	9.0
Day 365	13.7 / 12.2 [a]	12.5	12.4	11.0	10.0	10.9 [b]	9.0	8.5

Individual body weight (g) on day 365								
	18 209	14 054	13 102	9801	8787	11 486	10 240	8387
	11 226	9663	12 746	10 881	10 269	9273	8194	8300
	12 027	14 451	12 227	11 066	11 104	11 997	7524	9293
	13 215	11 696	11 342	12 287	9740	6927 [c]	9869	7855
Body weight gain								
Day 1–365 (g)	3175 / 1969 [d]	2276	2040	475	1717	2918	653	194
% of control	–	72/116 [d]	64/104 [d]	15/24 [d]	–	170	38	11
Mean feed consumption (% of control)								
Day 1–94	–	95.4	106.4	87.2	–	126.4	109.7	78.1
Day 94–365	–	91.6	97.8	100.8	–	112.7	100.7	97.2
Mean feed consumption (g/animal per day)								
Day 87–94	417.4	394.6	464.7	377.5	313.9	350.2	322.8	277.4
Day 360–366	425.5	399.5	422.0	493.5	325.0	210.4	281.3	331.1
Serum ALT (mU/mL)								
3 months	14	19	20	28	15	14	16	15
6 months	17	18	17	25	17	15	17	14
12 months	20	22	23	23	17	15	21	17
Serum cholesterol (mg/dL)								
3 months	157	135	197	118	188	197	158	153
6 months	189	164	211	131	186	196	187	171
12 months	167	154	184	118	186	219	151	159

ALT: alanine aminotransferase; bw: body weight; U: units; *: $P < 0.05$ (RM-ANOVA) *Source*: Cosse et al. (1993)

[a] Mean body weight excluding one male heavy dog.

[b] Mean value without the female with the fractured lower canine tooth (incidental accident).

[c] Lower as a result of dramatic weight loss associated with a fractured lower canine in one dog and its generally debilitated state.

[d] With/without one heavy control.

The NOAEL for one-year oral toxicity in dogs was 5 mg/kg bw per day based on a significant decrease in body weight gains and reduced feed consumption at 12 mg/kg bw per day (Cosse et al., 1993).

(b) Dermal application

A 21-day dermal toxicity study was conducted in New Zealand White rabbits (LSR:(NZW); $n = 5$/sex per group; the control and highest dose groups had an additional five animals/sex to assess recovery) administered fenazaquin at concentrations of 0, 100, 315 or 1000 mg/kg bw per day. The rabbits were killed on day 21; the additional animals were kept without treatment for 14 more days. The area of dermal exposure was approximately 10% of the total body surface area for six hours/day. The test substance was directly applied to the treatment area, which was then covered with cheese cloth dampened with 1 mL of tap water/kg, and wrapped with semi-occlusive elastic wrap and secured with porous tape. Control animals were treated similarly with tap water alone. The test article was vacuumed from the test site on the treated animals. The rabbits underwent detailed clinical observations including for signs of dermal irritation, and body weight and feed consumption measurements. Haematological and blood biochemistry analyses were performed prior to treatment, on treatment day 20 and 10 days after treatment end. Gross pathological and histopathological examinations were conducted after the animals were killed, and organs were weighed.

All animals survived to study end. One male at 100 mg/kg bw per day was euthanized for humane reasons on day 6 because of an accidental fracture of lumbar vertebrae. Most treated animals developed dry skin after 15 days of treatment; this persisted through to the conclusion of both the treatment and

recovery periods. Treatment-related clinical signs were not observed. Very slight to slight dermal irritation at the test site was observed in animals treated with fenazaquin at 100 mg/kg bw per day and above. At the end of the 14-day recovery period, dermal irritation was no longer observed in males, and there was a trend toward resolution of the irritation in females.

No treatment-related changes were observed in body weights, body weight gains, feed consumption, ophthalmology, haematology, blood biochemistry, pathology, organ weights or macroscopic and microscopic analyses.

The NOAEL for dermal toxicity in rabbits was 1000 mg/kg bw per day, the highest dose tested (Wright, Poulsen & Francis, 1992).

(c) Exposure by inhalation

No studies were available.

2.3 Long-term studies of toxicity and carcinogenicity

Hamster

Because the results of pharmacokinetic, acute toxicity and short-term toxicity studies showed that mice were less susceptible to the toxicity of fenazaquin than rats and hamsters (see Tables 1, 6 and 7), the hamster was the second species chosen to evaluate carcinogenicity.

In a carcinogenicity study, fenazaquin (purity 97.28–98.0%; batch no.vACD 13041) was administered by gavage to Syrian golden hamsters (Lak:LVG(SYR); $n = 80$/sex per group) at 2, 15, 30 (for males) or 35 mg/kg bw per day (for females) for 78 weeks. Due to the logistic challenges of conducting studies with large numbers of animals, this study was divided into two, with 50, 40, 40 and 40 animals at 0, low, mid and high doses in both part 1 and part 2. There were 100 animals/sex in the control group. Clinical signs, body weights and deaths were recorded for all animals. Blood was drawn ($n = 14$/sex per dose) at 12 months for haematological evaluation, and all surviving hamsters underwent haematology and blood biochemistry evaluation at scheduled kill. All the animals were necropsied, and organ weights were measured. Organs and tissues were fixed for histopathological evaluation.

During the study, an unacceptably high incidence of fatal enteritis caused by *Clostridium difficile* occurred in animals in all groups including the controls. The hamsters were treated with vancomycin hydrochloride (40 mg/kg bw on the first day and 20 mg/kg bw per day thereafter). Caecal tympanites, a side-effect of the vancomycin treatment, seriously affected 27 animals and resulted in 17 fatalities.

The appearance or behaviour of the hamsters was not influenced by fenazaquin treatment. Survival was not adversely influenced by treatment and was generally higher than 50% in all male treated groups. Higher mortalities in control and low-dose females were considered to be mainly due to a higher incidence of systemic amyloidosis compared to the other females (15 and 35 mg/kg bw per day). Significantly decreased mean body weights and body weight gain relative to controls were observed in mid- and high-dose males and females.

The following parameters were evaluated at 12 months and at necropsy: erythrocyte count, haemoglobin, PCV, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), erythrocyte morphology, total and differential leukocyte counts and thrombocyte count. Administration at 15 mg/kg bw per day and above was associated with minimally decreased thrombocyte counts and minimal alterations in leukocytic parameters in males and females. Statistically significant changes in clinical chemistry parameters at terminal kill included reduced activity of alkaline phosphatase (ALP), ALT and AST in mid- and high-dose males; increased total protein in mid-dose males; increased albumin in mid- and high-dose males and high-dose females; and reduced globulin in high-dose males and females. Consequently, albumin/globulin ratio in mid- and high-dose males and high-dose females was increased. Blood urea nitrogen and creatinine were decreased in mid- and high-dose females. Fasted glucose in high-dose females was increased. These changes in haematological and clinical chemistry parameters were not considered toxicologically significant due to the effects on growth, the low magnitude of the differences and/or abnormal values in control animals; they were considered secondary effects.

Absolute and relative kidney, liver and spleen weights were statistically significantly decreased in both sexes as were thyroid gland in mid- and high-dose females and adrenal gland in mid- and high-dose males. The decreases in these organ weights likely reflect the decreased amyloidosis observed in the mid- and high-dose groups compared with controls and the low-dose group.

Dose-related decreases in the incidence and severity of amyloidosis were observed in mid- and high-dose males and high-dose females. The decreases were postulated to be a secondary effect related to a reduction in nutrient intake associated with reduced body weight gain, and were not considered adverse. Incidence of enterotoxic enteritis (an inflammation caused by *C. difficile* infection) was increased in both sexes at mid and high dose. Consistent lower body weights in mid-dose males and females might have been a result of the infection. Overall, neoplasms were uncommon. The only statistically significant increase in neoplasms was adrenocortical adenoma in females at 35 mg/kg bw per day, which was noted when the incidences of the part 1 and part 2 studies were combined (Table 13).

The pooled incidences were 2/100, 2/80, 6/80 and 8/80 at 0, 2, 15 and 35 mg/kg bw per day, respectively. However, the incidences of the part 1 and part 2 studies were not significantly increased in pair-wise comparisons when considered individually. The incidences were 1/50, 1/40, 6/40 or 3/40 in the part 1 study and 1/50, 1/40, 0/40 or 5/40 in the part 2 study at 0, 2, 15 or 35 mg/kg bw per day, respectively. The incidences of adrenal tumours were not increased in males. Although the qualified historical control data were not provided, adrenocortical adenoma is the most frequently observed neoplasm in aged Syrian golden hamsters (Francis, 1995; McInnes, Ernst & Germann, 2013). A recent study reported incidences of adrenal cortical adenoma in control groups of 35/60 to 42/60 in male and 27/60 to 39/60 in female Syrian golden hamsters examined at 24 months of age; in the same article, female Syrian golden hamsters evaluated at 12 months showed an 8% (4/50) incidence of adrenocortical adenomas (McInnes, Ernst & Germann, 2013). No increased malignancy was observed in the adrenal tumours in both part 1 and part 2 studies, when analysed separately or when combined. The Meeting noted that the survival rate in females at 35 mg/kg was much higher than in the concurrent control (52% vs 17%) and that most of the adrenal tumours were observed in females. Survival of females was known to be lower than males (McConell et al., 1983; Annex 1, reference 132). Therefore, unexpected differences between groups in survival rates would likely lead to the slight increase in incidences of adrenocortical adenomas in females at 35 mg/kg bw. Treatment with fenazaquin was not considered to lead to a toxicologically significant increase in neoplasms, with the results considered to be equivocal or not carcinogenic.

Table 13. Treatment-related observations in a carcinogenicity study in hamsters

Parameter	Measure per dose level of fenazaquin							
	Males				Females			
	0 mg/kg bw per day	2 mg/kg bw per day	15mg/kg bw per day	30 mg/kg bw per day	0 mg/kg bw per day	2 mg/kg bw per day	15 mg/kg bw per day	35 mg/kg bw per day
Survival rate [a] (%)	62	69	74	68	17	15	51	52
Body weight [b]								
Pre-dosing (g)	83.5	83.9	83.3	83.8	83.7	83.8	83.7	83.5
(%)	–	100	100	100	–	100	100	100
Week 4 or 5 (g)	109.5	109.5	108.4	109.5	128.4	130.4	128.3	122.3*
(%)	–	100	100	100	–	102	100	95
Week 8 (g)	126.5	126.0	121.1**	120.9**	144.5	149.0	142.6	133.7**
(%)	–	100	96	96	–	103	99	93
Week 12 (g)	137.8	136.7	130.2**	126.3**	157.0	160.7	152.4	140.3**
(%)	–	99	95	92	–	102	97	89
Week 25 (g)	149.2	149.7	136.1**	121.2**	164.9	165.9	155.6**	141.4**
(%)	–	100	91	81	–	100	94	86
Week 42 (g)	144.5	142.6	131.2**	112.4**	146.0	146.1	140.4	126.5**
(%)	–	99	91	78	–	100	96	87

Parameter	Measure per dose level of fenazaquin							
	Males				Females			
	0 mg/kg bw per day	2 mg/kg bw per day	15mg/kg bw per day	30 mg/kg bw per day	0 mg/kg bw per day	2 mg/kg bw per day	15 mg/kg bw per day	35 mg/kg bw per day
Week 51 (g)	144.5	142.4	133.5**	114.5**	144.2	143.7	144.3	125.5**
(%)	–	99	92	79	–	100	100	87
Week 68 (g)	139.6	142.3	133.4	118.4**	128.9	128.0	132.8	128.0
(%)	–	102	96	85	–	99	103	99
Week 76 (g)	136.4	135.0	130.6	116.6**	119.5	128.3	130.7	128.3
(%)	–	99	96	85	–	107	109	107
Body weight gain [b]								
Start to week 12 (g)	54.2	52.8	47.1**	42.5**	73.3	76.9	68.7	56.7**
(%)	–	97	87	78	–	105	94	77
Start to week 25 (g)	65.7	65.9	52.9**	37.1**	81.0	82.0	71.7**	57.5**
(%)	–	100	81	56	–	101	89	71
Start to week 42 (g)	61.0	58.6	47.5**	28.0**	61.7	62.2	56.1	42.4**
(%)	–	96	78	46	–	101	91	69
Start to week 51 (g)	61.1	58.4	50.0**	30.0**	60.0	59.5	60.2	41.1**
(%)	–	96	82	49	–	99	100	69
Start to week 68 (g)	55.9	58.4	49.9	33.9**	44.2	45.2	49.4	44.4
(%)	–	104	89	61	–	102	112	100
Start to week 78 (g)	52.9	51.0	46.8	31.9**	35.8	42.4	46.6	44.7
(%)	–	96	88	60	–	118	130	125
Amyloidoses [c]								
All grades	46	41	16	6	90	85	91	65
Severe degree	26	18	8	1	67	59	50	14
Enterotoxic enteritis	18	24	38	58	14	12	21	53
Adrenocortical adenoma [d,e]								
Total	8/100	12/80	12/80	5/80	2/100	2/80	6/80	8/80*
Part 1	4/50	3/40	5/40	3/40	1/50	1/40	6/40	3/40
Part 2	4/50	8/40	7/40	2/40	1/50	1/40	0/40	5/40
Adrenocortical adenocarcinoma [d]								
Total	0/100	1/80	0/80	0/80	0/100	0/80	2/80	2/80
Part 1	0/50	1/40	0/40	0/40	0/50	0/40	2/40	2/40
Part 2	0/50	0/40	0/40	0/40	0/50	0/40	0/40	0/40

bw: body weight; no.: number; *: $P > 0.03$ (Peto survival adjusted trend test)

[a] Survival rate at 18 months.

[b] Expressed in units and as % of the control value.

[c] Expressed as % of the number of animals examined.

[d] Expressed as the number of animals with the finding / no. of animals examined.

[e] Including multifocal, haemorrhagic or bilateral type.

Sources: Francis, Boss & Gries (1992b); Francis (1995)

The Meeting considered the data on females insufficiently reliable to assess toxicity and carcinogenicity because of the high mortality of the controls. The study was also considered to be confounded due to amyloidosis and the long-term use of vancomycin hydrochloride to treat a serious *C. difficile* infection.

The NOAEL for long-term toxicity in male hamsters was 2 mg/kg bw per day based on lower body weights and decreased body weight gain at 15 mg/kg bw per day.

The NOAEL for carcinogenicity in male hamsters was 30 mg/kg bw per day, the highest dose tested (Francis, Boss & Gries, 1992b).

Rat

In a combined chronic toxicity study and carcinogenicity study, fenazaquin (purity 98.0%, batch no. ACD 13041; purity 98.5%, batch no. 435MH8) was administered to Fischer 344 (SPF) albino rats (n = 60/sex per group) at dietary concentrations of 0, 10, 100, 200 or 400 (males)/450(females) ppm (equal to 0, 0.46, 4.5, 9.2 and 18.3 mg/kg bw per day for males and 0, 0.57, 5.7, 11.5 and 25.9 mg/kg bw per day for females, respectively) for 24 months. The test article was distributed homogeneously in the diet and was stable for the duration of use.

Due to scheduling difficulties, this study was divided into two, with each conducted with equal numbers of animals per sex and group but housed in a separate room. These duplicate studies were initiated approximately two weeks apart. Blood from 20 rats/sex per group was drawn at 6, 12 and 18 months and at scheduled kill for investigation of haematological and clinical chemistry parameters. Urine from 10 rats/sex per group at 6, 12 and 18 months were used for urine analysis. Clinical signs, body weights, feed consumption and mortality were monitored in all animals throughout the study. All animals underwent ophthalmological examination prior to treatment initiation and study end. At study end, all surviving animals were necropsied and their organs weighed. Organs and tissues were fixed for histopathological evaluation.

The treatment-related changes are summarized in Table 14. Appearance, behaviour and survival of the rats were not influenced by treatment. No treatment-related abnormality was detected in ophthalmological examination. Body weights were slightly lower in males and females at 200 ppm and above throughout the study. Total body weight gains were 86% and 80% of control values in males and females at 400 ppm, respectively. Average feed consumption was slightly reduced after two years in males at 200 ppm and above and in females at 450 ppm compared to age-matched controls.

Haematological analyses showed that group mean PCV, haemoglobin and red blood cell counts were slightly lower in males at 100 ppm and above. However, because the haematological changes were minimal and inconsistent at all sample time points and there was no clear dose–response pattern, they were not considered treatment related. Compound-related clinical chemistry changes included decreased serum cholesterol and triglyceride concentrations in males and females at 200 ppm or above (17–39% below control values).

Table 14. Treatment-related observations in a chronic toxicity and carcinogenicity study in rats

Parameter/ time	Measure per dietary concentration of fenazaquin									
	Males					Females				
	0 ppm	10 ppm	100 ppm	200 ppm	400 ppm	0 ppm	10 ppm	100 ppm	200 ppm	450 ppm
Body weight [a]										
Pre-dosing (g)	114.0	110.2	111.8	109.4*	109.1*	89.1	89.2	90.1	89.7	88.4
(%)	–	97	98	96	96	–	100	101	101	99
Week 4 (g)	241.9	234.7	233.6*	226.4**	213.0**	147.0	147.7	144.9	140.8**	134.9**
(%)	–	97	97	94	88	–	100	99	96	92
Week 8 (g)	303.0	294.8	294.3	286.5**	270.1**	173.0	174.4	170.2	165.2**	157.2**
(%)	–	97	97	95	89	–	101	98	95	91
Week 12 (g)	342.8	333.4	332.2	325.4**	309.6**	187.8	188.0	185.0	179.7**	169.9**
(%)	–	97	97	95	90	–	100	99	96	90
Week 25 (g)	408.7	400.8	400.1	385.3**	364.6**	213.1	217.7	210.7	201.4**	191.9**
(%)	–	98	98	94	89	–	102	99	95	90

Parameter/ time	Measure per dietary concentration of fenazaquin									
	Males					Females				
	0 ppm	10 ppm	100 ppm	200 ppm	400 ppm	0 ppm	10 ppm	100 ppm	200 ppm	450 ppm
Week 52 (g)	452.8	444.7	443.2	426.9**	399.3**	246.2	254.7*	244.6	227.4**	211.4**
(%)	–	98	98	94	88	–	104	100	93	85
Week 77 (g)	476.0	462.2	457.0*	437.4**	408.6**	292.1	298.9	292.0	272.9**	243.8**
(%)	–	97	96	92	86	–	102	100	93	83
Week 104 (g)	396.8	389.9	395.6	378.3	351.1*	294.2	305.9	297.4	289.5	251.8**
(%)	–	98	100	95	88	–	104	101	98	86
Body weight gain – overall, from study start to termination [a]										
(g)	282.3	279.3	284.9	270.1	242.6*	205.5	215.6	207.4	199.9	163.4*
(%)	–	99	101	96	86	–	105	101	97	80
Feed consumption – overall, from study start to termination [a]										
(g/day)	18.4	18.2	18.3	17.5**	16.6**	13.6	14.0*	13.6	13.0**	12.0**
(%)	–	99	100	95	90	–	103	100	96	88
RBC ($\times 10^6$/mL)										
6 months	9.71	9.56	9.54	9.37*	9.29*	8.67	8.85*	8.64	8.62	8.61
12 months	9.43	9.48	9.34	9.23	9.26	8.30	8.53	8.47	8.46	8.35
18 months	8.58	9.06	8.73	8.24	9.36	8.40	8.42	8.24	8.24	8.14
24 months	6.31	6.77	6.64	8.40*	8.04*	7.84	7.82	7.71	7.89	7.97
Haemoglobin (g/dL)										
6 months	16.28	16.14	15.96*	15.74*	15.65*	15.88	16.13	15.79	15.79	15.65
12 months	16.02	15.99	15.80	15.44	15.59	15.37	15.74	15.70	15.65	15.38*
18 months	15.46	16.13	15.73	14.70*	16.27	15.73	15.67	15.44	15.50	15.17*
24 months	12.10	12.62	12.60	15.09*	14.38*	14.85	14.68	14.70	14.93	15.06
PCV (%)										
6 months	50.4	49.8	49.4	48.8*	48.5*	48.3	49.3*	48.2	48.2	48.3
12 months	49.8	50.3	49.7	48.1*	49.1*	46.6	47.9	47.8	47.8	47.4
18 months	49.2	50.7	49.6	46.5*	51.3	48.0	48.2	47.4	48.0	47.4
24 months	39.0	39.5	39.9	47.1*	45.5*	44.61	44.38	44.18	15.06	45.59
Serum cholesterol (mg/dL)										
6 months	95.0	91.5	84.7*	79.2*	74.2*	125.2	122.5	103.9*	98.8*	88.9*
12 months	114.5	110.6	101.5	91.8*	92.6*	124.8	122.1	108.2*	101.8*	102.1*
18 months	168.1	164.8	149.6	138.9*	102.6*	129.9	126.1	113.9*	105.4*	103.9*
24 months	241.8	220.6	213.8	164.9*	155.0*	158.9	176.7	154.6	127.4*	116.6*
Serum triglyceride (mg/dL)										
6 months	335.5	295.3	306.0	270.9*	240.5*	223.9	232.9	195.1	179.3*	156.4*
12 months	335.0	350.2	307.0	317.6	292.1	275.5	284.9	240.8	256.7	223.0
18 months	291.8	255.7	247.6	248.5	208.4	296	334	245	266	215*
24 months	316	257	284	238	188	222	257	230	145	93*

(*Continued on next page*)

Parameter / time	Measure per dietary concentration of fenazaquin									
	Males					Females				
	0 ppm	10 ppm	100 ppm	200 ppm	400 ppm	0 ppm	10 ppm	100 ppm	200 ppm	450 ppm
Focal hepatocellular atypia – Incidence [b,c]										
Study 1 + 2 [d]										
Overall	13/60	16/60	28/60#	31/60#	33/60#	22/60	25/60	28/60	25/60	29/60
(%)	(22)	(27)	(47)	(52)	(55)	(37)	(42)	(47)	(42)	(48)
Study 1 [e]	4/30	9/30	13/30#	13/30#	17/30#	13/30	13/30	13/30	12/30	15/30
Study 2 [e]	9/30	7/30	15/30	18/30#	16/30	8/30	12/30	16/30	10/30	14/30
Mild	13	13	19	25	20	16	19	26	15	27
Slight	0	3	9	5	12	5	6	1	10	2
Moderate	0	0	0	1	1	1	0	1	0	0

PCV: packed cell volume; ppm: parts per million; RBC: red blood cells;
*: $P < 0.05$ (Dunnett two-tailed t-test); **: $P < 0.01$ (Dunnett two-tailed t-test); #: $P < 0.05$ (chi-square test)

[a] Expressed as % of mean control value.

[b] Called hepatocellular altered foci in the recent criteria.

[c] Expressed as no. of animals with the finding / no. of animals examined and in parentheses, as a % of the number of animals examined.

[d] Statistical significance from the original report.

[e] Statistical significance calculated by the monographer.

Source: Cocke et al. (1992)

Males at 400 ppm had significantly reduced absolute liver and heart weights, considered to be related to lower terminal body weights. Although statistically significant changes were observed in several organ weights, they were not treatment related because of the lack of a dose–response relationship. No treatment-related macroscopic findings were noted. Microscopic examination found that focal incidences of hepatocellular atypia in males were statistically significantly increased at 100 ppm and above in the duplicate studies, but dose-related increases in intensity of this lesion were not clear. In the original report, the study pathologist recognized the focal basophilic atypia as the usual type seen in ageing rats of this strain and that there was sometimes accompanying vacuolation. In addition, these lesions were characterized as a small cluster of hepatocytes within a lobule with tinctorial properties different from the surrounding hepatic parenchyma. The description of basophilic focal hepatocellular atypia in the original report appears to be the same as a basophilic focus of hepatocellular alteration using more recent criteria. This term was proposed towards the end of the 1980s (Ward, 1980) and has been widely accepted since the 1990s (Maronpot et al., 1986; Eustis et al., 1990; Thoolen et al., 2010). The basophilic type was known to be the most common and most frequent change in the liver of aged F344 rats (Eustis et al., 1990). Incidences of focal hepatocellular atypia in control F344 rats in two-year feeding or gavage studies conducted by the National Toxicology Program (NTP Technical Reports, 2016) during 1991–1993 (years similar to the Cooke et al., 1990 study) varied but averaged about 35% in males or 58% in females (Table 15). This change is nonneoplastic, but is believed to represent a preneoplastic step in rodent hepatocarcinogenesis. In addition, several hepatotoxicants are known to induce various types of hepatocellular altered focus (foci of cellular alteration). The historical control range at the institution where this study was conducted was not provided; however, incidences of this effect in all groups were within the ranges of control NTP groups (Table 15). The incidences of the male control group in this study (13/60, 21%) were lower than the average of the 32 NTP studies (34.5% during the 1991–1993 period), and those incidences at 200 and 400 ppm (31/60, 51.7%; 33/60, 55%, respectively) appeared to be approaching the upper range of the NTP controls. The incidence at 100 ppm was 28/60 (43%). The incidence in females at each dose in this study is similar to the control incidences seen in the NTP studies. The Meeting considered the changes in hepatocellular atypia (currently termed basophilic altered focus) were of uncertain significance as the incidence of this lesion is highly variable in this strain and this lesion was not accompanied by any other pathological changes in the rat studies; however, the elevated occurrence at the two highest doses could be treatment related as they exceeded

the upper bound of the range of control incidences in the NTP studies, though the increased intensity of lesion was not clear. No treatment-related effects were observed in male reproductive tracts including the testes or epididymis. The incidence in atrophy decreased with increasing dose. No treatment-related neoplastic lesions were noted in all organs and tissues examined in both sexes.

Table 15. Incidence of basophilic focus of hepatocellular alteration in F344 rats from 1991–1993 NTP two-year feeding or gavage studies and a textbook

Study	Route	Year reported	Incidence [a]	
			Males	Females
NTP study number [b]				
Study 1 [c]	Gavage	1991	2/60 [d]	4/60
Study 2	Gavage	1991	13/50	16/50
Study 3	Gavage	1991	32/50	35/50
Study 4	Gavage	1991	46/60	45/60
Study 5 [c]	Gavage	1992	12/53	37/53
Study 6	Gavage	1992	21/50	36/50
Study 7	Gavage	1992	7/50	–[e]
Study 8 [c]	Gavage	1992	20/50	36/50
Study 9	Feeding	1992	4/50	1/50
Study 10	Feeding	1992	12/50	36/50
Study 11 [c]	Feeding	1992	21/50	38/50
Study 12	Feeding	1992	16/50	40/50
Study 13	Feeding	1992	10/50	34/50
Study 14 [c,d]	Feeding	1992	14/50	24/50
Study 15	Feeding	1992	34/48	42/50
Study 16	Feeding	1992	17/50	37/50
Study 17	Gavage	1993	12/60 [c]	39/60
Study 18	Gavage	1993	27/50	42/50
Study 19	Gavage	1993	32/50	43/50
Study 20 [c]	Gavage	1993	17/50	21/50
Study 21	Gavage	1993	16/59	39/60
Study 22	Gavage	1993	3/58	3/60
Study 23	Gavage	1993	20/60	28/60
Study 24	Feeding	1993	6/50	38/50
Study 25	Feeding	1993	27/50	3/50
Study 26	Feeding	1993	17/50	40/50
Study 27	Feeding	1993	1/50	0/50
Study 28 [c]	Feeding	1993	32/50	39/50
Study 29	Feeding	1993	16/70	42/70
Study 30	Feeding	1993	9/60	32/60
Study 31	Feeding	1993	12/70	45/70
Study 32	Feeding	1993	27/60	38/60
Total 32 studies (1991–1993) [f]				
Average (%)			34.5	57.7
SD (%)			21.2	27.1
Range [g]			1/50–46/60	0/50–43/50

Study	Route	Year reported	Incidence [a]	
			Males	Females
Four studies in 1991				
Average (%)			42.5	45.9
SD (%)			33.8	32.5
Range [g]			2/60–46/60	4/60–45/60
Twelve studies in 1992				
Average (%)			31.5	65.3
SD (%)			16.5	32.5
Range [g]			4/50–34/48	1/50–42/50
Sixteen studies in 1993				
Average (%)			34.8	55.5
SD (%)			21.8	28.7
Range [g]			1/50–37/50	0/50–43/50
Incidence of basophilic hepatocellular foci in F344 rats (%) [g]				
6 months of age			25	46
9 months of age			50	74
12 months of age			67	77
15 months of age			88	94
18 months of age			100	100
24 months of age			98	100

[a] Expressed as the no. of rats bearing basophilic foci / the no. of rats examined.

[b] NTP Technical Reports (2016), accessed 18 May 2017.

[c] Rats bearing basophilic focus, focal and basophilic multifocal or multiple combined.

[d] Reported as "cytologic alternation or cytologic alteration, multiple" or "focal cellular change".

[e] Only male data reported.

[f] Eustis et al. (1990). Total of 408 F344 rats examined.

[g] Presented as no. of rats with findings / number of rats examined.

Sources: Eustis et al. (1990); NTP Technical Reports (2016)

The NOAEL for long-term toxicity was 100 ppm (equal to 4.5 mg/kg bw per day) based on increased incidence of focal hepatocellular atypia in males at 200 ppm (equal to 9.2 mg/kg bw per day).

The NOAEL for carcinogenicity was 400 ppm (equal to 18.3 mg/kg bw per day), the highest dose tested.

There was no evidence of carcinogenicity associated with fenazaquin treatment in rats (Cocke et al., 1992).

The Meeting concluded that fenazaquin is not carcinogenic in rats and male hamsters.

2.4 Genotoxicity

Fenazaquin was evaluated for possible mutagenic/genotoxic effects in in vitro test systems using bacterial and mammalian cells and in vivo test systems using rats and mice.

Fenazaquin did not have mutagenic or genotoxic effects in vitro in bacterial cells and in primary rat hepatocytes. In mammalian cell assays, fenazaquin was equivocal or negative for gene mutations and structural chromosomal aberrations. An increase in polyploidy was seen in Chinese hamster ovary (CHO) cells treated in vitro.

In in vivo test systems, fenazaquin did not induce unscheduled DNA repair, sister chromatid exchanges or micronuclei. Overall, the data indicated that fenazaquin is unlikely to be genotoxic in vivo.

The results of genotoxicity tests of fenazaquin in in vitro and vivo are summarized in Table 16.

Table 16. Summary of genotoxicity studies with fenazaquin

End-point	Test system	Concentration	Purity, Lot/ batch no.	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* (TA98, TA1535, TA100, TA1537) *Escherichia coli* (WP2*uvr*A$^-$)	188, 375, 750, 1500, 3000 µg/plate ± S9	98% ACD13041	Negative	Francis, Scheuring & Richardson (1989)
Gene mutation	Mouse lymphoma (L5178Y)	0.05, 0.1, 0.5, 1, 2.5, 5, 10 µg/mL (−S9) 0.5, 1, 2, 4, 6, 8, 10, 12 µg/mL (+S9)	98% ACD13041	Equivocal (6–8 µg/mL, +S9)	Francis, Richardson & Michaelis (1989)
Unscheduled DNA synthesis	Hepatocytes, Fischer 344 rat (male)	0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 µg/mL	98% ACD13041	Negative	Francis, Garriot & Yount (1989)
Chromosomal aberrations	CHO cells	0.1, 0.5, 1 µg/mL (−S9) 40, 50, 60µg/mL (+S9) (4 h treatment)	98% ACD13041	Negative for SCA Equivocal for polyploidy (−S9) Negative for SCA, positive for polyploidy (+S9)	Francis, Garriott & Kindig (1989)
Chromosomal aberrations	CHO cell line (WBL)	0.5, 1 and 2 µg/mL (−S9); 10, 20, and 30 µg/mL (+S9)	97.5% 0082	**18-h harvest**: Negative for SCA and negative for polyploidy (−S9); **18-h harvest**: Negative/ equivocal for SCA and negative for polyploidy (+S9) **30-h harvest**: Negative for SCA and negative for polyploidy (−S9); **30-hr harvest**: Equivocal for SCA, positive for polyploidy/ endoreduplication (+S9)	Jenkinson & Wright (1995)

(*Continued on next page*)

End-point	Test system	Concentration	Purity, Lot/ batch no.	Results	Reference
In vivo/in vitro					
Unscheduled DNA synthesis	Hsd/Ola Sprague Dawley rat (male)	180, 600 mg/kg bw (single gavage, after 2 and 14 h of treatment)	99.3% RMM 1836; 97.8% 435 MH8	Negative	Proudlock (1993)
In vivo					
Micronucleus	Bone marrow cells from ICR mice (n = 5/sex)	400, 800, 1600 mg/kg bw (males) 400, 800, 1200 mg/kg bw (females) (twice gavage, after 24 h treatment)	98% ACD13041	Negative for males and females	Francis, Parton & Garriott (1989), reviewed by Lovell (1995)
Micronucleus	Bone marrow cells from ICR mice (n = 6 females)	500, 1000, 2000 mg/kg bw (twice gavage, after 24 h treatment)	99.9% H29803 0416	Negative	Beevers (2007)
Sister chromatid exchange	Bone marrow cells from CD-1 mice (n = 5 males)	500, 1000, 2000 mg/kg bw (single gavage, after 21 h treatment)	98% ACD13041	Negative	Francis, Garriott & Brunny (1989)

bw: body weight; CHO: Chinese hamster ovary; S9: 9000 × g supernatant fraction from rat liver homogenate; SCA: specific combining ability

(a) In vitro studies

Fenazaquin produced mixed, largely negative results in genotoxicity tests in vitro. Fenazaquin did not induce mutations in the standard test battery used to assess mutation in bacteria. It was also negative in an unscheduled DNA synthesis test using rat hepatocytes and was largely negative for structural chromosomal aberrations in CHO cells.

Fenazaquin was not mutagenic in the absence of metabolic activation in the mouse lymphoma assay (*tk* locus; Francis, Richardson & Michaelis, 1989). However, increases in the mutation frequency were observed at the high doses (≥6 µg/mL) in the presence of metabolic activation. The increase in mutation frequency at 6 and 8 µg/mL was associated with high toxicity (≥87%) that approached the upper range of that considered acceptable for evaluation (90%). At the next lower concentration of 4 µg/mL, the total survival was approximately 62.5%, with a mutation frequency of approximately 1.6-fold of the control. The results seen in the presence of metabolic activation (S9 mix) may have been a secondary effect related to cytotoxicity. The study authors considered the increase in mutation frequency as positive for genotoxicity. However, the OECD recently adopted new criteria for improving the biological relevance of the mouse lymphoma assay (OECD guideline 476). Based on these new criteria, while a positive dose–response relationship in mutation frequency was seen, the induced mutation frequency did not exceed the Global Evaluation Factor of 90 for the agar version of the assay. As a result, the test results would not be considered positive; rather, it would be more appropriate to consider the test results equivocal.

Fenazaquin did induce polyploidy in two in vitro studies using CHO cells. Of note, effects were seen primarily at an intermediate concentration and were reduced at higher concentrations. With regard to the relevance of these findings, the International Conference on Harmonisation guideline (ICH, 2011, p.13) states that:

> polyploidy is a common finding in chromosome aberration assays in vitro… polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation; it is also commonly associated with increasing cytotoxicity. If polyploidy, but no structural chromosome breakage, is seen in an in vitro assay, generally a negative in vivo micronucleus assay with assurance of appropriate exposure would provide sufficient assurance of lack of potential for aneuploidy induction.

As shown in Table 16, fenazaquin was negative in two separate micronucleus assays in vivo and is therefore unlikely to be associated with an induction of aneuploidy in vivo.

(b) In vivo studies

Positive results were not seen in genotoxicity studies in vivo. Fenazaquin did not induce unscheduled DNA repair, sister chromatid exchanges or micronuclei in short-term tests in mice or rats.

The Meeting concluded that fenazaquin is unlikely to be genotoxic in vivo.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, fenazaquin (purity 98.4%; batch no. 271MH8) was administered by gavage to male and female Crl:CD®(SD)BR rats (n = 30/sex per dose group; six weeks old at study start) over two generations with one litter per generation. Fenazaquin was administered at doses of 0, 1, 5 and 25 mg/kg bw per day in aqueous 10% acacia at a constant dosing volume of 5 mL/kg bw. The doses were based on the results of a range-finding study in which excess salivation and reduced feed consumption and body weight gain were observed at doses of 11 and 27.5 mg/kg bw per day in addition to impaired righting reflex observed in one male during premating at 27.5 mg/kg bw per day. Females showed impaired reflex, decreased motor activity and ataxia during premating, excess salivation during gestation, impaired righting reflex, decreased motor activity, pale eyes and ears, and bradypnoea during lactation at 27.5 mg/kg bw per day.

In this study, fenazaquin was administered during premating (70 days), cohabitation (21 days), gestation (females only) and lactation (females only). Parental males were necropsied after the cohabitation period in both generations. F_1 and F_2 pups were culled to five/sex per group on day 4, with dosing of F_1 pups starting on day 22 postpartum. F_1 males were killed after the cohabitation period while F_1 females and F_2 litters were killed on lactation day 21. Gestation day 0 was defined as the day a copulatory plug was detected or a vaginal smear tested positive for sperm. Lactation day 1 was the day of parturition. All F_0 and F_1 parental animals were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded. Mating performance during the cohabitation period, duration of gestation, litter size (number of live and dead pups) and pup viability was examined for all F_0 and F_1 female rats. At necropsy, reproductive organs (females: vagina, uterus, ovaries; males: testes, epididymides, seminal vesicles, prostate), urinary bladder and tissues/organs with gross lesions were examined for histopathological changes. Testes and epididymides were weighed prior to fixation. F_1 and F_2 pups were checked for viability, physical signs including gross external anomalies, nursing and nesting behaviour until postpartum day 21. Culled F_1 and F_2 pups with gross lesions were fixed in 10% formalin for histopathological examination. The head of each pup was cross-sectioned and the brain examined for hydrocephaly.

Treatment did not affect mortality. In clinical signs, the incidences of excess salivation observed after oral treatment were statistically significantly increased in F_0 and F_1 males and females at 25 mg/kg bw per day. In F_0 males and females, the salivation first occurred 20 or 14 days, respectively, after study commencement, and continued throughout treatment. At 5 mg/kg bw per day, the salivation was considered not toxicologically significant because of its sporadic occurrence and the lack of statistical significance. Other treatment-related clinical signs were not observed.

Changes in body and testes weights of F_0 and F_1 animals are shown in Table 17. In F_0 males at 25 mg/kg bw per day, body weights were slightly but not statistically significantly lower during the premating and cohabitation period, and their body weight gains were statistically significantly lower during premating week 1 only. In females at 25 mg/kg bw, body weights were increased during the lactation period. No treatment-related changes were observed in other treated groups.

Table 17. Weight changes in a multigeneration reproductive toxicity study in rats

Parameters	Measure per dose level of fenazaquin							
	Males				Females			
	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	25 mg/kg bw per day	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	25 mg/kg bw per day
Body weight gain (g)								
No. of F_0 rats examined	30	30	30	30	30	30	30	30
Premating days 1–8	58.4	58.1	59.9	54.1**	27.8	25.3	26.5	27.1
Premating days 8–15	52.3	53.4	56.1	48.3	26.3	26.5	27.1	25.9
Premating days 15–22	43.6	44.6	42.3	40.3	24.9	23.9	24.1	23.0
Premating days 22–29	34.9	37.0	37.6	35.8	17.3	18.0	18.3	15.9
Premating days 36–43	23.6	25.9	26.2	24.1	13.1	14.1	12.7	15.9
Premating days 50–57	19.7	19.6	19.5	17.9	7.1	9.0	7.1	7.0
Premating days 64–70	16.1	18.0	18.3	17.6	9.4	9.8	10.2	10.7
Premating days 99–104	9.8	9.8	12.6	10.5	–	–	–	–
Total [a]	373.3	387.9	389.4	362.8	156.2	157.4	157.7	153.0
No. of F_1 rats examined	39	40	39	40	40	40	40	40
Premating days 1–8	45.9	47.6	45.9	40.4**	39.9	40..2	39.9	36.3**
Premating days 8–15	59.6	61.9	60.2	56.9	46.9	44.8	46.6	44.4
Premating days 15–22	62.9	64.0	65.2	64.1	37.3	36.2	35.7	38.4
Premating days 22–29	64.9	63.2	64.6	59.2*	27.3	24.2	26.2	23.0
Premating days 36–43	44.6	45.2	48.4	44.3	23.7	21.6	22.6	20.1
Premating days 50–57	34.9	35.8	33.6	33.5	12.2	14.7	14.8	11.9
Premating days 64–71	23.8	24.7	24.5	15.8	7.8	7.0	9.3	7.6
Total [b]	464.7	468.2	473.7	447.6	246.5	246.4	243.5	235.6
Gestation days 0–6	–	–	–	–	30.4	23.7*	21.6**	25.6
Postcohabitation days 1–8	18.1	18.2	19.6	18.0	–	–	–	–
Testes weight (g)								
No. of F_0 rats examined	30	30	30	29	–	–	–	–
Absolute weight (g)	5.54	5.71	5.33	5.56	–	–	–	–
Relative weight (% bw)	0.93	0.94	0.87	0.95	–	–	–	–
No. of F_1 rats examined	38	39	36	39	–	–	–	–
Absolute weight (g)	5.59	5.65	5.57	5.40*	–	–	–	–
Relative weight (% bw)	0.96	0.95	0.93	0.98	–	–	–	–

bw: body weight; *: $P \leq 0.05$; **: $P \leq 0.01$

[a] Premating day 1–108 for males, 1–70 for females.

Source: Christian, Hoar & Hoberman.(1991)

Body weights in F_1 males at 25 mg/kg bw per day were significantly decreased throughout the premating and cohabitation periods though statistical significance was not attained during the second half of the premating period. Weekly and overall body weight gains at 25 mg/kg bw per day were lower. Body weights in F_1 females at 25 mg/kg bw per day were statistically significantly decreased during premating days 8–64 but not statistically significantly lower thereafter. The body weight gains were significantly decreased on days 1–8 of the premating period only. Thereafter, they were comparable to those in the controls. During gestation and lactation, body weights as well as their gains in treated groups were comparable to those in the controls. The reduced maternal body weight gains seen during gestation days 0–6 were considered not biologically significant due to the lack of a clear dose–response relationship. In F_0 parents, feed consumption in males at 25 mg/kg bw per day was significantly decreased during week 1 of the premating period, but increased thereafter. In females at 25 mg/kg bw per day, there were no notable changes in feed consumption throughout the study. In F_1 parents, feed consumption in males at 25 mg/kg bw per day was significantly decreased during days 8–15 of the premating period only and comparable to controls thereafter. In females in all treated groups, feed consumption was comparable to the controls during premating and during gestation and lactation.

No treatment-related findings were seen in necropsied F_0 and F_1 parents and pups. There were no treatment-related histopathological changes in the organ tissues examined, including reproductive organs in both generations. The very slight (3%) but statistically significant decrease in absolute weights of the testes in F_0 males was not considered to be toxicologically significant.

Fenazaquin treatment did not influence the reproductive performance of the parental F_0 and F_1 animals or the development of the fetuses. Gestational and litter parameters remained unaffected by treatment compared to concurrent controls.

The NOAEL for parental toxicity was 5 mg/kg bw per day based on body weight depression, lower body weight gain and clinical signs at 25 mg/kg bw per day. The NOAEL for effects on reproduction and offspring was 25 mg/kg bw per day, the highest dose tested (Christian, Hoar & Hoberman, 1991).

(b) Developmental toxicity

Rat

In a developmental toxicity study, fenazaquin (purity 98%; batch no. ACD13041) in 10% (w/v) aqueous acacia solution was administered by gavage to pregnant Crl:CD®(SD)BR rats (n = 25/group) at doses of 0, 3, 10 or 40 mg/kg bw per day during organogenesis from gestation days 6 through 17 at a dosing volume of 10 mL/kg bw. Dose levels were selected on the basis of the results of a dose range-finding study where pregnant female Crl:CD®(SD)BR rats (n = 5/group) were administered doses of 0, 5, 10, 30 or 100 mg/kg bw per day by gavage on gestation days 6–17. Mortality was observed at 100 mg/kg bw per day; at 30 and 100 mg/kg bw per day, maternal body weight gain and feed consumption was decreased and at 100 mg/kg bw, decreased fetal weights were characterized as having an adverse effect on development.

In the present study, all dams were observed daily for mortality and clinical signs. Maternal body weights and feed consumption were measured on gestation days 0, 6, 10, 14, 18 and 20 and at scheduled kill. On gestation day 20, all dams were killed. Uteri were weighed and examined. The number of corpora lutea, implantations, live and dead fetuses and early and late resorptions was determined. The percentage of preimplantation loss was calculated as the percentage of corpora lutea in excess of implantations per total implantations. Detectable implantation scars were defined as early resorptions. Live fetuses were individually weighed and any fetuses weighing less than 30% of control means were designated as runts. Approximately one half of all live fetuses from each litter were fixed in Bouin's solution for visceral examination. The remaining fetuses were eviscerated and processed for skeletal examination. All variations, deviations and malformations were recorded.

The major toxicities in this study are summarized in Table 18. All pregnant dams survived until the scheduled kill on gestation day 20. There were no treatment-related clinical signs. At 40 mg/kg bw per day, feed consumption was significantly reduced from gestation days 6 to 18. The increases seen on gestation days 0–5 and 18–19 were not considered toxicologically significant. No significant changes in body weight were seen. Body weight gains of dams at 40 mg/kg bw per day were significantly reduced from gestation days 6 to 17 but were increased on gestation days 0–5 and 18–19. Relative uterine weight was also statistically significantly reduced at 40 mg/kg bw per day.

Table 18. Maternal findings in a developmental toxicity study in rats with fenazaquin

Findings / Gestation day	Weight per dose level of fenazaquin			
	0 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day	40 mg/kg bw per day
Feed consumption (g)				
0–5	21.32	22.42	22.54	23.05*
6–9	15.08	15.86	15.39	12.85*
10–13	16.39	17.04	16.81	14.25*
14–17	17.94	18.28	18.21	16.26*
18–19	26.62	27.54	28.56	29.39*
Body weight (g)				
0	218.3	224.3	220.7	221.9
6	253.6	263.7	259.3	261.1
10	272.2	280.9	275.1	268.2
14	292.7	302.6	296.7	283.3
18	338.7	350.0	342.5	323.9
20	368.4	382.4	372.2	361.4
Body weight gain (g)				
0–5	35.2	39.4	38.5	39.2
6–9	18.6	17.2	15.8	7.0*
10–13	20.4	21.7	21.6	15.1*
14–17	46.0	47.4	45.8	40.5*
18–19	29.7	32.3	29.6	37.5*
Total	150.0	158.0	151.5	139.5*
Relative uterine weight (g)	74.5	80.5	76.1	63.4*

bw: body weight; *: $P \leq 0.05$

Source: Francis & Higdon (1989)

All pregnant dams delivered live fetuses and no abortions or total implantation loss were observed. No treatment-related effect was noted on the total number of corpora lutea, implantations and pre- and postimplantation losses. The number of live and dead fetuses and the litter size in each dam was also not affected by treatment. There was no dose-dependent effect on mean fetal weight (males, females and combined). The sex ratio and the total number and percentage of runts in each litter were not affected by fenazaquin administration. Fenazaquin treatment had no statistically significant and/or dose-independent influence on the total number and percentage of deviations, variations and malformations whether compared on an individual (fetus) or litter basis.

The NOAEL for maternal toxicity was 10 mg/kg bw per day based on the reduced feed consumption and decreased body weight gains at 40 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 40 mg/kg bw, the highest dose tested (Francis & Higdon, 1989).

Rabbit

Fenazaquin (purity and batch no. not stated) in 10% (w/v) aqueous acacia solution was administered by gavage to pregnant New Zealand White rabbits (strain: Hra:(NZW); $n = 20$/group) at doses of 0, 3, 13 or 60 mg/kg bw per day during organogenesis from gestation day 6 to 18 at a dosing volume of 2 mL/kg bw. Dose levels were selected based on the results of a doserange-finding teratology study in which pregnant female New Zealand White rabbits ($n = 5$/group) received fenazaquin by gavage at doses of 0, 10, 30, 60 or 100 mg/kg bw per day on gestation days 6–18. No mortality was observed at any dose level. Decreases in feed consumption with no effect on maternal body weight gain were noted

at 30, 60 and 100 mg/kg bw per day. Soft stool was evident at 60 and 100 mg/kg bw per day. An increase in early fetal resorptions was noted at 30, 60 and 100 mg/kg bw per day, reaching statistical significance at 60 mg/kg bw per day.

In the present study, the finding of viable sperm in the vaginal lavage, indicative of successful mating, was designated gestation day 0. All does were checked for mortality and clinical signs daily. Feed consumption and body weights were measured on gestation days 0, 6, 13, 19, 24 and 28 and at scheduled kill. On gestation day 28, all dams were killed. Uteri were weighed and an internal examination was conducted and abnormalities recorded. The number of corpora lutea, implantations, live and dead fetuses and early and late resorptions was determined. The percentage of preimplantation loss was calculated as the percentage of corpora lutea in excess of implantations per total implantations. Uteri without recognizable implantation sites were stained with 10% aqueous ammonium sulfide solution. Detectable implantation scars were defined as early resorptions. Live fetuses were individually weighed and those weighing 30% less than control means were designated as runts. Individual fetuses received an external, visceral and skeletal examination, and their sex was determined by internal examination. Late resorptions and aborted fetuses were examined externally only. Approximately one half of all live fetuses from each litter were fixed in Bouin's solution for visceral examination. The remaining fetuses were eviscerated and processed for skeletal examination. All variations, deviations and malformations were recorded.

The major findings are summarized in Table 19. One pregnant doe at 13 mg/kg bw per day and six at 60 mg/kg bw per day died or were euthanized when moribund. The causes of death in 4/6 of the high-dose rabbits were related to mechanical difficulties during dose administration or handling. The one rabbit at 13 mg/kg bw per day and 2/6 rabbits at 60 mg/kg bw per day were euthanized due to abortions on gestation days 23–26. All affected animals showed weight loss and/or decreased feed consumptions during or after the treatment period. No abnormalities were found on internal examination of the aborted fetuses. The results indicate that the treatment did not influence the mortality of the does.

Feed consumption of the pregnant does at 60 mg/kg bw was slightly (about 20%) but statistically significantly lowered during gestation days 6–12. No statistically significant difference in feed intake was observed between does at 0 and 60 mg/kg bw per day during the overall treatment period. Body weights of does at 60 mg/kg bw per day tended to be consistently lower compared to controls, but their magnitude was small (within 10%) and not statistically significant. A slight but statistically significant decrease in feed consumption just after the beginning of treatment at 60 mg/kg bw per day was considered treatment related.

In maternal reproductive parameters, the percentage of early resorptions was somewhat increased (9.62%, 4.53%, 22.39% and 33.15% at 0, 1, 3, 13 and 60 mg/kg bw per day, respectively). The increase at the high dose resulted primarily from three litters with resorptions and was not statistically significant.

The nonsignificant decrease in litter size per dam was related to a slight increase in litters with total resorptions. There was no statistically significant difference between control and treated animals in the number of implantation sites. The percentage of preimplantation loss was 28.0%, 42.72%, 28.41% and 43.53% at 0, 3, 13 and 60 mg/kg bw per day, respectively. No clear dose–response relationship was seen and historical control data (at least six studies demonstrating preimplantation losses of > 43.5% in controls) indicate that the higher degree of preimplantation loss at 3 and 60 mg/kg bw per day were not likely to be treatment related.

In fetal parameters, the percentage of male and female fetuses, fetal weights, percentage of fetal runts, and normal and anomalous fetuses were not affected by treatment with fenazaquin. The occurrences in variations, deviations and malformations were sporadic and unrelated to treatment. There was no evidence of a teratogenic effect of fenazaquin treatment up to 60 mg/kg bw per day.

Table 19. Observations in a developmental toxicity study in rabbits

Parameters	Measure per dose level of fenazaquin			
	0 mg/kg bw per day	3 mg/kg bw per day	13 mg/kg bw per day	60 mg/kg bw per day
Feed consumption (g)				
Gestation days 0–5	204.7	194.5	174.8	183.3
Gestation days 6–12	185.4	171.4	159.1	140.8 *
Gestation days 13–18	182.4	147.5	140.7	144.5
Gestation days 19–27	162.4	160.0	136.4	152.4
Gestation days 6–18 (% of pretreatment period days 0–5)	90	82	86	78
Body weight gain (kg)				
Gestation days 0–5	0.208	0.180	0.130 *	0.152
Gestation days 6–12	0.095	0.035	0.059	0.059
Gestation days 13–18	0.122	0.086	0.005 *	0.088
Gestation days 19–27	0.146	0.187	0.155	0.169
Total	0.572	0.489	0.351 *	0.469
Gestational parameters				
No. of females in study	20	19 [a]	20	19 [a]
No. of females that died [b]	0	0	1	6
No. of pregnant dams (%) [c]	17 (85)	12 (63)	18 (90)	16 (84)
No. of surviving pregnant dams (%) [c]	17 (85)	12 (63)	17 (85)	11 (58)
Mean uterus weight (g)	390.0	341	329	313
Mean no. of corpora lutea/dam	11.4	11.8	10.5	12.1
Mean no. of implantation sites/dam	8.1	6.3	7.2	6.0
Mean % preimplantation loss/dam	28.08	42.72	28.41	43.53
Mean no. of postimplantation losses/litter (%) [c,d]	1.1 (10.74)	0.6 (7.22)	1.6 (23.58)	0.8 (33.15)
Mean no. of early resorptions/litter (%) [c]	1.0 (9.62)	0.3 (4.53)	1.5 (22.39)	0.8 (33.15)
Mean no. of late resorptions/litter (%) [c]	0.1 (1.12)	0.3 (2.68)	0.1 (1.19)	0.0 (0.0)
Litters with resorptions (%)	7 (41.2)	3 (25.0)	10 (58.8)	6 (54.6)
Litters with total resorptions (%) [c]	1 (5.9)	0 (0.0)	2 (11.8)	3 (27.3)
Litter size/dam (%) [c,e]	7.0 (89.25)	5.8 (92.78)	5.6 (76.42)	5.2 (66.84)
Litter size/dam [f]	7.4	5.8	6.3	7.1
Fetal parameters				
Mean no. of viable fetuses/litter (%) [f,g]	7.4 (94.83)	5.8 (92.78)	6.3 (86.61)	7.1 (91.91)
Mean no. of dead fetuses/litter (%) [g]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

bw: body weight; no.: number; *: $P \leq 0.05$, significantly different from the vehicle control group

[a] One pregnant female from each group was removed from the study on gestation day 6 due to body weight loss and/or decrease in feed consumption. These dams were not considered in the calculation of percentage of total pregnant animals.

[b] Includes one mid-dose dam and two high-dose dams that aborted on gestation days 23–26. Four pregnant high-dose dams were euthanized in moribund condition (gavage error and handling difficulties).

[c] Expressed as no. of dams with the finding and, in parentheses, as a percentage of the no. of dams examined.

[d] Postimplantation losses: non-live implants (early + late resorptions) subtracted from total implantations.

[e] No. of fetuses in litters with total resorptions included.

[f] No. of fetuses in litters with total resorptions excluded.

[g] Data presented as number of viable or dead fetuses per litter and, in parentheses, this number as a percentage of the total fetuses per litter at that dose level.

Source: Francis & Higdon (1990)

The NOAEL for maternal toxicity was 13 mg/kg bw per day based on lower feed consumption on gestation days 6–12 at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 60 mg/kg bw per day, the highest dose tested. Fenazaquin was not teratogenic in rabbits at a NOAEL of 60 mg/kg per day, the highest dose tested (Francis & Higdon, 1990).

The Meeting concluded that fenazaquin is not teratogenic.

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In an acute neurotoxicity study, single oral doses of fenazaquin (purity 99.92%; lot/batch no. H29803-0416) were administered to Sprague Dawley rats (n = 10/group) at 0 (control), 20, 65 or 130 mg/kg bw in males and 0, 20, 60 or 120 mg/kg bw in females. Mortality, clinical signs, body weight and feed consumption were monitored throughout the study. A functional observational battery (FOB) and motor activity evaluation were performed prior to treatment initiation, at the time of peak effect (one hour after dosing) and on days 8 and 15. Animals were killed by in situ perfusion, and selected tissues and/or organs as well as the central and peripheral nervous system, skeletal muscles and eyes with retinas and optic nerves from five animals/sex in the control and high-dose groups underwent histopathologic examination.

There were no unscheduled deaths during the study. Adverse clinical signs, such as mild dehydration, urine-stained abdominal fur, soft or liquid faeces and ungroomed coat, were observed one hour after dose administration and were no longer apparent shortly after. Significant reductions in body weight and feed consumption values were observed at 65 and 130 mg/kg bw in male rats and at 60 and 120 mg/kg bw in female rats. Significantly reduced mean body temperature and motor activity measures were observed in the high-dose males at the one-hour evaluation. No effect on motor activity was observed in female rats at any time point. A significant decrease in mean body temperature was found in the high-dose female rats. There were no treatment-related effects on FOB or motor activity. No treatment-related findings were observed in macroscopic and microscopic examination of the central and peripheral nervous system, the eyes, optic nerve and skeletal muscle.

The NOAEL for acute neurotoxicity in rats was 120 mg/kg bw, the highest dose tested. The NOAEL for systemic toxicity was 20 mg/kg bw based on decreased body weight and feed consumption at 60 mg/kg bw. Fenazaquin had no acute neurotoxicity (Barnett, 2012).

Delayed and subacute neurotoxicity

No study on delayed or subacute neurotoxicity was submitted. Based on the structure of fenazaquin and the results of all available short- and long-term studies, fenazaquin is not expected to pose a risk for delayed or subacute neurotoxicity.

The Meeting concluded that fenazaquin is not neurotoxic.

(b) Immunotoxicity

In an immunotoxicity study, fenazaquin (purity 99.92%; lot/batch no. H29803-0416) was administered to female Sprague Dawley rats (n = 10/group) by gavage at 0, 15, 30 or 37.5/45 mg/kg bw per day on weekdays for 28 days. The highest dose of 45 mg/kg bw per day proved to be lethal following repeated treatment in rat; the dose was reduced to 37.5 mg/kg bw per day from day 8 onwards. For the positive control group, 10 females received 30 mg/kg bw of cyclophosphamide by a single intravenous injection one day prior to the 28-day spleen cell harvest. All animals received a single intravenous injection of the antigen, sheep red blood cells (sRBC), five days prior to spleen cell harvest. The animals were monitored regularly for viability/mortality and for signs of morbidity or reaction to treatment. After 28 days, all animals were killed and the spleen and thymus weighed.

On day 1, two animals at 30 mg/kg dose died, apparently due to aspiration of the test substance, and were replaced. Another animal in this dose group died on day 2. On day 2, three animals at 45 mg/kg per day dose were found dead and on day 3 another animal in this group was

found dead. After reducing the dose to 37.5 mg/kg bw, there were no other deaths in this or any other group over the course of the study. There were no treatment-related differences in body weights, feed consumption or organ weights. The test substance had no effect on the humoral immune system. It should be noted that two of the four surviving animals at the 45/37.5 mg/kg bw dose could not be evaluated for immunoglobulin M (IgM) plaque formation because of a total lysis of the sRBC in the chamber.

The NOAEL for immunotoxicity in rats was 37.5 mg/kg bw, the highest dose tested. Fenazaquin did not exhibit immunotoxicity in the sRBC assay (Curry, 2011).

The Meeting concluded that fenazaquin is not immunotoxic.

(c) Studies on metabolites

F-1, F-2, F-3, AN-1 and NA-1

Major metabolites in rat faeces are F-1 (the form of fenazaquin where one of the methyl groups on the alkyl side chain is oxidized), F-2 (a further oxidized form of the metabolite F-1 with completely oxidized side chain, named fenazaquin acid) and F-3 (an F-2 metabolite with an oxidized quinazoline ring in position 2, named 2-hydroxy-fenazaquin acid). The major metabolites in rat urine are AN-1 (an acid nonconjugate) and NA-1 (a neutral aglycone) (Fig. 3; Appendix 1). A small amount of parent compound was found in the faeces. Biotransformation data based on radioisotope labelling indicated that fenazaquin was rapidly absorbed, metabolized to the metabolites described above, and eliminated through the urine, bile and, primarily, faeces. Very small amounts of fenazaquin were excreted in the urine.

Although insufficient toxicity data on the metabolites were submitted, integration of biochemical aspects of this compound suggest that the toxic effects of the metabolites would be adequately detected in the tests conducted on the parent compound.

2-Oxy-fenazaquin (4-[2-(4-tert-butyl-phenyl)-ethoxy]-quinazoline-2-ol)/2-hydroxy-fenazuin acid (F-3)

No data on the toxicity of this metabolite were submitted.

This metabolite was not detected in rats, but its presence is suggested by oxidative metabolism and presence of F-3 (2-hydroxy-fenazaquin acid). Taken together, it is anticipated that the toxic effects of the metabolites would be adequately detected in the tests conducted on the parent compound.

4-OH

4-OH (4-hydroxyquinazoline) is a minor metabolite in rats (<1% of faecal radioactivity) and in plants (<10% TRR). Acute oral toxicity and 4-week short-term oral toxicity studies in rats were submitted. Both studies were conducted in accordance with GLP.

Acute oral toxicity study

Female RccHan:WIST (SPF) rats ($n = 6$) were treated sequentially with 4-OH (purity 99.8%; lot no. 1176335) by single oral gavage administration at dose levels of 95 ($n = 2$ rats), 300 ($n = 3$ rats) or 950 ($n = 1$ rat) mg/kg bw. 4-OH was formulated in polyethylene glycol 300 (PEG 300) at concentrations of 9.5, 30 or 95 mg/mL, respectively, and administered at a dosing volume of 10 mL/kg body weight. Clinical signs as well as mortality/viability were recorded before treatment, within 30 minutes and approximately 1, 2, 3 and 5 hours after treatment and then twice daily on days 2–15. Body weights were recorded on day 1 (prior to administration) and on days 8 and 15. All animals were necropsied and macroscopically examined.

The two rats at 95 mg/kg bw and one at 300 mg/kg survived until the end of the observation period. Two rats at 300 mg/kg and one at 950 mg/kg bw were killed in extremis on day 1. In animals that survived treatment, mild clinical signs such as slightly increased activity, slightly ruffled fur and slight tachypnoea were observed after treatment on day 1 and up to day 3. No clinical signs were observed in these animals after day 4. In the animals killed in extremis, marked clinical signs such as collapse, dragging of fore and hind limbs, moderately to markedly decreased activity, slightly to moderately ruffled fur, ptosis and/or clear lachrymation of both eyes, slight to marked tachypnoea, prostration, hunched posture and moderately reduced body temperature were observed prior to euthanasia. Body weight change of the surviving animals was not affected by treatment.

No macroscopic abnormalities were found in the surviving animals at necropsy. The stomach of all the animals killed in extremis on day 1 contained fluid. In addition, the animals at 950 mg/kg bw were noted as having light brown, discoloured kidneys.

The estimated LD_{50} of 4-OH in female rats was 300 (95% CI: 50.13–1220) mg/kg bw, the one dose with partial response (Sieber, 2011a).

Short-term toxicity studies

Metabolite 4-OH (purity 99.8%; lot no. 1176335) was administered daily by gavage to SPF-bred Wistar rats (n = 5/sex per group) at dose levels of 0, 10, 30 or 100 mg/kg bw per day for 28 days. A satellite group (n = 5/sex per group) received 4-OH at 0 or 100 mg/kg bw per day by gavage, followed by a 14-day recovery period. The rats in the control group were treated with polyethylene glycol 300 (PEG 300) only. Clinical signs, cage-side observations, feed consumption and body weights were recorded periodically during the acclimatization, treatment and recovery periods. FOB, locomotor activity and grip strength tests were performed in week 4. At the end of the dosing period or the treatment-free recovery period, blood samples were withdrawn for haematology and plasma chemistry analyses. Urine samples were collected for urine analysis. All animals were necropsied. Histological examinations of organs and tissues from all animals at 0 and 100 mg/kg bw, selected animals from other dose groups, and all gross lesions from all animals were performed.

All animals survived the scheduled treatment and recovery periods. No clinical signs were detected in any animal throughout treatment or recovery. In detailed clinical observations conducted weekly, no abnormalities were detected in any animal throughout the treatment or recovery periods. In FOB, no abnormalities were detected in any animal throughout the scheduled treatment or recovery periods. No differences were detected in grip strength between test item-treated groups and controls. In locomotor activity, no differences were detected between treated males and controls. Females at 100 mg/kg bw showed a slight but statistically significantly increase in locomotor activity during the first 10-minute interval (363, 385, 455 and 539 at 0, 10, 30 and 100 mg/kg bw, respectively); this was not statistically significantly increased at the remaining measuring intervals. The increase at first interval led to a statistically significantly increase in total locomotor activity (1306, 1164, 1217 and 1797 at 0, 10, 30 and 100 mg/kg bw, respectively). The data were highly variable, and this effect was not seen in males. Taken together, the increase at 100 mg/kg bw in females was considered not toxicologically significant. In feed consumption, no treatment-related effect was observed. Body weights of males at 100 mg/kg bw were slightly but not statistically significantly depressed during the treatment period. Body weight gains in males at this dose were approximately 15–20% lower, reaching statistical significance on day 15. This reduction was reversible after two weeks of recovery. The slight decrease in body weight gain was considered treatment related. In females, no difference in body weight gain was observed. In ophthalmological examination, treatment-related findings were not detected. In haematology, haemoglobin levels were slightly (within 5%) increased in males at 100 mg/kg bw and females at 30 and 100 mg/kg bw without a dose–response relationship. These changes were not considered toxicologically significant because haemoglobin levels were increased rather than decreased and there was no dose–response relationship. No treatment-related findings were seen in clinical biochemistry and urine analysis parameters. At the scheduled kill, a few statistically significant changes were observed. The change in relative testes weights in males at 100 mg/kg bw were considered not toxicologically significant because an increase rather than a decrease was seen and there were no corresponding histopathological findings. Increased relative weights of liver, kidneys and uterus in females at 100 mg/kg bw were considered not treatment related because the increases in liver and kidney weights were slight (approximately 10%) and there were no corresponding histopathology and clinical pathology findings. The increase in uterine weight was not accompanied by histopathological changes in related organs; it was thought to be most likely related to estrous cyclicity and not to treatment. None of the changes were present after two weeks of recovery. No treatment-related macroscopic or microscopic findings were detected.

The NOAEL for short-term oral toxicity in rats was 30 mg/kg bw based primarily on the slight and reversible reduction in body weight gain at 100 mg/kg bw (Sieber, 2011b).

Ames test

The potential of 4-OH (purity 99.8%; lot no. 1176335) to induce gene mutations in the plate incorporation test and the preincubation test were determined using the *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and the *Escherichia coli* strain WP2uvrA.

No substantial increase in gene mutations was seen in any of the five tester strains following treatment with 4-OH in either the presence or absence of metabolic activation (S9 mix).

In conclusion, 4-OH did not induce gene mutations by base pair changes or frameshifts in the tester strains used (Sokolowski, 2011).

Tertiary butylphenylethanol (TBPE)

TBPE is a plant metabolite of fenazaquin and its possible formation is implied due to general oxidative metabolism and the presence of metabolite AN-1. Toxicity data submitted included from an acute oral and subcutaneous toxicity study in rats, eye and skin irritation studies in rabbits, a hypersensitization study in guinea-pigs, a short-term oral toxicity in rats and genotoxicity studies in vitro and in vivo. All studies were performed in accordance with GLP.

Acute oral and subcutaneous toxicity studies in rats, along with studies of irritation to eye and skin and hypersensitization, are summarized in Table 20.

Table 20. Acute toxicity, irritation and sensitivity studies with TBPE

Route (method)	Species/ strain	Purity (%) / batch no.	Result	Reference
Oral (gavage)	CD rats	88.9 ACPR-343-2-13	LD_{50}: > 2000 mg/kg bw in both sexes	Johnson (1992a)
Dermal	CD rats	88.9 ACPR-343-2-13	LD_{50}: > 2000 mg/kg bw in both sexes	Johnson (1992b)
Skin irritation	NZW rabbits	88.9 ACPR-343-2-13	Slight irritant	Johnson (1992c)
Eye irritation	NZW rabbits	88.9 ACPR-343-2-13	Serious irritant	Johnson (1992d)
Dermal sensitization (Magnusson–Kligman maximization test)	Dunkin Hartley guinea pig	88.9 ACPR-343-2-13	Not sensitizing	Johnson (1992e)

bw: body weight; LD_{50}: median lethal dose; TBPE: tertiary butylphenylethanol

Acute toxicity

The acute oral toxicity of TBPE was investigated in CD rats (n = 5/sex per group) at 2000 mg/kg bw. The animals were fasted overnight prior to dosing. The test material was administered in maize oil at a dosing volume of 10 mL/kg. Mortality and signs of reaction to treatment were recorded over a 14-day observation period. The animals were killed and necropsied on day 15.

One male rat was found dead and another was euthanized on the first day after dosing. Antemortem signs included lethargy, hunched posture, piloerection, decreased motor activity, staggering gait, prone position, unconsciousness and slow deep respiration. Treatment-related clinical signs in the surviving animals were similar to those of the decedents with the exception of unconsciousness and slow deep respiration and with the addition of hair loss and ungroomed appearance. The surviving animals appeared normal 6 days after dosing and achieved expected body weight gains. No remarkable finding was observed at necropsy.

The oral LD_{50} was over 2000 mg/kg bw (Johnson, 1992a).

The acute dermal toxicity of TBPE was investigated in CD rats (n = 5/sex per group) by applying TBPE to the closely clipped dorsum of each animal at a dose of 2000 mg/kg bw. This was covered by an occlusive dressing for 24 hours. Systemic or local signs of reaction were recorded over a 14-day observation period. The animals were killed and necropsied on day 15.

There were no deaths. Systemic signs of reaction to treatment were irritability, decreased motor activity, ungroomed appearance, serous discharge from the eyes, pigmented staining of the snout and hunched posture, all of which were resolved in all animals by day 6 after dosing. Very slight to moderate erythema, very slight to slight oedema, eschar formation, slight or moderate exfoliation, loss of elasticity, loss of flexibility, sensitivity to touch and brown discoloration were seen at the site of application; these signs were generally more marked in females than males but had fully resolved by day 15 after dosing. Overall body weight gains were generally slightly less than expected for rats of this age and strain. Necropsy revealed no significant macroscopic lesions.

Under the conditions of this study, the acute percutaneous LD_{50} of TBPE was greater than 2000 mg/kg bw (Johnson, 1992b).

Skin irritation

Skin irritation was assessed by applying 0.5 g of TBPE to the closely clipped dorsa of New Zealand White rabbits ($n = 3$). The treatment site was then covered by a semi-occlusive dressing for 4 hours. Dermal reactions were assessed 1, 24, 48 and 72 hours and 6, 9, 12 and 15 days after the dressings were removed.

Very slight to slight erythema and oedema were observed in all rabbits during the first 6 days after bandage removal and persisted in one rabbit for up to 12 days after treatment. Exfoliation was observed in two rabbits on day 6 and in one rabbit on day 12. The test site in all rabbits appeared normal 15 days after treatment.

Although the study author classified TBPE as a dermal "non-irritant", the results indicate slight irritation to the skin of rabbits (Johnson, 1992c).

Eye irritation

The potential of TBPE to cause damage to the conjunctiva, iris and/or cornea was assessed by instilling 0.1 g TBPE into the eye of one New Zealand White rabbit. Ocular reactions were examined 1, 24, 48 and 72 hours after instillation.

The study authors reported observing a diffuse beefy-red appearance to the conjunctiva, very slight or moderate discharge, very slight or slight chemosis, stippling of the cornea, very slight or slight opacity and iritis during the first 72 hours after instillation. Areas of haemorrhage on the upper surface of the conjunctiva were also apparent at 72 hours. Instillation of the test material caused a slight initial pain response.

In the light of the severe responses observed in this sentinel rabbit, no further animals were tested. Based on these results, TBPE was classified as an eye irritant and considered to have the potential to cause serious ocular damage (Johnson, 1992d).

Sensitivity

Delayed contact hypersensitivity of TBPE in guinea-pigs was assessed using the Magnusson–Kligman maximization test. The closely clipped dorsa of Dunkin Hartley guinea-pigs ($n = 10$/sex) were intradermally injected with Freunds Complete Adjuvant, 1% w/v TBPE in paraffin oil and 1% w/v TBPE in the adjuvant on day 1. After 7 days, the same area of skin was treated with topical application of 50% w/v TBPE in paraffin oil and the test site was covered by an occlusive dressing for 48 hours. On day 22, all animals received an occluded application of paraffin oil on the left flank and 50% w/v TBPE in paraffin oil and 10% w/v TBPE in paraffin oil to two sites on the right flank. The occlusive dressings were removed on the following day and the condition of the test sites was assessed approximately 24 and 48 hours later.

Challenge application of 50% w/v TBPE in paraffin oil caused a significant response (eschar formation) in five test and two control animals. Although the incidence was slightly higher in test than control animals, the nature of the response was considered consistent with primary irritation rather than hypersensitivity. Challenge application of 10% w/v TBPE in paraffin oil caused no significant dermal response. Challenge with paraffin oil alone caused a significant response (slight erythema) in one control animal. Slight erythema over the entire flank was apparent in five other control animals, but this was considered to be a nonspecific incidental reaction.

Under the conditions of this study, repeated administration of TBPE did not cause delayed contact hypersensitivity in guinea pigs (Johnson, 1992e).

Short-term oral toxicity study

CD rats (*n* = 5/sex per group) received TBPE (purity 88.9%; batch no. ACPR-343-2-13) by gavage at dose levels of 20, 150 or 400 mg/kg bw per day for four weeks. The test material was administered in maize oil at a dosing volume of 5 mL/kg bw. Another group (*n* = 10/sex) received the vehicle alone and acted as a contemporaneous control. At the end of the treatment period, surviving animals were killed and necropsied. Selected tissues were taken from all animals and processed for microscopic examination.

The major toxic effects observed in this study are summarized in Table 21.

Three males and four females at 400 mg/kg bw per day were euthanized in extremis between days 17 and 30. These animals had severe body weight loss, staggering gait, muscle flaccidity, reduced body temperature and underactivity. Necropsy findings included emaciation, abnormal gastrointestinal contents and enlarged and/or pale kidneys. A control female died following routine blood sampling on day 29. There were no other unscheduled deaths. Treatment-related clinical signs in the surviving rats at 400 mg/kg bw per day were essentially the same as those observed in decedents, but were less severe. Treatment-related clinical signs at 150 mg/kg bw per day included salivation at dosing and occasional underactivity and hunched posture. There were no signs of reaction to treatment at 20 mg/kg per day. Body weight gain, feed consumption and feed utilization efficiency at 400 mg/kg bw per day were less than those of controls, particularly from treatment week 2. Water consumption at 150 or 400 mg/kg per day was greater than that of control rats. PCV, haemoglobin concentrations and erythrocyte counts in rats at 400 mg/kg bw per day and in female rats at 150 mg/kg bw per day were generally slightly lower than those of control animals. Haematological parameters at 20 mg/kg bw per day were comparable to those in controls. In blood biochemistry, plasma ALP activities of male rats at 20 mg/kg bw and above and female rats at 150 mg/kg bw per day were higher than those of control animals. The ALP change at 20 mg/kg bw per day was not considered biologically significant because the mean value fell within the testing laboratory's historical control range and there was no corresponding hepatotoxicity at this dose. ALT activities of males at 400 mg/kg bw were increased. In urine analysis, the urinary volume of rats at 150 or 400 mg/kg bw per day was higher and the urinary specific gravity lower than those of control animals. Ketones were observed in the urine of male rats at 150 mg/kg bw per day and above and in female rats at 400 mg/kg bw per day. Fewer crystals were observed in the urinary deposit of rats treated at 150 or 400 mg/kg per day. Urine analysis at 20 mg/kg bw per day was unaffected by the treatment.

Liver weights were increased in males at 150 mg/kg bw per day and above, and in females at 20 mg/kg bw per day and above. The increased liver weight seen at the 20 mg/kg bw dose was considered an adaptive change. Testicular weights at 400 mg/kg bw per day were decreased. Relative kidney weights at 150 and 400 mg/kg per day were slightly increased. One male at 400 mg/kg bw per day had small testes and all surviving animals at this dose were emaciated at necropsy. Microscopic examination showed that the kidneys of all animals (surviving and euthanized in extremis) at 400 mg/kg bw per day and of two males at 150 mg/kg bw per day had papillary necrosis, amorphous deposits in tubules and Bowman's spaces, dilated tubules and tubular vacuolation and/or degeneration with dose dependency. The livers in both sexes at 400 mg/kg bw per day and in males at 150 mg/kg bw per day showed dose-dependent microvesicular vacuolation. The testes of all males at 400 mg/kg bw per day showed moderate to marked bilateral degeneration of the tubular germinal epithelium. Minimal spermatid giant cells were observed in the testes at 400 mg/kg bw per day. One male at 150 mg/kg bw per day showed a minimal bilateral degeneration of the tubular germinal epithelium. Intensity of those changes in liver, kidney and liver appeared to be more severe in rats euthanized in extremis than those that survived to scheduled kill. Although the possibility of secondary effect of severe body weight reduction on maturation of spermatogenesis was suggested, the possibility of direct damage to the testes could not be eliminated. The profiles of testicular toxicity suggested no damage to spermatogonia. The described changes in the kidney, testes and liver were not observed in animals at 20 mg/kg bw per day.

The NOAEL for short-term oral toxicity of TBPE in rats was 20 mg/kg bw based on adverse effects on the kidney, testes and liver in males at 150 mg/kg bw per day (Johnson, 1992f).

Table 21. Effects of TBPE in a four-week gavage study in rats

Parameter	Measures per dose [a]							
	Males				Females			
	0 mg/kg bw	20 mg/kg bw	150 mg/kg bw	400 mg/kg bw	0 mg/kg bw	20 mg/kg bw	150 mg/kg bw	400 mg/kg bw
No. examined (no. killed in extremis)	10	5	5	5 (3)	10 (1)	5	5	5 (4)
Final body weight (g)	346	332	335	241	233	235	214	190
Body weight gain (g)	197	186	185	94***	93	91	83	49***
ALP (IU/L)	220	321*	351**	387**	133	141	207***	321
ALT (IU/L)	32	38	46	113***	31	32	28	48
Urea (mg %)	25	24	32**	50***	33	26	29	29
Creatinine (mg %)	0.4	0.4	0.4	0.5*	0.5	0.5	0.4	0.3
Kidney papillary necrosis	0	0	1	2 (2)	0 (0)	0	0	1 (4)
Minimum	–	–	1	1 (1)	–	–	–	–
Slight	–	–	–	1	–	–	–	1
Moderate	–	–	–	(1)	–	–	–	(3)
Marked/severe	–	–	–	–	–	–	–	(1)
Kidney tubular dilatation	0	0	2	2 (3)	0 (0)	0	0	0 (4)
Minimum	–	–	1	1 (1)	–	–	–	–
Slight	–	–	1	–	–	–	–	–
Moderate	–	–	–	1 (1)	–	–	–	(1)
Marked/severe	–	–	–	(1)	–	–	–	(3)
Kidney vacuolation/ degeneration of tubules	0	0	2	1 (1)	0 (0)	0	0	1 (4)
Minimum	–	–	2	1 (1)	–	–	–	–
Slight	–	–	–	–	–	–	–	1 (2)
Moderate	–	–	–	–	–	–	–	(2)
Testes seminiferous tubule degeneration	0	0	1	2 (3)	–	–	–	–
Minimum	–	–	1	–	–	–	–	–
Moderate	–	–	–	1 (1)	–	–	–	–
Marked/severe	–	–	–	1 (2)	–	–	–	–
Giant cells of spermatid	0	0	0	1 (3)	–	–	–	–
Minimum	–	–	–	1 (3)	–	–	–	–
Liver microvesicular vacuolation	0	0	2	0 (3)	0 (0)	0	0	1 (4)
Slight	–	–	2	–	–	–	–	1
Moderate	–	–	–	1	–	–	–	–
Marked/severe	–	–	–	2	–	–	–	4

ALP: alkaline phosphatase; ALT: alanine aminotransferase; IU: International Units; TBPE: tertiary butylphenylethanol; no.: number; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, statistically significantly different from controls; –: not examined

[a] Values in parentheses are for animals killed in extremis.

Source: Johnson (1992f)

Genotoxicity: Ames test

TBPE (purity 88.9%; batch no. ACPR-343-2-13) was examined for mutagenic activity in four histidine-dependent autotrophs of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537, using pour plate assays. The procedures used comply with the OECD Test Guideline 471 (issued 1983) and Annex V of the sixth amendment (79/831/EEC) to the European Community Directive 67/548/EEC (Method B.14). Each test, in each strain, was conducted on two separate occasions. The studies, which were conducted in the presence or absence of a metabolic activation system derived from rat liver (S9 mix), tested a range of levels of 2-(4-*tert*-butylphenyl)ethanol, from 25 to 2500 µg per plate. The levels were selected following a preliminary toxicity test in strain TA98. All tests included solvent (dimethyl sulfoxide) controls with and without the S9 mix.

No increases in reversion to prototrophy were obtained with any of the four bacterial strains at the 2-(4-*tert*-butylphenyl)ethanol levels tested, either in the presence or absence of the S9 mix. Inhibition of growth, observed as a reduction in the number of revertant colonies, occurred in all strains following exposure to TBPE at 2500 µg per plate. Slight precipitation was seen on plates containing 2500 µg TPBE.

Marked increases in the number of revertant colonies were induced by the known mutagens benzo[a]pyrene, 2-nitrofluorene, 2-aminoanthracene, 9-aminoacridine and sodium azide when examined under similar conditions.

It was concluded that TBPE was devoid of mutagenic activity under the conditions of the test (May, 1992).

Genotoxicity: Micronucleus test

The effects of TBPE (purity 88.9%; batch no. ACPR-343–2-13) on chromosome structure in bone marrow cells was investigated following acute oral administration to mice. Chromosome damage was measured indirectly by counting micronuclei.

A preliminary toxicity test was conducted, using doses of 312.5, 625, 1250 or 2500 mg/kg bw. Two of four mice at 2500 mg/kg and two mice of four at 1250 mg/kg bw were euthanized in extremis. Subsequently, male and female mice were given a single dose of TBPE at 40, 200 or 1000 mg/kg bw. In all cases, TBPE was mixed with maize oil. Concurrent vehicle and positive control groups were similarly dosed with maize oil or chlorambucil (30 mg/kg bw), respectively. Subgroup (n = 5/sex per group) were killed 24 hours after treatment; further lots (n = 5/sex) given TBPE at 1000 mg/kg bw or the vehicle control were killed 48 and 72 hours after treatment. Bone marrow smears from each animal were made on glass slides, which were stained and prepared for examination. Using a light microscope, at least 2000 erythrocytes per animal were examined for the presence of micronuclei. Calculated values of micronuclei per 1000 polychromatic erythrocytes were analysed statistically using the Mann-Whitney U test. The ratio of polychromatic (newly formed): normochromatic (mature) cells was also calculated for each animal to assess bone marrow toxicity.

No real indication of bone marrow toxicity, as evidenced by depression of bone marrow proliferation, was noted in any group treated with TBPE. In addition, no adverse reactions were recorded for any animal treated with TBPE at 40 and 200 mg/kg bw. However, at 1000 mg/kg bw, adverse reactions to treatment included hunched posture (29/30 animals), underactivity (16/30), piloerection (9/30), slow respiration (3/30) and prone posture (3/30). The three females showing slow respiration and prone posture were killed in extremis approximately 5.5 hours after dosing. Frequencies of micronucleated polychromatic erythrocytes in animals killed 24, 48 or 72 hours after administration of 2-(4-*tert*-butylphenyl)ethanol were similar to those in concurrent vehicle controls. This lack of treatment-related effect was apparent in both sexes and was confirmed by statistical analysis. Statistically significant increases over control values were, however, seen in positive control group animals given chlorambucil at 30 mg/kg bw ($P < 0.01$).

There was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hours after oral administration of TBPE. The test procedure was highly sensitive to the chromosome-damaging action of chlorambucil (Edwards, 1992).

TBPE was not genotoxic in vitro and in vivo.

Based on the available data and comparisons of toxicity with the parent compound (see Table 22), the Meeting concluded that TBPE was unlikely to be of greater toxicity than fenazaquin. The Meeting concluded that the acceptable daily intake (ADI) and acute reference dose (ARfD) of fenazaquin can be applied to TBPE (see Table 22).

Table 22. Comparison of toxicity profiles and severity of fenazaquin and TBPE [a]

Parameters	Fenazaquin	TBPE
LD_{50} (mg/kg bw)	134 for males, 138 for females At 500 mg/kg bw, all males and females were dead, mostly within 5 hours	> 2000 mg/kg bw
4-week study, rats		NOAEL = 20 mg/kg bw, LOAEL = 150 mg/kg bw At 400 mg/kg bw, 3/5 males and 4/5 females died Kidney: papillary necrosis (1/5, minimum); tubular dilatation: (2/5, minimum/slight); tubular degeneration (2/5 minimum) Testis: Tubular degeneration (1/5, minimum) Liver: Microvesiculation (2/5, slight) Severe renal/hepato/testicular toxicity, and decreased body weight at 400 mg/kg bw (Johnson, 1992f)
13-week study, rats	NOAEL = 10 mg/kg bw, LOAEL = 30 mg/kg bw bw, FI↓	–
13-week study, rats, 2	NOAEL = 9.7 mg/kg bw, LOAEL = 28.7 mg/kg bw bw, FI↓	–
Genotoxicity	Negative in vitro/in vivo	Negative in vitro/in vivo
ADI	4.5 mg/kg bw (lowest NOAEL in 2-year rat study; safety factor of 100	If ADI should be established, based on NOAEL of 20 mg/kg bw in 4-week rat study; SF; 100

ADI: acceptable daily intake; bw: body weight; LD_{50}: median lethal dose; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; SF; safety factor; TBPE: tertiary butylphenylethanol

[a] Highest exposure: 0.0046 µg/kg bw per day.

Fenazaquin dimer

The predominant residues in plants were fenazaquin and its dimer, a photolysis product. No toxicity study on the dimer was submitted, however, the dimer is likely to have limited bioavailability after ingestion due to its relatively high molecular weight (molecular weight of fenazaquin, 306.4) and lipophilic properties.

3. Observations in humans

There was no information about adverse health effects in manufacturing plant personnel. There were no clinical cases or poisoning incidents reporting the results of accidental exposure to fenazaquin or on the effects due to exposure on the general population.

A search of the open literature on effects of fenezaquin on human health or toxicity was conducted in PubMed. Although a few references of articles on fenezaquin were retrieved, none were appropriate for inclusion in the toxicological evaluation.

Data from the mammalian toxicity and metabolism studies together with the low residue levels, which have been reported to be present on treated produce, indicate that adverse effects following exposure under normal agricultural or horticultural use or through consumption of treated crops are unlikely to occur.

Comments

Biochemical aspects

In studies conducted using [^{14}C]fenazaquin, C_{max} was reached in plasma at 8 hours after a single oral dose of 1, 10 or 30 mg/kg bw to rats. The absorption in rats was estimated to be 60% of a 1 mg/kg bw dose. Fenazaquin and/or its metabolites did not accumulate in tissues. The major route of excretion was via faeces (71.9–88.9% after seven days). Excretion in the urine ranged from 16.4% to 20.9%. The majority of fenazaquin was excreted within 48 hours after dosing.

The biotransformation of fenazaquin in the rat was predominantly by cleavage of the ether bond, resulting in the formation of the respective alcohol (4-hydroxyquinazoline or 4-OH) and carboxyl acid (AN-1) derivatives. Other biotransformation reactions included oxidation of one of the methyl groups on the alkyl side chain to produce either alcohol (F-1) or carboxylic acid (F-2) metabolites. Finally, hydroxylation at the *O*-ether alkyl moiety of F-1 or F-2 resulted in the metabolites F-1A and 2-hydroxyfenazaquin acid (F-3), respectively. Major metabolites were AN-1, NN-2 complex and NN-3 complex in urine and F-2 and F-3 in faeces (> 7% for all metabolites) (Althaus & Magnussen, 1992).

Fenazaquin was more rapidly absorbed in mice and hamsters than in rats and more rapidly excreted by mice than by rats and hamsters. The half-life in mice was about one-tenth to one-twentieth of that in rats and hamsters, respectively.

Toxicological data

The oral LD_{50} of fenazaquin was 134 mg/kg bw in rats (Wright, Davis & Francis, 1992a), 1480 mg/kg bw in mice (Wright, Davis & Francis, 1992b) and 812 mg/kg bw in hamsters (Wright, Davis & Francis, 1992c). The dermal LD_{50} in rabbits was more than 5000 mg/kg bw (Francis, Rock & Clair, 1989a). The LC_{50} in rats was greater than 1.9 mg/L (Francis, Herman & Wolff, 1990). Fenazaquin was not irritating in rabbit skin and eyes (Francis, Rock & Clair, 1989a,b). Fenazaquin was not sensitizing in guinea pigs (Jones, 1994).

The main toxic effects of fenazaquin in short- and long-term toxicity studies in hamsters, rats and dogs were decreased body weight gain and feed consumption. Testicular toxicity was observed in hamsters.

In a 90-day oral toxicity study in hamsters, fenazaquin was administered by gavage at doses of 0, 5, 25, 50 (females only), 75 (males only), 100 (females only) or 150 (males only) mg/kg bw per day. The NOAEL was 25 mg/kg bw per day based on lower body weights and body weight gains at 50 mg/kg bw per day (Francis, Boss & Gries, 1992a).

Two 90-day oral toxicity studies were conducted in rats. In the first, fenazaquin was administered by gavage to rats at doses of 0, 1, 3, 10 or 30 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day based on decreased body weight, body weight gain and feed consumption at 30 mg/kg bw per day (Francis, Ward & Gries, 1992).

In the second 90-day study in rats, fenazaquin was administered in the diet at 0, 15, 45, 150 or 450 ppm (equal to 0, 1.0, 3.0, 9.6 and 28.7 mg/kg bw per day for males and 0, 1.2, 3.5, 11.5 and 33.0 mg/kg bw per day for females, respectively). The NOAEL was 150 ppm (equal to 9.6 mg/kg bw per day) based on decreased body weight, body weight gain and feed consumption at 450 ppm (equal to 28.7 mg/kg bw per day) (Cocke, Francis & Gries, 1992).

In a 90-day oral toxicity study in dogs, fenazaquin was administered in the diet at 0, 1, 5 and 15 mg/kg bw per day. The NOAEL was 5 mg/kg bw based on decreased body weights and feed consumption at 15 mg/kg bw (Cosse et al., 1992).

In a 12-month oral toxicity study in dogs, fenazaquin was administered by gavage at 0, 1, 5 and 12 mg/kg bw per day. The NOAEL was 5 mg/kg bw per day based on decreased body weight gain and feed consumption at 12 mg/kg bw (Cosse et al., 1993).

The overall NOAEL for oral toxicity in dogs was 5 mg/kg bw per day.

In a 78-week carcinogenicity study in hamsters, fenazaquin was administered by gavage at 0, 2, 15, 30 (for males) and 35 mg/kg bw per day (for females). The Meeting considered the data on females insufficiently reliable to assess toxicity and carcinogenicity because of the high mortality of controls. The study was also considered confounded due to amyloidosis and the long-term use of vancomycin hydrochloride to treat a serious *C. difficile* infection (McConell et al., 1983; Annex 1, reference 132). The NOAEL for systemic toxicity in male hamsters was 2 mg/kg bw per day based on lower body weights and decreased body weight gain at 15 mg/kg bw per day. The NOAEL for carcinogenicity in male hamsters was 30 mg/kg bw per day, the highest dose tested (Francis, Boss & Gries, 1992b).

In a combined chronic toxicity and carcinogenicity study in rats, fenazaquin was administered in the diet at 0, 10, 100, 200 or 400 (males) / 450 (females) ppm (equal to 0, 0.46, 4.5, 9.2 and 18.3 mg/kg bw per day for males and 0, 0.57, 5.7, 11.5 and 25.9 mg/kg bw per day for females, respectively) for 24 months. The NOAEL for long-term toxicity was 100 ppm (equal to 4.5 mg/kg bw per day) based on significantly decreased body weight gain at 200 ppm (equal to 9.2 mg/kg bw per day). The Meeting considered the changes in hepatocellular atypia (currently termed basophilic altered focus) were of uncertain significance as the incidence of this lesion is highly variable in this strain and this lesion was not accompanied by any other pathological changes in the rat studies.

The NOAEL for carcinogenicity in rats was 18.3 mg/kg bw per day, the highest dose tested (Cocke et al., 1992).

The Meeting concluded that fenazaquin is not carcinogenic in rats and male hamsters.

Fenazaquin was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave negative or equivocal responses in most of the in vitro studies and was negative in the in vivo studies (Francis, Garriott & Kindig, 1989; Francis, Garriott & Yount, 1989; Francis, Parton & Garriott, 1989; Francis, Richardson & Michaelis, 1989; Francis, Scheuring & Richardson, 1989; Proudlock, 1993; Jenkinson & Wright, 1995; Lovell, 1995; Beevers, 2007).

The Meeting concluded that fenazaquin is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and the absence of carcinogenicity in rats and male hamsters, the Meeting concluded that fenazaquin is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats, fenazaquin was administered by gavage at 0, 1, 5 or 25 mg/kg bw per day. The NOAEL for parental toxicity was 5 mg/kg bw per day based on decreased body weight gain and body weights and clinical signs at 25 mg/kg bw per day. The NOAEL for effects on reproduction and on offspring was 25 mg/kg bw per day, the highest dose tested (Christian, Hoar & Hoberman, 1991).

In a developmental toxicity study in rats, fenazaquin was administered by gavage at 0, 3, 10 and 40 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day based on decreased body weight gain and feed consumption at 40 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 40 mg/kg bw per day, the highest dose tested (Francis & Higdon, 1989). There was no evidence of teratogenicity.

In a developmental toxicity study in rabbits, fenazaquin was administered by gavage at 0, 3, 13 and 60 mg/kg bw per day. The NOAEL for maternal toxicity was 13 mg/kg bw per day based on lower feed consumption on gestation days 6–12 at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 60 mg/kg bw per day, the highest dose tested (Francis & Higdon, 1990). There was no evidence of teratogenicity.

The Meeting concluded that fenazaquin is not teratogenic.

In an acute neurotoxicity study in rats, a single dose of fenazaquin was administered by gavage at 0 (control), 20, 65 or 130 mg/kg bw in males and 0, 20, 60 and 120 mg/kg bw in females. The NOAEL for acute neurotoxicity in rats was 120 mg/kg bw, the highest dose tested. The NOAEL for systemic toxicity was 20 mg/kg bw based on decreased body weight and feed consumption at 60 mg/kg bw (Barnett, 2012).

The Meeting concluded that fenazaquin is not neurotoxic.

In an immunotoxicity study in rats, fenazaquin was administered by gavage at 0, 15, 30 or 37.5/45 (from day 8) mg/kg bw per day for 28 days. The NOAEL for immunotoxicity in this rat study was 37.5 mg/kg bw, the highest dose tested (Curry, 2011).

The Meeting concluded that fenazaquin is not immunotoxic.

Toxicological data on metabolites and/or degradates

4-Hydroxyquinazoline (4-OH)

4-OH is a minor metabolite in rats and a plant metabolite. Oral LD_{50} of 4-OH in rats was 300 mg/kg bw (Sieber, 2011a).

In a four-week oral toxicity study in rats administered 4-OH at 0, 10, 30 and 100 mg/kg bw by gavage, the NOAEL was 30 mg/kg bw per day based on slight reductions in body weight gains at 100 mg/kg bw per day (Sieber, 2011b).

4-OH was negative in an in vitro bacterial reverse mutation assay.

Based on the available data and comparisons of toxicity with the parent fenazaquin, the Meeting concluded that 4-OH was unlikely to be of greater toxicity than fenazaquin. The Meeting concluded that the ADI and ARfD of fenazaquin can be applied to 4-OH.

Tertiary butylphenylethanol (TBPE)

The oral and dermal LD_{50} of TBPE, a plant metabolite, was greater than 2000 mg/kg bw in rats (Johnson, 1992a). TBPE was a slightly irritating to the skin (Johnson, 1992c) and severely irritating to the eye of rabbits (Johnson, 1992d). TPBE was not sensitizing in the skin of guinea-pigs (Johnson, 1992e).

In a four-week oral toxicity study in rats administered TBPE at 0, 20, 150 and 400 mg/kg bw per day, the NOAEL was 20 mg/kg bw per day based on toxic effects on the kidney, testis and liver at 150 mg/kg bw per day (Johnson, 1992f).

TBPE was not genotoxic in vitro and in vivo (Edwards, 1992; May, 1992).

Based on the available data and comparisons of toxicity with the parent compound, the Meeting concluded that TBPE was unlikely to be of greater toxicity than fenazaquin. The Meeting concluded that the ADI and ARfD of fenazaquin can be applied to TBPE.

Fenazaquin dimer

The Meeting concluded that a fenazaquin dimer, a photoproduct, was unlikely to be of toxicological concern given its higher molecular weight leading to expected low absorption.

2-Hydroxyfenazaquin acid (F-3)

Although no toxicity studies on the livestock metabolite F-3 were submitted, it is present as a major metabolite in rat faeces. It is therefore covered by studies of the parent compound, fenazaquin.

2-Oxy-fenazaquin

Although no toxicity studies on 2-oxy-fenazaquin, a plant and soil metabolite, were submitted, oxidative metabolism and the presence of F-3, a faecal metabolite in rats, suggests the formation of 2-oxy-fenazaquin in the rat. The toxicity of 2-oxy-fenazaquin should therefore be covered by studies of the parent compound, fenazaquin.

Human data

There were no reports of fenazaquin-associated adverse effects in manufacturing plant personnel.

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

Toxicological evaluation

The Meeting established an ADI for fenazaquin of 0–0.05 mg/kg bw based on the NOAEL of 4.5 mg/kg bw per day for decreased body weight gains in males at 9.2 mg/kg bw per day in a combined chronic toxicity and carcinogenicity study in rats. A safety factor of 100 was applied.

This ADI was supported by the overall NOAEL of oral toxicity studies in dogs and the NOAEL of the two-generation reproductive toxicity study in rats.

The lowest NOAEL in the database was 2 mg/kg bw per day for decreased body weight gains at 15 mg/kg bw per day in male hamsters in an 18-month carcinogenicity study. The Meeting noted that the lower LOAEL of 9.2 mg/kg bw per day in the database was obtained from the long-term study in rats, the species most sensitive to fenazaquin toxicity. The Meeting concluded the long-term study in rats was more appropriate for establishing the ADI for fenazaquin.

The Meeting established an ARfD for fenazaquin of 0.1 mg/kg bw based on the NOAEL of 10 mg/kg bw per day for decreased body weight gains at 40 mg/kg bw per day early in a rat developmental toxicity study. A safety factor of 100 was applied.

The Meeting concluded that the ADI and ARfD for fenazaquin could be applied to TBPE and 4-OH.

Levels relevant to risk assessment of fenazaquin

Species	Study	Effect	NOAEL	LOAEL
Hamster	Eighteen-month study of toxicity and carcinogenicity [a]	Toxicity	2 mg/kg bw per day (males only)	15 mg/kg bw per day
		Carcinogenicity	30 mg/kg bw per day [b] (males only)	–
Rat	Two-year study of toxicity and carcinogenicity [c]	Toxicity	100 ppm, equal to 4.5 mg/kg bw per day	200 ppm, equal to 9.2 mg/kg bw per day
		Carcinogenicity	400 ppm, equal to 18.3 mg/kg bw per day [b]	–
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	25 mg/kg bw per day [b]	–
		Parental toxicity	5 mg/kg bw per day	25 mg/kg bw per day
		Offspring toxicity	25 mg/kg bw per day [b]	–
	Developmental toxicity study [a]	Maternal toxicity	10 mg/kg bw per day	40 mg/kg bw per day
		Embryo/fetal toxicity	40 mg/kg bw per day [b]	–
	Acute neurotoxicity study [a]	Neurotoxicity	120 mg/kg bw per day [b]	–
Rabbit	Developmental toxicity study [a]	Maternal toxicity	13 mg/kg bw per day	60 mg/kg bw per day
		Embryo/fetal toxicity	60 mg/kg bw per day [b]	–
Dog [e]	Ninety-day and 1-year studies of toxicity [c,d]	Toxicity	5 mg/kg bw per day	12 mg/kg bw per day

[a] Gavage administration. [b] Highest dose tested. [c] Dietary administration. [d] Two or more studies combined.

Estimate of acceptable daily intake (ADI; applies to fenazaquin, TBPE and 4-OH)

0–0.05 mg/kg bw

Estimate of acute reference dose (ARfD; applies to fenazaquin, TBPE and 4-OH)

0.1 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 fenazaquin

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	60% absorbed; T_{max} within 8 h at low dose
Dermal absorption	< 1%
Distribution	Widely distributed
Potential for accumulation	No indication of accumulation in tissues
Rate and extent of excretion	Rapidly excreted (> 90% within 48 h at low dose)
Metabolism in animals	Oxidation + hydroxylation
Toxicologically significant compounds in animals and plants	Fenazaquin, TBPE, 4-OH
Acute toxicity	
Rat, LD_{50}, oral	134 mg/kg bw
Rabbit, LD_{50}, dermal	> 5000 mg/kg bw [a]
Rat, LC_{50}, inhalation	> 1.9 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Guinea pig, dermal sensitization	Non-sensitizing (modified Buehler and Magnusson–Kligman)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight gain (hamster, rat, dog)
Lowest relevant oral NOAEL	5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rabbit) [a]
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased body weight gain (rat)
Lowest relevant NOAEL	4.5 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in hamster (male only) or rat [b]
Genotoxicity	No evidence of genotoxicity in vivo [b]
Reproductive toxicity	
Target/critical effect	Body weight, clinical signs (rat)
Lowest relevant parental NOAEL	5 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	25 mg/kg bw per day (rat) [a]
Lowest relevant reproductive NOAEL	25 mg/kg bw per day (rat) [a]
Developmental toxicity	
Target/critical effect	Body weight effect (rat)
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	40 mg/kg bw per day (rat) [a]

Neurotoxicity	
Acute neurotoxicity NOAEL	120 mg/kg bw (rat) [a]
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	37.5 mg/kg bw per day (rat) [a]
Studies on toxicologically relevant metabolites	
4-OH	Oral LD_{50} = 300 mg/kg bw (rat) Four-week oral toxicity study NOAEL: 30 mg/kg bw based on effects on body weights at 100 mg/kg bw per day (rat) No genotoxicity in vitro
TBPE	Oral LD_{50}: > 2000 mg/kg bw (rat) [a] Severely irritating to eye Four-week oral toxicity study NOAEL: 20 mg/kg bw based on effects on kidney and liver at 150 mg/kg bw per day (rat) No genotoxicity in vitro and in vivo
Human data	–

[a] Highest dose tested.

[b] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI [a]	0–0.05 mg/kg bw	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD [a]	0.1 mg/kg bw	Developmental toxicity study (rat)	100

[a] Applies to fenazaquin, TBPE and 4-OH, expressed as fenazaquin.

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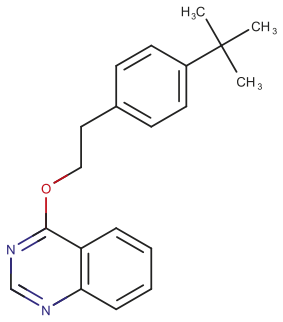
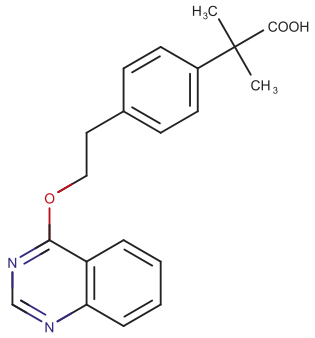
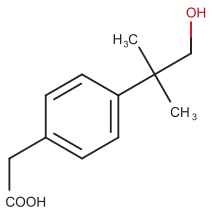
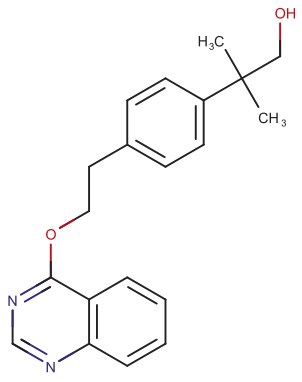
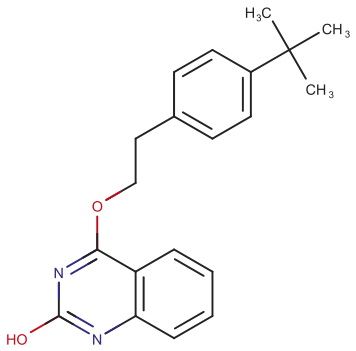
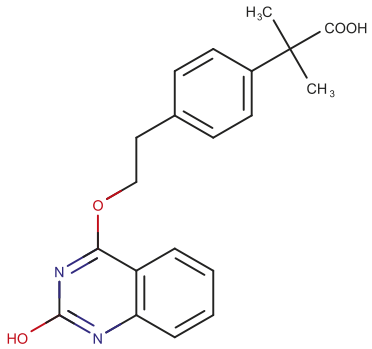
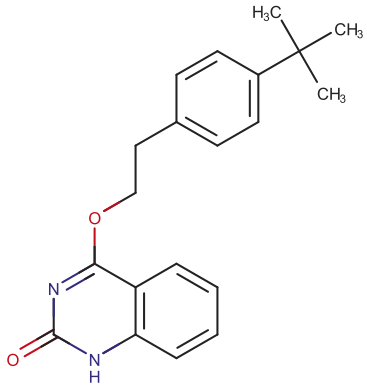
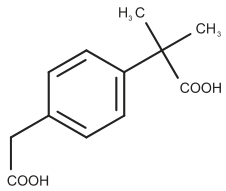
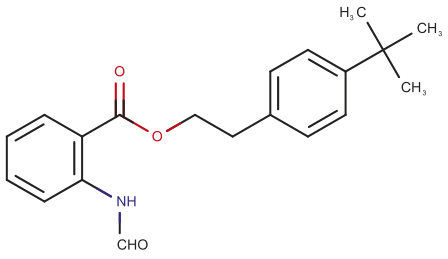
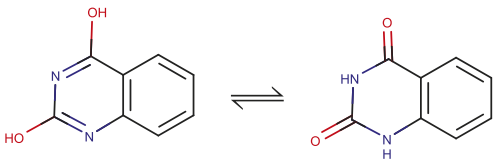
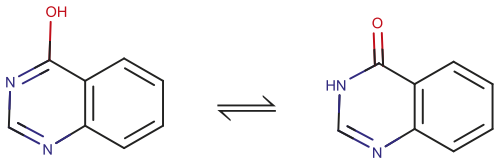
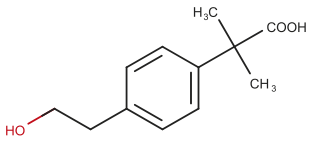
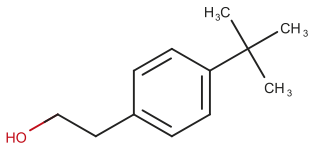
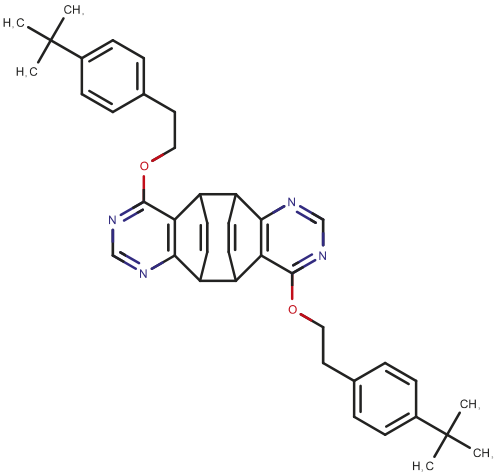
Appendix 1: List of metabolites and degradates of fenazaquin

List of metabolites and degradates of fenazaquin			
Common name	Chemical name	Chemical structure	Occurrence in
Fenazaquin	4-[2-(4-tert-Butyl-phenyl)-ethoxy]-quinazoline		Apples, oranges, grapes, maize, rat, goats, hens, soil
Fenazaquin acid (Rat metabolite in feces, F-2)	2-methyl-2-{4-[2-(quinazolin-4-yloxy)ethyl]phenyl}propanoic acid		Maize, grapes, rat, hen, soil
Metabolite B (Rat metabolite in urine, AN-1)	2-[4-(1-hydroxy-2-methylpropan-2-yl)phenyl]acetic acid		Rat
Metabolite C (Rat metabolite in feces, F-1)	2-Methyl-2-{4-[2-(quinazolin-4-yloxy)-ethyl]-phenyl}-propan-1-ol		Apples, grapes, rat

2-Hydroxy-fenazaquin	4-[2-(4-tert-butylphenyl)ethoxy]quinazolin-2-ol/ Exists in tautomeric equilibrium with 2-oxy-fenazaquin		Maize, goats, soil
2-Hydroxy-fenazaquin acid (Rat metabolite in feces, F-3)	2-(4-{2-[(2-hydroxyquinazolin-4-yl)oxy]ethyl}phenyl)-2-methylpropanoic acid		Apples, oranges, goats, rats
2-Oxy-fenazaquin	4-[2-(4-tert-butylphenyl)ethoxy]-1,2-Dihydroquinazolin-2-one Exists in tautomeric equilibrium with 2-hydroxy-fenazaquin		Maize, soil
Metabolite G	2-[4-(carboxymethyl)phenyl]-2-methylpropanoic acid		Goats, soil
Metabolite H	2-(4-tert-butylphenyl)ethyl 2-formamidobenzoate		Grapes, soil

Dihydroxyquinazoline	Equilibrium exists between the two tautomeric forms: quinazoline-2,4-diol and 1,2,3,4-tetrahydroquinazoline-2,4-dione		Apples, grapes
4-Hydroxyquinazoline (Rat metabolite in urine, 4-OH)	Equilibrium exists between the two tautomeric forms: quinazoline-4-ol and 3,4-dihydroquinazolin-4-one		Apples, grapes, maize, rats, goats, soil
Metabolite K	2-[4-(2-hydroxyethyl)phenyl]-2-methylpropanoic acid		Grapes
Tertiarybutylphenylethanol (TBPE)	2-(4-tert-butylphenyl)ethan-1-ol		Apples, grapes, maize, soil
Fenazaquin Dimer	7,15-bis[2- (4- tert-butylphenyl)ethoxy]-4,6,12,14-tetraazapentacyclo[8.6.2.2^{2,9}.0^{3,8}.0^{11,16}]icosa-3(8),4,6,11(16),12,14,17,19-octaene		Apples

FENPYRAZAMINE

First draft prepared by
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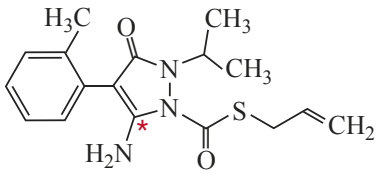
Explanation

Fenpyrazamine is the International Organization for Standardization–approved common name for allyl 5-amino-2,3-dihydro-2-isopropyl-3-oxo-4-(*o*-tolyl)pyrazole-1-carbothioate (International Union of Pure and Applied Chemistry [IUPAC] name), with the Chemical Abstracts Service number 473798-59-3. Fenpyrazamine is a fungicide whose mode of action is by inhibition of 3-ketoreductase in the ergosterol biosynthesis pathway.

Fenpyrazamine (Fig. 1) 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).

Figure 1. Structure of fenpyrazamine with radiolabel positions for the metabolism studies



* Indicates position of radiolabel

Source: Redrawn from Dohn (2007)

Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion, as well as the toxicokinetics, of fenpyrazamine were investigated in Wistar rats. Summaries of the relevant data are presented below.

The metabolism of fenpyrazamine was investigated using fenpyrazamine labelled at the 5-pyrazolyl position (Fig. 1). The test item was a mixture of ^{14}C-labelled and unlabelled fenpyrazamine. Repeated-dose metabolism studies were performed with the same mixture of ^{14}C-labelled and unlabelled fenpyrazamine over the multiple days of administration. Radiolabelled fenpyrazamine was administered via oral gavage in 0.5% aqueous methylcellulose. The study designs are summarized in Table 1.

Table 1. Dosing groups for metabolism experiments with [pyrazolyl-5-^{14}C]fenpyrazamine in rats

Test	Dose of labelled material (mg/kg bw per day)	No. of rats/ sex	Remarks	Reference
Pharmacokinetics of [pyrazolyl-5-^{14}C] fenpyrazamine	3	8	A single analytical dose at 3.06 ± 0.15 mg/kg bw by oral gavage. Blood sampling via cannulae at 10 time points over 120 hours. Killed at 120 hours.	Dohn (2007)
Pharmacokinetics of [pyrazolyl-5-^{14}C] fenpyrazamine	300	8	A single analytical dose at 300.0 ± 7.3 mg/kg bw by oral gavage. Blood sampling via cannulae at 10 time points over 120 hours. Killed at 120 hours.	Dohn (2007)
Tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	3	12	A single analytical dose of [pyrazolyl-5-^{14}C] fenpyrazamine at 2.99 ± 0.12 mg/kg bw by oral gavage. Subgroups of 3 rats/sex were killed at 0.25, 1, 4 and 12 hours.	Quistad & Kovatchev (2007a)
Tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	300	12	A single analytical dose at 306 ± 9 mg/kg bw by oral gavage. Subgroups of 3 rats/sex were killed at 0.5, 6, 24 and 72 hours.	Quistad & Kovatchev (2007a)
Metabolism and excretion of [pyrazolyl-5-^{14}C] fenpyrazamine	3	4	A single analytical dose at 3.12 ± 0.15 or 3.18 ± 0.05 mg/kg bw in males and females, respectively, by oral gavage. Killed at 7 days.	Dohn, Kavatchev & Estigoy (2007)

(*Continued on next page*)

Test	Dose of labelled material (mg/kg bw per day)	No. of rats/ sex	Remarks	Reference
Metabolism and excretion of [pyrazolyl-5-^{14}C] fenpyrazamine	300	4	A single analytical dose at 273.8 ± 11.4 or 282.6 ± 6.8 mg/kg bw in males and females, respectively, by oral gavage. Killed at 7 days. Due to a failure in urine collection, 6/8 of the high-dose animals (4 males, 2 females) were used only for tissue distribution determination (the data on the dose suspension indicated that the animals were properly dosed). An additional group of 6 animals (4 males, 2 females) was used to obtain the correct number of high-dose samples for material balance determination (excretion).	Dohn, Kavatchev & Estigoy (2007)
Metabolism, excretion and tissue distribution of single dose of [pyrazolyl-5-^{14}C] fenpyrazamine	3 × 1	3	A single analytical dose at 3.46 or 3.67 mg/kg bw in males and females, respectively, by oral gavage, to compare against repeated-dose groups. Killed 24 hours following dose.	Quistad & Kovatchev (2007b)
Metabolism, excretion and tissue distribution – [pyrazolyl-5-^{14}C] fenpyrazamine	Repeated-dose administration 3 × 6	3	Six daily analytical doses at 3.12 ± 0.22 or 3.20 ± 0.25 mg/kg bw in males and females, respectively, by oral gavage. Killed 24 hours following final dose.	Quistad & Kovatchev (2007b)
Metabolism, excretion and tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	Repeated-dose administration 3 × 10	3	Ten daily analytical doses at 3.07 ± 0.17 or 3.19 ± 0.15 mg/kg bw in males and females, respectively, by oral gavage. Killed 24 hours following final dose.	Quistad & Kovatchev (2007b)
Metabolism, excretion and tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	Repeated-dose administration 3 × 14	3	Fourteen daily analytical doses at 3.04 ± 0.18 or 3.16 ± 0.19 mg/kg bw in males and females, respectively, by oral gavage. Killed 24 hours following final dose.	Quistad & Kovatchev (2007b)
Metabolism, excretion and tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	Repeated-dose administration 3 × 14	3	14 daily analytical doses of 3.04 ± 0.15 or 3.16 ± 0.19 mg/kg bw in males and females respectively by oral gavage. Killed 5 days following final dose.	Quistad & Kovatchev (2007b)
Metabolism, excretion and tissue distribution of [pyrazolyl-5-^{14}C] fenpyrazamine	Repeated-dose administration 3 × 14	3	Fourteen daily analytical doses of 3.05 ± 0.11 or 3.13 ± 0.17 mg/kg bw in males and females, respectively, by oral gavage. Killed 10 days following final dose.	Quistad & Kovatchev (2007b)

bw: body weight; no.: number

1.1 Absorption, distribution and excretion

Studies of [pyrazolyl-5-^{14}C]-labelled fenpyrazamine indicated that absorption was rapid and extensive. Low doses of fenpyrazamine were almost completely metabolized, while at the high dose, approximately 4% of the administered dose was eliminated unabsorbed in the faeces. Maximum blood and plasma concentrations (C_{max}) occurred at one and six hours following administration of the low and high doses, respectively. Elimination followed first order kinetics with half-lives ranging between two and three hours after administration of the low dose and 14 and 17 hours after administration of the high dose (Table 2). Further evidence of saturation of the elimination process was seen in the area under the concentration–time curve (AUC) values, which were 150- to 170-fold greater after administration of [pyrazolyl-5-^{14}C]fenpyrazamine at 300 mg/kg bw than after administration at 3 mg/kg bw. There was no evidence of sex differences.

Table 2. Pharmacokinetic parameters of radiolabelled fenpyrazamine at low and high dose

Pharmacokinetic parameter	Measure of per dose of ^{14}C-S2188							
	3 mg/kg bw				300 mg/kg bw			
	Male		Female		Male		Female	
	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
C_{max} (µg/g)	1.5	1.5	2.0	1.7	68.4	65.2	52.1	45.0
T_{max} (h)	1	1	1	1	6	6	6	6
$t_{½}$ (h) [a]	2.66	2.76	2.43	2.55	15.1	16.6	14.0	14.6
DT_{50} (h)	3.66	3.76	3.43	3.55	21.1	22.6	20.0	20.6
$t_{1/10}$ (h)[a]	8.83	9.16	8.08	8.47	50.3	55.2	46.5	48.4
DT_{90} (h)	9.83	10.16	9.08	9.47	56.3	61.2	52.5	54.4
R^2	0.98	0.99	0.99	0.99	0.96	0.97	0.92	0.91
$AUC_{0–120}$ (h) [b]	13.16	14.34	13.02	12.47	2139	2184	1909	1822
$AUC_{120–\infty}$ (h)	0.2	0.17	0.07	0.09	107	146	78	77
AUC_{total} (h)	13.36	14.51	13.09	12.56	2246	2330	1987	1899

bw: body weight; C_{max}: maximum concentration in blood or plasma; T_{max}: time to reach C_{max}

$AUC_{0–120}$: area under the concentration–time curve for up to 120 hours after administration of dose;

$AUC_{120–\infty}$: area under the concentration–time curve from 120 hours after administration of dose;

AUC_{total}: area under the total concentration–time curve; $t_{½}$: kinetic half-life; $t_{½}$ + 1 hour (start of decline curve);

DT_{50}: time from dose administration to decline to 50% of peak concentration ($t_{½}$ + 1 hour [start of decline curve]);

DT_{90}: time from dose administration to decline to 10% of peak concentration ($t_{½}$ + 1 hour [start of decline curve]);

R^2: square of correlation coefficient of linear regression lines used to calculate $t_{½}$ and $t_{1/10}$;

$t_{1/10}$: time from peak value to decline to 10% of peak value; $t_{1/10}$ + 1 hour (start of decline curve);

[a] Calculated from 1-hour, 2-hour, 6-hour and 1-hour data by linear regression using Ln (mean blood or mean plasma concentration).

[b] Calculated by the Trapezoidal Rule.

Source: Dohn (2007)

Investigation of tissue distribution after administration of a single oral dose indicated that elimination of the radioactivity was essentially complete with less than 0.5% of the administered dose retained in the tissues (Tables 3 and 4). Peak radioactivity was reached in the tissues one hour after dosing with a single oral low dose, and six hours after dosing with a single oral high dose. In both dose groups, peaks in the stomach, including contents, and gastrointestinal tract occurred later than in the rest of the tissues due to elimination via faecal excretion. At the one-hour peak in low-dose animals, the highest concentrations of radioactivity were found in the stomach and small intestines, liver and kidney. At 0.5 h in high-dose animals, highest radioactivity concentrations were found in the stomach and small intestine (including contents), liver and kidney, but also in pancreas, sciatic nerve, caecum and large intestine (including contents). At the six-hour peak concentration, higher radioactivity amounts than in liver and kidney were measured in adrenal gland, bone marrow, fat, lymph nodes and pituitary (both in males), but the amount declined rapidly until scheduled kill. There were no significant sex differences.

In animals given repeated doses, the concentration of radioactivity in most tissues was maximized up to approximately day 7 and then remained constant during the rest of the dosing period. Radioactivity residues in the tissues declined with the cessation of dosing, and there was no evidence of bioaccumulation.

Table 3. Distribution in tissues after administration of low dose of fenpyrazamine

Tissue	Distribution in tissues (µg/g) with time							
	Males				Females			
	0.25 h	1 h	4 h	12 h	0.25 h	1 h	4 h	12 h
Adrenal gland	0.63	0.84	0.48	0.11	0.88	1.68	0.92	0.13
Bone	0.26	0.47	0.22	0.05	0.27	0.58	0.47	0.07
Bone marrow	1.82	1.26	0.56	0.00	1.13	1.33	1.83	0.11
Brain	0.14	0.28	0.14	0.02	0.18	0.55	0.36	0.05
Caecum	0.41	0.88	2.92	2.09	0.93	1.48	2.89	2.31
Eye	0.19	0.34	0.28	0.05	0.17	0.56	0.48	0.11
Fat	0.69	0.38	0.16	0.06	0.89	0.39	0.26	0.05
Hair/skin	0.36	0.52	0.37	0.10	0.35	0.88	0.64	0.12
Heart	0.58	0.78	0.44	0.07	0.68	1.28	0.79	0.11
Kidneys	2.43	3.27	1.87	0.48	1.32	2.88	2.26	0.27
Large intestine	0.88	1.100	0.80	0.92	0.13	1.45	1.50	0.78
Liver	3.46	3.17	1.59	0.55	3.28	3.64	2.04	0.67
Lungs	0.69	0.94	0.56	0.21	0.84	1.33	0.85	0.14
Lymph nodes	0.50	1.15	0.57	0.10	0.63	1.39	0.84	0.10
Muscle	0.08	0.63	0.34	0.05	0.11	1.03	0.76	0.09
Ovaries	NA	NA	NA	NA	0.40	1.04	0.79	0.10
Pancreas	0.66	1.19	0.46	0.10	0.71	1.41	0.88	0.11
Pituitary	1.04	0.97	1.91	0.11	0.91	2.26	1.22	0.19
Prostate	0.57	1.27	0.55 [a]	0.20	NA	NA	NA	NA
Salivary gland	0.48	0.73	0.39	0.09	0.60	1.19	0.75	0.11
Sciatic nerve	0.36	0.62	0.46	0.14	0.38	1.04	0.75	0.11
Small intestine	5.55	6.10	3.40	0.41	6.14	7.21	2.80	0.47
Spinal cord	0.15	0.32	0.19	0.03	0.18	0.57	0.35	0.05
Spleen	0.48	0.66	0.35	0.07	0.58	1.16	0.72	0.11
Stomach	91.07	25.72	20.20	0.69	73.65	25.35	9.26	0.32
Testes	0.15	0.48	0.36	0.07	NA	NA	NA	NA
Thymus	0.35	0.57	0.31	0.05	0.46	0.99	0.66	0.10
Thyroid	0.76	1.17	0.67	0.09	0.91	1.17	0.64	0.10
Uterus	NA	NA	NA	NA	0.54	2.62	1.50 [a]	0.16
Whole blood	0.77	0.93	0.69	0.15	0.71	1.44	0.89	0.18
Blood cells	0.73	1.02	0.59	0.14	0.74	1.66	1.03	0.18
Plasma	0.83	1.25	0.82	0.18	0.65	1.31	0.82	0.16
Stomach contents	93.53	41.65	82.47	3.38	222.15	46.26	55.65	1.021
Small intestine contents	5.83	8.45	9.62	1.83	5.13	5.92	9.90	1.60
Caecum contents	0.10	0.49	6.07	7.76	0.23	1.32	5.46	5.33
Large intestine contents	0.17	0.66	2.43	12.33	0.29	1.22	2.21	6.30
Carcass	0.38	0.61	0.35	0.10	0.52	0.92	0.65	0.11

NA: not analysed

[a] Mean of two animals – all other values are means of three animals.

Source: Quistad & Kovatchev (2007a)

Table 4. Distribution in tissues after administration of high dose of fenpyrazamine

Tissue	Distribution in tissues (µg/g) with time							
	Males				Females			
	0.5 h	6 h	24 h	72 h	0.5 h	6 h	24 h	72 h
Adrenal gland	15.6	150.7	7.3	2.2	22.2	110.9	30.1	1.3
Bone	4.1	37.7	5.0	1.7	5.0	25.6	10.9	0.9
Bone marrow	0.0	173.1	0.0	0.7	16.0	155.0	9.8	0.4
Brain	2.6	47.1	1.5	0.2	4.9	37.5	10.6	0.1
Caecum	26.1	760.9	39.8	2.6	22.2	596.3	89.0	1.7
Eye	3.3	45.1	7.8	1.9	4.9	35.3	13.4	1.3
Fat	4.0	184.2	5.5	0.8	6.5	168.4	34.4	0.4
Hair/skin	6.5	101.9	9.2	4.3	9.1	79.4	19.6	2.9
Heart	8.2	74.9	6.1	2.0	11.7	59.2	20.3	1.4
Kidneys	28.9	118.2	19.7	3.1	22.6	86.9	31.5	1.8
Large intestine	20.0	174.1	23.4	3.4	15.4	144.2	55.8	2.1
Liver	43.0	140.2	23.6	5.2	44.8	115.3	51.0	4.3
Lungs	11.5	74.9	9.4	3.8	27.0	57.3	20.3	2.2
Lymph nodes	7.7	161.7	6.4	1.4	15.2	86.7 [a]	36.4	0.7
Muscle	6.5	64.0	3.3	1.2	8.1	48.7	15.0	0.7
Ovaries	NA	NA	NA	NA	10.6	105.0	28.0	1.3
Pancreas	35.2	100.3	7.6	2.3	23.2	106.3	25.6	1.1
Pituitary	16.5	234.2	0.0	0.0	27.3	88.2	35.7	0.0
Prostate	8.9	105.1	12.1	2.4	NA	NA	NA	NA
Salivary gland	7.2	93.5	6.4	2.4	10.4	67.7	21.6	1.2
Sciatic nerve	122.9	11.6	8.9	5.1	61.5	31.8	28.1	0.5
Small intestine	185.7	263.9	27.3	3.4	348.7	154.3	81.6	1.7
Spinal cord	2.7	48.5	1.8	0.4	5.6	39.2	10.3	0.0
Spleen	8.3	63.5	6.3	2.3	12.6	50.2	18.5	1.3
Stomach	3059.5	2284.8	928.2	23.4	2106.7	1927.6	1770.0	10.2
Testes	3.2	62.9	5.2	1.7	NA	NA	NA	NA
Thymus	5.3	58.3	4.1	1.4	8.3	46.1	15.6	0.8
Thyroid	7.5	65.8	12.5	4.4	16.1	50.0	17.6	2.4
Uterus	NA	NA	NA	NA	9.0	61.3	19.6	1.5
Whole blood	11.0	85.6	13.8	6.0	11.3	58.6	24.9	3.2
Blood cells	10.8	90.2	13.1	5.5	11.9	62.9	25.2	3.0
Plasma	11.0	71.1	13.8	6.0	10.8	55.2	20.8	2.6
Stomach contents	6695.9	4311.6	3534.5	16.8	12 399.0	8848.2	4677.2	21.2
Small intestine contents	1096.9	525.7	62.6	3.5	2751.5	445.2	143.7	1.7
Caecum contents	9.9	1268.0	104.5	2.8	7.1	3349.2	121.9	1.9
Large intestine contents	7.8	1598.5	150.0	4.0	9.0	815.1	169.5	2.8
Carcass	5.8	68.4	7.4	2.6	9.3	54.3	17.0	1.1

NA: not analysed

[a] Mean of two animals – all other values are means of three animals.

Source: Quistad & Kovatchev (2007a)

Excretion was rapid. Low doses were almost completely eliminated within 24 hours and high doses within 48 hours. Urine was the most important route of elimination with more than 80% excreted in the urine in either sex and in low or high doses. Elimination via CO_2 was almost insignificant.

In the repeated-dose studies, 96.5% of the administered radioactivity was excreted by 10 days after the last of 14 doses in males. In females, 97.2% of the administered radioactivity was excreted in the same time frame. As in the single-dose studies, the majority of the radioactivity was recovered in the urine (up to 78.7%) and the faeces (up to 13.6%). Again, there was no evidence of bioaccumulation.

1.2 Biotransformation

The principal routes of metabolism in the rat involved an initial elimination of the allylsulfanylcarbonyl group by hydrolysis to produce 5-amino-1,2-dihydro-2-isopropyl-4-(*o*-tolyl)pyrazol-3-one (S-2188-DC), followed by dealkylation of S-2188-DC to 5-amino-1,2-dihydro-4-(*o*-tolyl)pyrazol-3-one (MPPZ) and hydroxylation to S-2188-CH_2-DC and S-2188-OH in small amounts (Fig. 2a; Fig. 2b).

Figure 2a. Proposed metabolic pathway of fenpyrazamine in the rat [a]

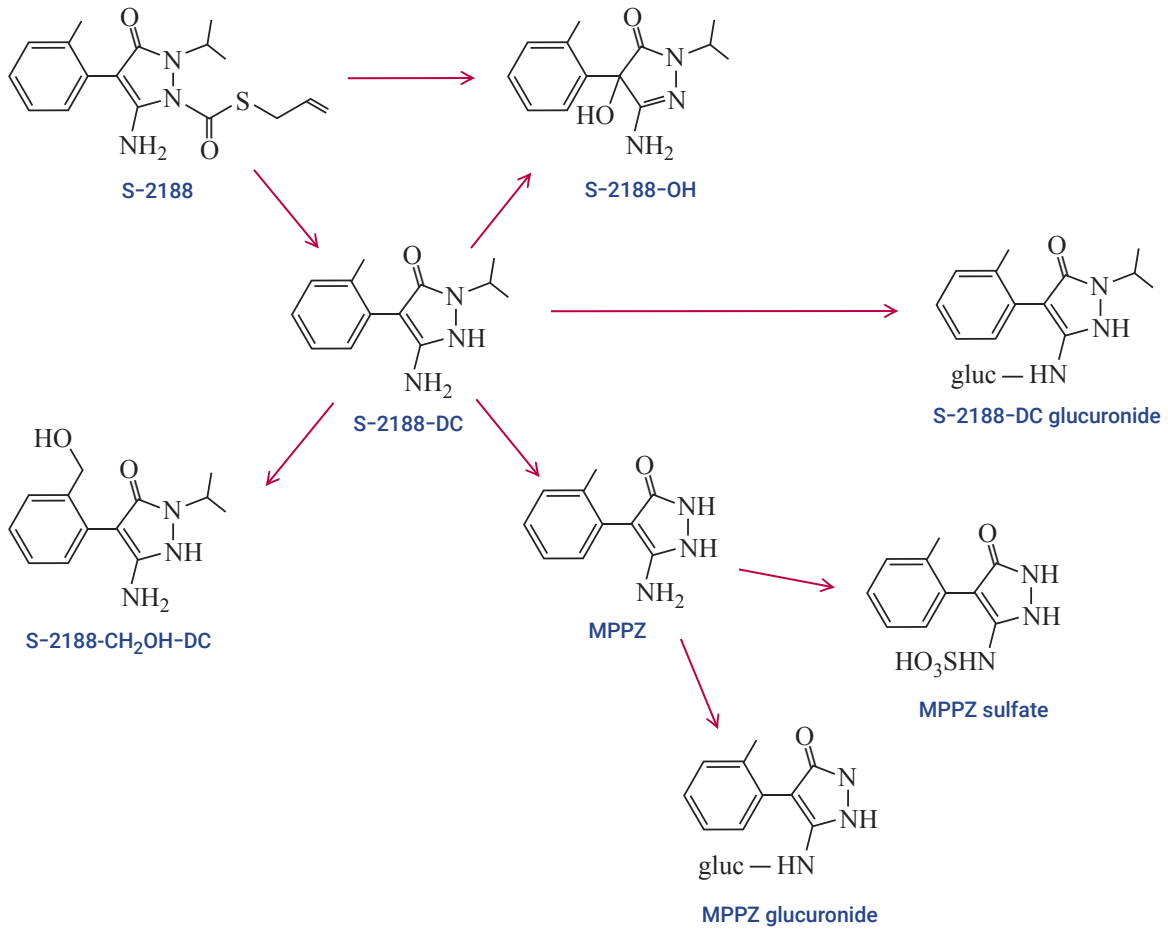
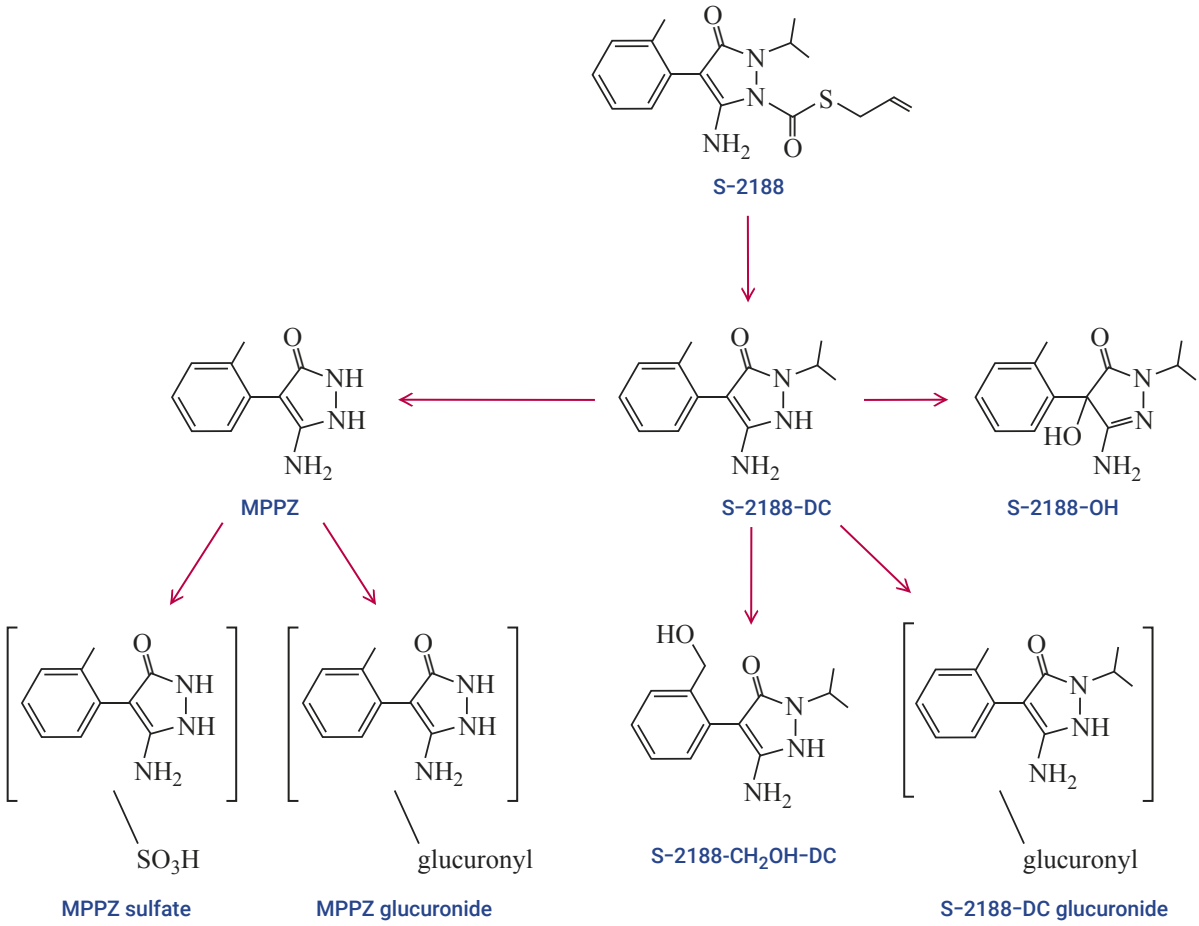


Figure 2b. Details of the MPPZ and S-2188-DC metabolism pathways



MPPZ: 5-amino-1,2-dihydro-4-(*o*-tolyl)pyrazol-3-one; S-2188: fenpyrazamine;

S-2188-DC: 5-amino-1,2-dihydro-2-isopropyl-4-(*o*-tolyl)pyrazol-3-one

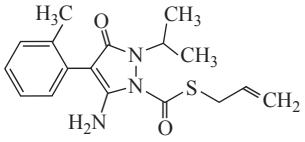
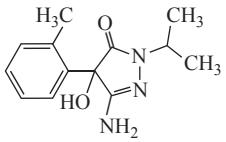
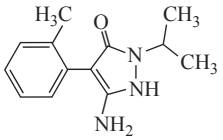
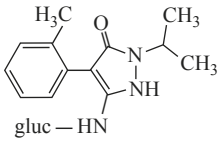
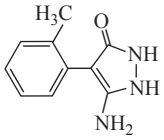
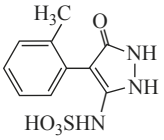
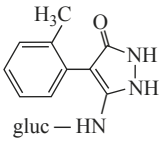
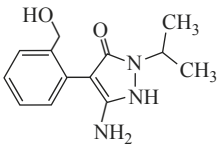
[a] The position of the conjugation with sulfate and glucuronic acid has not been determined.

Source: Dohn, Kavatchev & Estigoy (2007)

In the urine, the major pathway of excretion, the relative amounts of identified metabolites were similar in animals in the repeated-dose studies in both sampling periods (days 2–4 and days 13–15) and in both sexes (Table 5). In males, MPPZ sulfate and 5-amino-1,2-dihydro-4-(2-hydroxymethylphenyl)-2-isopropyl-pyrazol-3-one (S-2188-CH_2OH-DC) contributed the most to the radioactivity identified in urine, while S-2188-DC was present at less than 10%. In urine of females, MPPZ, MPPZ sulfate and S-2188-DC contributed evenly to the identified radioactivity. The animals in the single low-dose studies exhibited a very similar pattern to those in the repeated-dose study. In single high-dose males and females, S-2188-DC was by far the major component of excreta. In the faeces of repeated-dose animals, S-2188-CH_2OH-DC was the major metabolite in males (maximum applied radioactivity: 25.1%) and MPPZ the major metabolite in females (maximum applied radioactivity: 26.6%).

An investigation of metabolites in various target tissues in the tissue distribution study indicated differences in metabolite degradation between the sexes and doses. In both sexes and dose groups, fenpyrazamine was a minor metabolite in the plasma, liver and kidney. In the plasma, S-2188-DC, the major metabolite in both sexes, was found at higher concentrations in females. An unknown metabolite, RT 31 min, was present in high amounts in low-dose males, but it was not found in low-dose females. In high-dose animals, sex differences in metabolites were less pronounced. Following repeated dosing, a mixture of S-2188-CH_2OH-DC and MPPZ constituted the major identified metabolite in the plasma of males and females. In the liver, other unknown metabolites were present at a maximum 30% and 17% in low-dose males and females and 13% and 15% in high-dose males and females, respectively. Patterns in the kidney were similar to those in the liver.

Table 5. Metabolites identified in excreta of rats after single and repeated dose of radiolabelled fenpyrazamine

Substance	IUPAC name	Structure	% of applied radioactivity (maximum values)	
			Single dose	Repeated dose
Fenpyrazamine (parent)	*S*-allyl 5-amino-2,3-dihydro-2-isopropyl-3-oxo-4-(*o*-tolyl)pyrazole-1-carbothioate		4.3	1.1
S-2188-OH	5-amino-2,4-dihydro-4-hydroxy-2-isopropyl-4-(*o*-tolyl)pyrazol-3-one		0.7	2.7
S-2188-DC	5-amino-1,2-dihydro-2-isopropyl-4-(*o*-tolyl) pyrazol-3-one		44.9	34.6
S-2188-DC glucuronide [a]	–		5.3	– [b]
MPPZ	5-amino-1,2-dihydro-4-(*o*-tolyl)pyrazol-3-one		11.1	26.6 [c]
MPPZ sulfate [a]	–		31.0	36.3
MPPZ glucuronide [a]	–		13.6	– [b]
S-2188-CH_2OH-DC	5-amino-1,2-dihydro-4-(2-hydroxymethylphenyl)-2-isopropyl-pyrazol-3-one		18.4	32.3 [c]

IUPAC: International Union of Pure and Applied Chemistry;
MPPZ: 5-amino-1,2-dihydro-4-(*o*-tolyl)pyrazol-3-one

[a] The position of the conjugation with sulfate and glucuronic acid has not been determined.

[b] Not identified.

[c] Sum of MPZZ and S-2188-CH2OH-DC.

Source: Quistad & Kovatchev (2007b)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Fenpyrazamine is of low acute oral, dermal and inhalation toxicity.

In an acute oral toxicity study, animals were given a limit dose of fenpyrazamine (purity 94.7%) at 2000 mg/kg bw. There were no deaths and no clinical signs of toxicity. All animals gained weight throughout the observation period. There were no treatment-related gross pathological changes at necropsy (Deguchi, 2007a).

In an acute dermal toxicity study, animals were given a limit dose of fenpyrazamine (purity 94.7%) at 2000 mg/kg bw. There were no deaths and no treatment-related clinical signs of toxicity or signs of irritation at the dose site. All animals gained weight throughout the observation period. There were no gross pathological changes at necropsy (Deguchi, 2007b).

In an acute inhalation toxicity study, animals were given a limit dose of fenpyrazamine (purity 94.7%) at 4.84 mg/L (4840 mg/m^3). A control group of males and females was exposed to air only. There were no deaths. Wet fur was noted in all males in the hour after exposure. All clinical signs had abated by the second hour after exposure in control males and the third hour after exposure in test males. All animals gained weight throughout the observation period and weights were comparable to controls. There were no clinical signs of toxicity in either female group. There were no compound-related changes at gross necropsy. While the mass median aerodynamic diameter (MMAD) was reported as above 7 µm, the sponsor attested that the technical grade active ingredient was unable to be milled to a smaller size and the inhalation toxicity study was indicative of the toxicity of the compound (Deguchi, 2007c).

Table 6. Acute toxicity of fenpyrazamine

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	BrlHan:WIST@ Jcl(GALAS)	F	Oral	94.7	LD_{50} > 2000 mg/kg bw (F)	Deguchi (2007a)
Rat	BrlHan:WIST@ Jcl(GALAS)	M + F	Dermal	94.7	LD_{50} > 2000 mg/kg bw	Deguchi (2007b)
Rat	BrlHan:WIST@ Jcl(GALAS)	M + F	Inhalation	95.7	LC_{50} > 4.84 mg/L (4840 mg/m^3)	Deguchi (2007c)

bw: body weight; F: females; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: males

(b) Dermal irritation

In a dermal irritation study, 0.5 g of fenpyrazamine (purity 94.7%) was moistened with distilled water and applied on a 2.5 cm^2 lint patch to the dorsal skin, clipped free of fur, of male New Zealand White rabbits (n = 3). All scores at the 1-, 24-, 48- and 72-hour marks were zero.

Fenpyrazamine was considered non-irritating to the skin of rabbits (Odawara, 2007a).

(c) Ocular irritation

In an eye irritation study, 0.1 mL of fenpyrazamine (purity 94.7%) was instilled into the conjunctival sac of one eye of male New Zealand White rabbits (n = 6). In three animals, the eyes were unwashed and in the other three animals, the eyes were washed 30 seconds after instillation. The opposite eyes in both groups were left as untreated controls. In the unwashed group, two of three animals exhibited Grade 1 redness, chemosis and discharge. By 24 hours post instillation, one animal exhibited Grade 1 redness. There were no signs of irritation noted by 48 hours after instillation. In the washed group, Grade 1 redness was observed in two of three animals and Grade 1 conjunctivitis was observed in one animal 1 hour after instillation. At 24 hours post instillation, one animal exhibited Grade 1 redness and discharge. No signs of irritation were noted by 48 hours after instillation.

Fenpyrazamine is considered non-irritating to the eyes of the rabbit (Odawara, 2007b).

(d) Dermal sensitization

In a dermal sensitization study using a maximization protocol, young adult female Slc:Hartey guinea pigs (n = 20) were each injected on day 1 with 5% fenpyrazamine (purity 94.7%) in corn oil, 1:1 (volume per volume; v/v) mixture of Freund's complete adjuvant and distilled water and 5% fenpyrazamine in 1:1 (v/v) mixture of adjuvant and distilled water. One week after the first induction, lint patches saturated with 0.4 mL of 50% fenpyrazamine in acetone were applied for 48 hours. Challenge was 14 days after the topical application, with 0.2 mL of 25% w/v fenpyrazamine in acetone applied to the test sites with 24-hour exposure. An additional group of 10 guinea pigs was treated in the same manner, except they were exposed to the test material during the challenge phase only. The concentrations of fenpyrazamine used were based on a preliminary assay of a group of three animals.

At challenge, two of 20 challenge animals had slight erythema at 24 and 48 hours after exposure to 25% fenpyrazamine in acetone. Under the conditions of this study, there was no evidence of a dermal sensitization response to fenpyrazamine (Odawara, 2007c).

2.2 Short-term studies of toxicity

(a) Oral administration

Short-term oral toxicity was evaluated in the mouse, rat and dog.

Mouse

In a 14-day range-finding oral toxicity study, CD-1 (SPF) mice (n = 6/dose level) received fenpyrazamine (purity 98.5%) in the diet at a dose level of 0, 200, 700, 2000 or 7000 ppm (equal to 0, 36, 139, 353 and 1348 mg/kg bw per day for males and 0, 39, 145, 419 and 1429 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity. Clinical signs were observed three times on day 1 and twice daily from days 2 to 14. Body weight was recorded during acclimatization and on days 1, 4, 8 and 14. Feed consumption was recorded during acclimatization, from days 1–4, 4–8 and 8–14. Samples taken prior to terminal kill underwent clinical chemistry analyses. Gross examinations were performed and organs from all animals weighed. Bone marrow, kidneys, liver, spleen, thymus, thyroid and gross lesions in the control and 7000 ppm animals were histopathologically examined. Tissue samples from animals at 200, 700 and 2000 ppm were examined for changes at the high dose.

There were no effects on mortality, no clinical signs of toxicity and no gross findings at necropsy. Body weights and body weight gains in males at 7000 ppm were decreased compared to controls. While body weight gains were decreased in females at 2000 and 7000 ppm, in the absence of effects on body weight, the change was not considered adverse. Feed consumption was decreased in the first 4 days of treatment in males at 7000 ppm. Bilirubin was decreased in males at 2000 and 7000 ppm and in females at 7000 ppm. Cholesterol and triglycerides were increased at 7000 ppm in both sexes. In females, creatinine was decreased at 2000 and 7000 ppm. At 7000 ppm in females, alanine aminotransferase (ALAT) activity, globulin and γ-glutamyltransferase values were increased and alkaline phosphatase (ALP) activity and the albumin/globulin ratio were decreased. Other changes were not of sufficient magnitude to be considered adverse. Absolute and relative liver weights were increased in males and females at 2000 ppm and higher doses. Absolute and relative thymus weights were decreased in males at 2000 ppm and higher doses; however, in the absence of histopathological changes, the decreased weight was considered incidental. Absolute kidney weights were decreased at 7000 ppm in males; however, there was no effect on relative weights and the change was considered to be related to decreased body weights. Histopathological change consisted of increased incidence and severity of centrilobular hepatocellular hypertrophy in females at 2000 ppm and higher doses and in males at 7000 ppm. One male had haematopoietic foci in the liver at 7000 ppm. At 7000 ppm, one male had granuloma and one female coagulative necrosis. In the spleen, there was evidence of increased severity of extramedullary erythropoiesis starting at 2000 ppm in males and females and minimal lymphoid depletion at 7000 ppm in males and females. Further erythropoietic effects were seen in the bone marrow with minimally increased erythropoiesis in two females at 2000 ppm and in two males and one female at 7000 ppm. In the thyroid, there was an increase in minimal follicular hypertrophy at 7000 ppm (Sommer & Weber, 2005).

In a 13-week oral toxicity study, CD-1 (ICR)BR(SPF) mice (n = 12/dose level) received fenpyrazamine (purity 94.7%) in the diet at a dose level of 0, 200, 2000, 4000 or 6000 ppm (equal to 0, 28, 296, 640 and 1023 mg/kg bw per day for males and 0, 33, 363, 720 and 1098 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and feed consumption were measured twice during weeks 1–4 and weekly thereafter. Haematology and clinical chemistry parameters were analysed from samples taken prior to terminal kill. Gross examinations were performed and organs were weighed for all main study animals. Main study animals at 0 and 6000 ppm were histopathologically examined, as were all gross lesions, livers and kidneys of low- and mid-dose animals and any organ systems of animals that had treatment-related lesions at 6000 ppm.

At doses of 2000 ppm and higher, non-adverse changes such as increased feed intake, increased liver weights and increased hepatocellular hypertrophy were noted. At doses of 4000 ppm and higher, male mice had decreased body weight and decreased haematocrit (Table 7). At 6000 ppm, both males and females had decreased red blood cells (RBCs) and haemoglobin values and females had decreased body weight.

The no-observed-adverse-effect level (NOAEL) was 2000 ppm (equal to 296 mg/kg bw per day) in males based on decreased body weight and haematocrit at 4000 ppm (equal to 640 mg/kg bw per day) and 4000 ppm (equal to 720 mg/kg bw per day) in females based on decreased body weight, RBCs and haemoglobin at the lowest-observed-adverse-effect level (LOAEL) of 6000 ppm (1098 mg/kg bw per day) (Sommer et al., 2007).

Table 7. Results of 90-day oral toxicity study of fenpyrazamine in the mouse

Parameter	Measure per dose level				
	0 ppm	**200 ppm**	**2000 ppm**	**4000 ppm**	**6000 ppm**
Males (n = 12/dose level)					
Final body weight (g)[a]	38.9 ± 3.4	38.3 ± 3.2 (↓1)	37.2 ± 1.8 (↓4)	36.6 ± 3.6 (↓6)	36.3 ± 2.7 (↓7)
Body weight gain (%)[a]	32.3 ± 13.6	26.1 ± 8.8 (↓19)	22.3 ± 8.2* (↓31)	21.9 ± 6.7* (↓32)	21.4 ± 5.5* (↓34)
RBC count (× 10^9)	9.44	9.34	9.27	8.90 (↓6)	8.69 (↓8)
Haemoglobin	9.0	8.9	8.9	8.7	8.6 (↓4)
Haematocrit	0.45	0.45	0.43	0.41* (↓9)	0.41* (↓9)
Females (n = 12/dose level)					
Final body weight (g)[a]	31.1 ± 3.6	30.1 ± 1.5 (↓3)	30.1 ± 1.8 (↓3)	30.0 ± 1.3 (↓4)	29.6 ± 1.2 (5)
Body weight gain (%)[a]	22.9 ± 11.3	23.6 ± 7.9	23.2 ± 6.6	20.9 ± 6.7 (↓9)	21.1 ± 6.1 (↓8)
RBC count (× 10^9)	9.18	9.13	9.07	9.26	8.46 (↓8)
Haemoglobin	9.2	9.1	9.1	9.1	8.7 (↓5)
Haematocrit	0.44	0.44	0.44	0.43	0.41* (↓7)

ppm: parts per million; RBC: red blood cells; ↓: decrease; *: $P < 0.05$

[a] Results presented as group mean ± standard deviation and, in parentheses, the % change relative to the control.

Source: Sommer et al. (2007)

Rat

In an 13-week oral toxicity study, HanRcc:WIST(SPF) rats (n = 12/dose level) received fenpyrazamine (purity 97.7%) in the diet at concentrations of 0, 300, 600, 1000 or 3000 ppm (equal to 0, 19, 38, 64 and 196 mg/kg bw per day for males and 0, 21, 42, 69 and 207 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity.

Body weight and feed consumption were measured weekly. Ophthalmoscopic examinations were performed prior to administration and on week 13. A functional observational battery (FOB) including locomotor activity and haematology, clinical chemistry and urine analysis parameters were measured at week 13. All animals underwent a gross necropsy and organs were weighed. Histopathology was performed on control and high-dose animals, all gross lesions and bone marrow, liver, spleen and thyroid from the low- and mid-dose groups.

There were no unscheduled deaths and no clinical signs of toxicity. There was a statistically significant decrease in fore- (22%) and hindlimb (18%) strength in females at 3000 ppm; however, the change was considered incidental to treatment as the values were just outside the range of the concurrent controls. Body weight and body weight gains were decreased in males and females at 3000 ppm. Changes to haematology, clinical chemistry and urine analysis parameters were within historical control ranges. Absolute and relative liver weights were increased in males and females at 3000 ppm, and there was a concurrent increase in hepatocellular hypertrophy; however, in the absence of other clinical chemistry or liver histopathology changes, the increases were considered adaptive. There was an increase in thyroid follicular cell hypertrophy and increases in urinary bladder congestion and dilation in males at 3000 ppm.

The NOAEL was 1000 ppm (equal to 64 mg/kg bw per day in males and 68 mg/kg bw per day in females) based on decreased body weight and body weight gain in males and females and increased thyroid follicular cell hypertrophy and urinary bladder congestion and dilation in males at the LOAEL of 3000 ppm (equal to 196 mg/kg bw per day for males and 207 mg/kg bw per day for females) (Sommer et al., 2006).

Dog

In a 90-day oral toxicity study, beagle dogs (n = 4/dose level) received fenpyrazamine (purity 94.7%) in gelatine capsules at doses of 0, 25, 50 or 150 mg/kg bw per day. Animals were monitored for mortality, moribundity and clinical signs of toxicity twice daily. Detailed clinical observations were performed weekly along with body weight and feed and water consumption measurements. Ophthalmoscopic examinations were performed once pretest and at week 13. Haematology, clinical chemistry and urine analysis parameters were analysed at weeks 4, 8 and 13. Following terminal kill, all animals underwent gross necropsy; organs were weighed and histopathologically examined.

There were no unscheduled deaths and no effects on clinical signs, feed consumption, ophthalmoscopy, urine analysis parameters or gross necropsy. Mean bodyvweights and body weight gains were decreased by 13% and 76%, respectively, in males at 150 mg/kg bw per day; however, as one male dog lost weight throughout the study period, the change was considered adverse at 50 mg/kg bw per day. There were no effects on body weight or body weight gain in females. RBC counts, haemoglobin and haematocrit values were decreased throughout the study period in males. There was a statistically significant increase in mean corpuscular volume (MCV) in males and females at 150 mg/kg bw per day. Platelets were increased in males at 50 and 150 mg/kg per day; however, there were no effects on clotting parameters and the adversity of the change is unknown. The changes in males correlated with gelatinous bone marrow in males at 150 mg/kg bw per day. Liver enzymes, alanine aminotransferase and ALP, were increased at 150 mg/kg bw per day; however, the changes was within the magnitude of adaptive change. Likewise, the increase in hepatocellular hypertrophy at doses of 50 mg/kg bw per day for males and at 50 and 150 mg/kg bw per day for females is evidence of adaptive change. Liver changes in males at 150 mg/kg bw per day were considered adverse due to the presence of increased bilirubin along with hepatocellular hypertrophy and increased liver weights.

The NOAEL was 25 mg/kg bw per day for males based on body weight loss in one male at the LOAEL of 50 mg/kg bw per day. The NOAEL was 50 mg/kg bw per day for females based on increased MCV at the LOAEL of 150 mg/kg bw per day (Sato, 2008).

In a one-year oral toxicity study, beagle dogs (n =v4/group) received fenpyrazamine (purity 94.7%) in gelatine capsules at doses of 0, 5, 25 or 100 mg/kg bw per day. Animals were monitored for mortality, moribundity and clinical signs of toxicity twice daily. Detailed clinical observations were performed weekly along with body weight and feed consumption calculations, while water consumption was evaluated monthly. Ophthalmoscopic examinations were performed and haematology, clinical chemistry and urine analysis parameters were analysed once pretest and at weeks 13, 26, 39 and 52. Following the

terminal kill, all animals underwent gross necropsy, bone marrow examinations, determination of organ weights and histopathological examination.

There were no effects on clinical signs of toxicity, feed or water consumption, body weight or body weight gain. Detailed clinical observations, urine analysis and gross pathology were unaffected by treatment. Changes to haematological parameters consisted of increased platelets in females and increased MCV in males at 100 mg/kg bw per day. While the change in MCV only constitutes a 4% increase, it was statistically significant and consistent with the database. ALP values were increased by over 100% in males at 100 mg/kg bw per day, indicating an adverse effect, while values in females were increased by 22–62%, indicating an adaptive effect.

The NOAEL was 25 mg/kg bw per day based on increased MCV and ALP values in males and increased platelets in females at the LOAEL of 100 mg/kg bw per day (Sato, 2009).

(b) Dermal application

Short-term dermal toxicity was evaluated in the rat.

In a 28-day dermal toxicity study, fenpyrazamine (purity 94.7%) was applied to 10% of the body surface area, clipped free of fur, of Crl:CD(SD) rats (n = 10/sex per dose level) at a dose level of 0, 100, 300 or 1000 mg/kg bw per day. Animals were monitored twice daily for mortality and clinical signs of toxicity. Detailed clinical observations, body weights and feed consumption were assessed weekly. Ophthalmoscopic examinations were performed prior to administration and at week 4. All animals underwent a gross necropsy, and organs were weighed. Haematology and bone marrow assessments and clinical chemistry analyses were performed from blood samples taken at necropsy. control and high-dose animals underwent histopathological examination.

There was no effect on mortality, no clinical signs of toxicity and no ophthalmoscopic findings. There were no effects on body weights or feed consumption. There were no treatment-related changes in haematological parameters or clinical chemistry effects in either sex. Haemoglobin, haematocrit and prothrombin times were statistically significantly decreased in males at 1000 mg/kg bw per day; however, the changes were within the historical reference ranges supplied by the sponsor (Table 8) and were not seen in other rat studies. As such, the changes were considered incidental to treatment. Corrected heart weights were decreased in males at 1000 mg/kg bw per day; however, because the changes were within the historical control range and no concomitant changes were seen in the gross or histopathological examinations, the change was considered incidental to treatment.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Ogata, 2008).

Table 8. Results of the 28-day dermal toxicity study of fenpyrazamine in the rat

Observation	Measure per dose level [a]				HC [b]
	0 ppm	100 ppm	300 ppm	1000 ppm	
Males (n = 10/dose level)					
Haemoglobin (g/dL)	15.6 ± 0.4	15.4 ± 0.2	15.2 ± 0.4	15.2 ± 0.4* (↓2)	15.3 (14.0–17.6)
Haematocrit (%)	43.7 ± 1.1	42.7 ± 0.5	42.6 ± 1.3	42.4 ± 1.0* (↓3)	44.3 (40.8–50.3)
Prothrombin time (s)	17.5 ± 2.1	16.5 ± 2.5	15.5 ± 2.4	14.9 ± 1.6* (↓15)	15.2 (11.9–22.0)
Heart weight (g)	1.28 ± 0.13	1.30 ± 0.12	1.25 ± 0.12	1.17 ± 0.13 (↓9)	–
Heart (g/100 g bw)	0.37 ± 0.03	0.38 ± 0.03	0.35 ± 0.03	0.33 ± 0.02* (↓11)	0.35 (0.28–0.46)

bw: body weight; HC: historical control data; ppm: parts per million; ↓ decrease; *: $P < 0.05$

[a] Results presented as the group mean value ± standard deviation.

[b] Results presented as group means (range).

Source: Ogata (2008)

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In 78-week oral toxicity study, Crl:CD1 mice (n = 52/group) were given fenpyrazamine (purity 94.7%) at doses of 0, 100, 1500 or 3000 ppm in males (equal to 0, 11, 176 and 349 mg/kg bw per day) and 0, 100, 2000 or 4000 ppm in females (equal to 0, 14, 283 and 552 mg/kg bw per day). Animals were monitored for mortality and moribundity twice daily and clinical signs of toxicity daily; a detailed clinical examination including palpation for masses was performed weekly. Body weight and feed consumption were measured weekly until week 13 and every two weeks thereafter. Haematology, clinical chemistry and urine analysis parameters were measured in samples collected from unfasted mice at the termination of the satellite group and at terminal kill. All animals underwent a gross necropsy, and organs were weighed; histopathology was performed on control and high-dose animals and all gross lesions and liver and thyroid from the low- and mid-dose animals.

There were no treatment-related effects on mortality or clinical signs of toxicity. Feed consumption in both sexes and body weight and body weight gain in females were unaffected by treatment. In males, body weight was decreased in all treated groups in the satellite groups and body weight gain was decreased in the 1500 and 3000 ppm satellite groups; however, these decreases were not statistically significant, and there were no adverse changes by the end of the treatment period in the main study group. The body weight changes in males were not considered adverse.

There were no effects on clinical chemistry or urine analysis parameters. There were no haematological changes at week 52. At week 78, MCV values were increased in males at 1500 ppm and higher doses. RBC values were decreased at 3000 ppm in males and 4000 ppm in females. At the same doses, mean cell haemoglobin (MCH) was increased in males and haemoglobin and haematocrit were decreased in females. While the majority of the changes fell within the historical control range submitted by the sponsor, the changes were consistent with the rest of the database and indicate anaemia at 3000 ppm in males and 4000 ppm in females (Table 9).

Relative liver weights were increased after 52 weeks of treatment at 1500 and 3000 ppm in males and 2000 and 4000 ppm in females. Absolute liver weights were increased at 3000 ppm in males and at 2000 and 4000 ppm in females. At 78 weeks, absolute liver weights were increased at 1500 and 3000 ppm in males and at 2000 and 4000 ppm in females. Relative liver weights were increased in males and females at 1500 ppm and higher doses. At 78 weeks, livers were enlarged in 4000 ppm females and hepatocellular hypertrophy was noted at 3000 ppm in males and 4000 ppm in females. There was a slight increase in liver carcinomas in treated males at 78 weeks; however, as the incidence was low and there was no increase in adenomas or other histopathological changes other than an increase in hepatocellular hypertrophy, the change was considered incidental to treatment.

Spleen, heart and kidney weights were increased in females at 4000 ppm; however, in the absence of histopathological changes, the increased weights were not considered treatment related or adverse.

Table 9. Results of 78-week oral toxicity study of fenpyrazamine in the mouse

	Measure per dose level							
	Males				Females			
Observation	0 ppm	100 ppm	1500 ppm	3000 ppm	0 ppm	100 ppm	2000 ppm	4000 ppm
Week 52								
Liver								
Number examined	11	12	12	12	12	10	11	7
Absolute weight (g)	2.25 ± 0.25	2.15 ± 0.39	2.38 ± 0.49	2.64 ± 0.33* (↑17)	1.49 ± 0.17	1.58 ± 0.19	1.90 ± 0.23** (↑28)	2.03 ± 0.19** (↑36)
Relative weight (%)	4.36 ±.40	4.58 ± 0.85	5.17 ± 0.72* (↑19)	5.77 ± 0.80** (↑32)	4.53 ± 0.52	4.18 ± 0.60	5.61 ± 0.65* (↑24)	6.48 ± 0.57** (↑43)
Number enlarged	2	3	3	1	1	1	3	6*
Hepatocellular hypertrophy	–	1	1	1	–	–	3	6*

Observation	Measure per dose level							
	Males				Females			
	0 ppm	100 ppm	1500 ppm	3000 ppm	0 ppm	100 ppm	2000 ppm	4000 ppm
Week 78								
RBC ($10^6/\mu L$) [a]	8.12	8.45	7.98	7.53*	7.93	8.20	7.70	7.28*
HC	8.98 ± 1.14				8.19 ± 1.55			
HB (mmol/L)	8.0	8.2	8.0	7.8	8.2	8.1	7.9	7.4**
HC	8.4 ± 1.2				7.9 ± 1.6			
HCT (%)	37	39	38	37	38	38	37	35*
HC	42 ± 6				39 ± 7			
MCV (fL)	45.7	46.1	47.2*	48.5*	48.1	46.5	48.1	48.5
HC	46.4 ± 3.8				47.4 ± 5.0			
MCH (fmol)	0.99	0.96	1.00	1.04*	1.03	0.99	1.03	1.01
HC	0.92 ± 0.06				0.95 ± 0.06			
Liver								
Number examined	29	38	37	37	37	28	36	34
Absolute weight (g)	2.09 ± 0.33	2.28 ± 0.92	2.38 ± 0.55 (↑14)	2.71 ± 0.50** (↑30)	1.83 ± 0.92	1.64 ± 0.50	1.93 ± 0.28 (↑5)	2.61 ± 1.07** (↑43)
Relative weight (%)	4.78 ± 0.66	5.19 ± 2.18	5.36 ± 1.43 (↑12)	6.17 ± 0.96** (↑29)	4.92 ± 1.23	4.69 ± 1.11	5.54 ± 0.52 (↑13)	7.52 ± 2.8** (↑53)
Number enlarged	15	14	14	9	12	16	14	23*
Hepatocellular hypertrophy (number)	3	4	5	7	–	1	2	9
Adenoma (number)	4	5	9	5	1	–	–	1
Carcinoma (number)	–	1	2	2	–	–	–	–
Spleen								
Number examined	29	38	37	37	37	28	36	34
Absolute weight (g)	0.146 ± 0.162	0.149 ± 0.082	0.175 ± 0.107	0.142 ± 0.065	0.211 ± 0.207	0.195 ± 0.205	0.200 ± 0.127	0.251 ± 0.140 (↑19)
Relative weight (%)	0.348 ± 0.440	0.337 ± 0.183	0.409 ± 0.305	0.333 ± 0.170	0.577 ± 0.54	0.552 ± 0.513	0.576 ± 0.354	0.717 ± 0.368 (↑24)
Heart								
Number examined	29	38	37	37	37	28	36	34
Absolute weight (g)	0.243 ± 0.027	0.247 ± 0.031	0.253 ± 0.027	0.244 ± 0.033	0.179 ± 0.021	0.183 ± 0.022	0.184 ± 0.021	0.204 ± 0.044** (↑14)
Relative weight (%)	0.554 ± 0.051	0.566 ± 0.076	0.572 ± 0.115	0.560 ± 0.087	0.500 ± 0.062	0.530 ± 0.092	0.532 ± 0.060	0.593 ± 0.120** (↑19)

{*Continued on next page*)

Observation	Measure per dose level							
	Males				Females			
	0 ppm	100 ppm	1500 ppm	3000 ppm	0 ppm	100 ppm	2000 ppm	4000 ppm
Kidney								
Number examined	29	38	37	37	37	28	36	34
Absolute weight (g)	0.724 ± 0.108	0.732 ± 0.104	0.708 ± 0.123	0.676 ± 0.097	0.436 ± 0.072	0.450 ± 0.074	0.443 ± 0.06	0.487 ± 0.088** (↑12)
Relative weight (%)	1.653 ± 0.212	1.673 ± 0.219	1.598 ± 0.326	1.549 ± 0.233	1.216 ± 0.184	1.301 ± 0.227	1.277 ± 0.158	1.417 ± 0.230** (↑17)

HB: haemoglobin; HC: historical control data; HCT: haematocrit; MCH: mean cell haemoglobin; ppm: parts per million; MCV: mean corpuscular volume; RBC: red blood cells; ↑: increase; *: $P < 0.05$; **: $P < 0.01$

[a] Standard deviations for haematology parameters were not given in the study report.

Source: Sommer (2009a)

The NOAEL was 1500 ppm in males (equal to 176 mg/kg bw per day) and 2000 ppm in females (equal to 283 mg/kg bw per day) based on decreased RBC counts in males and females, decreased body weight and increased MCV and MCH in males and decreased haemoglobin and haematocrit in females. The LOAEL was 3000 ppm in males (equal to 349 mg/kg bw per day) and 4000 ppm in females (equal to 552 mg/kg bw per day) (Sommer, 2009a).

Rat

In a two-year oral toxicity study, HanRcc:WIST SPF rats ($n = 50$/sex per group) received fenpyrazamine (purity 94.7%) in the diet at doses of 0, 100, 300, 1200 or 2400 ppm (equal to 0, 4.3, 12.7, 52 and 107 mg/kg bw per day for males and 0, 5.2, 15.6, 64 and 130 mg/kg bw per day for females, respectively) with an additional 20/sex per group as a satellite interim kill group. Animals were monitored for mortality and moribundity twice daily and for clinical signs of toxicity daily; a detailed clinical examination including palpation for masses was performed weekly. Body weight and feed consumption were measured weekly until week 13 and every two weeks thereafter. A full FOB was performed on week 49. Ophthalmoscopic examinations of control and high-dose animals took place at pretest and at week 51. Haematology, clinical chemistry and urine analysis parameters were measured in samples collected from fasted animals in weeks 13, 26 and 52, and blood samples were taken from unfasted animals in weeks 78 and 104. All animals underwent a gross necropsy and organs were weighed; histopathology was performed on control and high-dose animals, all gross lesions, and liver and thyroid from the low- and mid-dose groups.

There were no effects on mortality, clinical signs of toxicity or ophthalmoscopy. Body weight was unaffected in the first year of treatment; however, there was a decrease in overall body weight compared to controls in males and females at 2400 ppm, and body weight gain was decreased compared to controls in females at 2400 ppm (Table 10). In females, motor activity was increased at 300 ppm and above and grip strength was decreased at 2400 ppm during the FOB at week 49; however, the increased activity was considered not treatment related as the same changes were not seen in the 90-day neurotoxicity study at similar doses (see section 2.6; Sommer, 2008b). The decreased grip strength at 2400 ppm was considered to be related to the decreased body weight and was treatment related and adverse.

In the haematological parameters, there was a statistically significant increase in prothrombin times in females at 1200 and 2400 ppm at weeks 13, 26 and 52. The 300 ppm group exhibited a statistically significant increase at week 26. While biological significance was only reached in females at 1200 and 2400 ppm at week 26, the consistent nature of the change indicated the changes were treatment related and adverse at all time points in these two groups. Changes in platelets did not have the same consistency in statistical significance, dose relation or biological significance, and there was no evidence of correlated effects on the organ systems; therefore, the increases were not considered to be treatment related. Neutrophils and eosinophils were decreased at 2400 ppm in week 26 in males, but unaffected

at week 52. All other statistically significant changes lacked biological significance, a dose response or both, and were considered unrelated to treatment.

Treatment-related and adverse changes to clinical chemistry parameters occurred in males and females at 1200 ppm and above. At weeks 13 and 26, blood glucose was decreased in females at 2400 ppm. At week 13, total bilirubin was decreased in males at 1200 ppm and above and in females at 2400 ppm, and triglycerides were increased in females at 2400 ppm. At weeks 26 and 52, globulin was increased in females at 2400 ppm. At week 52, total cholesterol was increased in females at 1200 ppm and above and creatinine was decreased in both males and females at 2400 ppm.

Urinary volume was increased in females at 300–2400 ppm at week 13, and there was a concurrent decrease in osmolality and increase in pH (approaching pH 7) at 1200 and 2400 ppm. The adversity of this change is unknown. There was an increase in urinary leukocytes in males at 1200 and 2400 ppm at week 13 and 26 and in females at 1200 ppm at week 26 and at 2400 ppm at week 52.

Absolute and relative liver weights were increased in males and females at 2400 ppm in animals terminated at 52 weeks. By week 104 at 2400 ppm, terminal body weights were decreased in males and females and only relative liver weights were increased. A number of relative organ weights were increased at 2400 ppm; however, changes to the brain, heart, kidneys and testes were considered unrelated to treatment due to the lack of gross or histopathological change. Relative spleen weights were decreased in females at 2400 ppm; however, in the absence of histopathological changes, the change was considered unrelated to treatment.

No changes were observed at gross necropsy. At the interim kill, there was a slight increase in fatty liver change at 2400 ppm in males. There was also an increase in hepatocellular hypertrophy starting at 100 ppm in males and at 1200 ppm in females; however, in the absence of any other liver changes at 100 ppm and in the presence of only changes to total bilirubin at 1200 ppm, the increase in hepatocellular hypertrophy was only considered adverse at 1200 ppm. At the terminal kill, hepatocellular hypertrophy was increased in males and females at 1200 ppm; however, there was no dose response, and the change was considered incidental to treatment. The incidence of fatty change was increased in females at 2400 ppm only. There was a statistically significant increase in vacuolated foci in males at 2400 ppm; however, the dose response was flattened and the change was considered incidental to treatment. Changes to the thyroid consisted of slight increases in diffuse and focal follicular hyperplasia in males at 2400 ppm and in follicular hypertrophy in females at 2400 ppm.

Table 10. Results of 2-year oral toxicity study of fenpyrazamine in the rat

	Measure per dietary dose level									
	Males					Females				
Observation	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm
Body weight and body weight gain										
Body weight (g)										
Week 52	576 ± 61.3	606 ± 74.7	618 ± 85.2	583 ± 53.9	577 ± 51.2	315 ± 39.3	319 ± 29.9	317 ± 36.4	297 ± 25.7	296 ± 38.2
Week 104	693 ± 100.6	710 ± 108.3	674 ± 85.0	703 ± 112.4	623** ± 91.0 (↓10)	412 ± 60.8	399 ± 48.7	412 ± 65.8	391 ± 53.4 (↓5)	360** ± 57.7 (↓13)
Body weight gain (g)										
Weeks 0–52	283 ± 37.1	300 ± 40.9	300 ± 63.7	289 ± 35.0	290 ± 25.2	153 ± 34.2	150 ± 22.6	155 ± 24.2	138 ± 18.4	133 ± 27.8
Weeks 0–104	354 ± 59.0	356 ± 57.4	346 ± 56.3	363 ± 80.2	328 ± 55.0	226 ± 44.9	221 ± 39.0	221 ± 48.5	209 ± 40.3	187** ± 44.7 (↓17)
FOB (Week 49)										
Grip strength (hind), kg	0.76 ± 0.15	0.74 ± 0.14	0.73 ± 0.16	0.75 ± 0.18	0.66 ± 0.12	0.58 ± 0.13	0.60 ± 0.08	0.55 ± 0.09	0.52 ± 0.07	0.49* ± 0.10 (↓16)

Observation	Measure per dietary dose level									
	Males					Females				
	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm
Haematology [a]										
Prothrombin time (relative)										
Week 13	0.81	0.82	0.82	0.83	0.76	0.84	0.85	0.85	0.90+ (↑7)	0.92+ (↑10)
Week 26	0.88	0.86	0.87	0.87	0.83+ (↓6)	0.81	0.84	0.87+	0.93+ (↑15)	0.94+ (↑16)
Neutrophils (g/L)										
Week 26	1.68	1.53	1.52	1.35	1.23* (↓27)	0.63	0.77	0.82	0.62	0.75
Week 52	2.06	1.86	1.57	1.44* (↓30)	1.44* (↓30)	0.9	0.78	0.92	0.86	0.84
Eosinophils (g/L)										
Week 26	0.14	0.13	0.11	0.11	0.1* (↓29)	0.06	0.07	0.07	0.07	0.07
Clinical chemistry										
Blood glucose (mmol/L)										
Week 13	4.79	5.04	4.95	4.6	4.36	4.54	4.88	4.52	4.49	3.92* (↓14)
Week 26	5.11	5.09	5.48	5.33	4.84	5.24	5.46	5.49	5.55	4.58* (↓13)
Week 52	4.94	5.47* (↑10)	5.3	5.45* (↑10)	4.53* (↓8)	4.67	5.66* (↑21)	5.49* (↑18)	5.78* (↑24)	5.34* *↑14)
Total bilirubin (µmol/L)										
Week 13	1.83	1.57 (↓14)	1.69 (↓8)	1.45 (↓21)	1.25 (↓32)	2.01	1.84	1.83	1.82	1.72 (↓14)
Triglycerides (mmol/L)										
Week 13	0.38	0.41	0.39	0.25* (↓26)	0.36	0.33	0.34	0.32	0.31	0.39 (↑18)
Cholesterol (mmol/L)										
Week 52	2.49	2.5	2.28	2.26	2.48	1.96	2.08	2.12	2.52 (↑29)	2.71 (↑38)
Creatinine (µmol/L)										
Week 26	28	28.7	29.1	28.2	25.4* (↓9)	34.9	34.2	33.8	32.7	31.8* (↓9)
Week 52	31.8	32.8	33.3	30.9	28.3* (↓11)	35.9	36.5	36.7	35.2	31.8* (↓11)
Urine analysis										
Volume (mL)										
Week 13	5.9	6.8	6.7	6.2	5.3 (↓10)	8.3	9.1	14 (↑69)	15.4* (↑86)	16.2* (↑95)
Osmolality (mOsm/kg)										
Week 13	1471	1429	1366	1369	1451	929	1042	813	631 (↓32)	457* (↓51)
pH										
Week 13	6.6	6.7	6.6	6.7	6.8	6.2	6.2	6.5	6.6 (↑6.5)	6.8 (↑9.7)

(*Continued on next page*)

Observation	Measure per dietary dose level									
	Males					Females				
	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm
Leukocytes (/µL)										
Week 26	78	75	74	100 (↑28)	180 (↑131)	14	14	18	28 (↑100)	31
Week 52	206	165	205	194	229	33	47	73	85	161 (↑388)
Organ weights										
Interim kill										
Terminal body weight (g)	562 ±58.5	587 ±79.8	603 ±84.8	561 ±54.8	558 ±52.6	305 ±36.9	309 ±31.1	305 ±34.7	286 ±24.5	289 ±32.9
Absolute liver weight (g)	14 ± 1.68	14.3 ± 2.58	14.3 ± 2.09	15 ± 1.36	16.6** ± 2.51 (↑19)	8.48 ± 1.38	8.42 ± 0.98	8.17 ± 1.008	8.33 ± 1.07	9.44* ± 1.38 (↑11)
Relative liver weight (%)	2.49 ± 0.18	2.43 ± 0.26	2.38 ± 0.18	2.69* ± 0.23	2.98* ± 0.38 (↑20)	2.78 ± 0.28	2.72 ± 0.20	2.69 ± 0.26	2.92 ± 0.28	3.28* ± 0.48 (↑18)
Terminal kill										
Terminal body weight (g)	683 ± 106.1	699 ± 106.4	675 ± 87.1	694 ± 110.1	613* ± 90.7 (↓10)	413 ± 57.1	386 ± 51.3	409 ± 63.8	382 ± 56.0	353** ± 58.5 (↓15)
Absolute liver weight (g)	16 ± 3.02	16.5 ± 2.69	16.1 ± 2.89	16.8 ± 2.47	17.4 ± 3.31	10.9 ± 2.42	10 ± 1.76	10.5 ± 1.84	9.7* ± 1.61	10.2 ± 1.86
Relative liver weight (%)	2.34 ± 0.27	2.38 ± 0.29	2.38 ± 0.34	2.44 ± 0.29	2.85** ± 0.43 (↑22)	2.64 ± 0.41	2.59 ± 0.31	2.58 ± 0.31	2.55 ± 0.36	2.9** ± 0.47 (↑10)
Histopathology										
Interim kill										
Liver										
Fatty change	8/20	11/20	14/20	8/20	16/20	5/20	2/20	7/20	10/20	11/20
Hepatocellular hypertrophy	1/20	3/20	7/20	10/20	8/20	2/20	0/20	4/20	6/20	12/20
Terminal kill										
Liver										
Fatty change	30/50	14/50	21/20	34/50	38/50	13/20	12/50	10/50	16/50	26/50*
Vacuolated foci	6/50	13/50	7/50	7/50	15/50*	6/50	1/50	1/50	3/50	3/50
Hepatocellular hypertrophy	0/50	2/50	2/50	7/50*	15/50*	1/50	2/50	1/50	7/50*	5/20
Thyroid										
Follicular hyperplasia, diffuse	5/50	5/16	8/18	7/19	11/49	3/49	1/14	1/15	8/18	5/49
Follicular hyperplasia, focal	1/50	0/16	0/18	1/19	3/49	3/49	0/14	0/15	1/18	5/49
Follicular hypertrophy, diffuse	1/50	0/16	1/18	1/19	2/49	0/49	0/14	0/15	0/18	3/49

FOB: functional observational battery; ppm: parts per million; *Source*: Sommer (2009b)
↓: decrease; ↑: increase; *: $P < 0.05$; **: $P < 0.01$

[a] Standard deviations for haematology parameters were not given in the study report.

Neoplastic changes were limited to males at 2400 ppm and consisted of a slight increase in liver carcinomas and C-cell and follicular cell carcinomas in the thyroid. The liver carcinomas were within historical control range, while the thyroid tumours were at the high end or outside of the historical control range (Table 11).

Table 11. Neoplasms in the two-year oral toxicity study of fenpyrazamine in rats [a]

Neoplasm	Number of neoplasms per dose level in males					
	0 ppm	100 ppm	300 ppm	1200 ppm	2400 ppm	HC
Liver						
Adenoma	1/50	1/50	1/50	1/50	1/50	0–4
Carcinoma	–	–	–	–	2/50	0–4
Thyroid						
C-cell adenoma	4/50	2/50	2/50	8/50	3/49	4–8
C-cell carcinoma	–	1/50	1/50	–	3/49	0–1
Follicular cell adenoma	2/50	4/50	2/50	3/50	3/49	0–6
Follicular cell carcinoma	–	1/50	1/50	–	3/49	0–3

HC: historical control data; ppm: parts per million

[a] Results shown as no. of neoplasms found / number of animals examined.

Source: Sommer (2009b)

The NOAEL was 1200 ppm (equal to 52 mg/kg bw per day for males and 64 mg/kg bw per day for females) based on decreased body weights compared to controls. The LOAEL was 2400 ppm (equal to 107 mg/kg bw per day for males and 130 mg/kg bw per day for females). Findings suggestive of thyroid carcinogenicity were observed at the high dose in male rats (Sommer, 2009b).

2.4 Genotoxicity

(a) In vitro studies

A range of GLP-compliant in vitro studies of the genotoxicity of fenpyrazamine was conducted to assess its potential for inducing chromosomal aberration, gene mutation and reverse mutation (Table 12). There was no evidence for genotoxicity or mutagenicity in the presence or absence of metabolic activation.

(b) In vivo studies

GLP-compliant unscheduled DNA synthesis and micronucleus assays were conducted to assess the potential of fenpyrazamine to damage DNA and impede repair in vivo (Table 12). There was no evidence of genotoxicity.

Table 12. Genotoxicity studies with fenpyrazamine

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 156, 313, 625, 1250, 2500 or 5000 µg/plate (±S9)	94.7	Negative	Kitamoto (2006a)
Mammalian cell gene mutation	Chinese hamster lung V79 cells HGPRT locus	0, 2.5, 6, 10, 20, 30, 40, 50 or 60 µg/mL –S9 for 4 h 0, 2, 12.5, 25, 50, 75, 100, 150, 175 or 200 µg/mL + S9 for 4 h 0, 25, 40, 55, 70, 85, 100, 115 or 130 µg/mL –S9 for 24 h 0, 20, 40, 60, 80, 100, 120, 140 or 160 µg/mL +S9 for 4 h	94.7	Negative	Wollny (2007)
Chromosomal aberrations	Chinese hamster lung CHL-IU cells	0, 105, 120 or 135 µg/mL – S9 for 6 h 0, 80, 120 or 160 µg/mL +S9 for 6 h 0, 40, 80, 120 or 160 +S9 0, 80, 120 or 160 µg/mL +S9	94.7	Negative	Kitamoto (2006b)
In vivo					
Mouse micronucleus	CD-1 mouse bone marrow, males and females	0, 500, 1000 or 2000 mg/kg bw – 24 h 0 or 2000 mg/kg bw – 48 h Harvest time: 24 and 48 h	94.7	Negative	Kitamoto (2007)

bw: body weight; HGPRT: hypoxanthine guanine phosphoribosyltransferase;
S9: 9000 × *g* supernatant fraction from liver homogenate from phenobarbital and 5,6-benzoflavone-treated rats (metabolic activation)

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a range-finding oral reproductive toxicity study, fenpyrazamine (purity 94.7%) was administered in the diet to young adult HanRcc:WIST(SPF) Wistar rats (n = 5/sex per group) at 0, 400, 1000 or 3000 ppm (equal to 0, 29, 75 and 245 mg/kg bw per day for males and 0, 32, 80, 254 mg/kg bw per day for females, respectively) for two weeks pre-pairing until 42 days after pairing in males and the end of the lactation period in females. Animals were monitored for mortality and clinical signs twice daily. Pre-mating parental animals were weighed weekly; thereafter, males were weighed weekly, while gestating females were weighed on gestation days 0, 7, 14 and 21 and dams were weighed on postnatal days 1, 4, 7, 14 and 21. Feed consumption was measured weekly (except during pairing) up to postnatal day 14. Day of birth was designated postnatal day 0.

Litters were examined as soon as possible for litter size, live births, stillbirths and any gross abnormalities. Pups were weighed on postnatal day 0 and/or 1, 4, 7, 14, 21 and 28; sex ratio was determined at first litter check and on postnatal day 21; and clinical signs and mortality were monitored daily. At postnatal day 4, litters were standardized to eight pups. Parental animals were killed just after weaning, and pups were maintained on treated diets one week past weaning and killed on postnatal day 28. Parental animals underwent gross necropsy, with the number of implantation sites in the uteri counted. Liver, thyroid and gross lesions were histopathologically examined. Dead pups, culled pups and pups killed at the end of the study underwent gross necropsy. Pup body weight, brain, spleen and thymus weights were measured in at least one male and female per litter.

There were no unscheduled deaths and no clinical signs of toxicity in the parental animals. Body weights were unaffected by treatment in the pre-pairing period. Body weight gain was decreased compared to controls in males at all doses following mating. Body weight and body weight gain were decreased in high-dose females during gestation. Feed intake was decreased in pre-mating males and gestating females. There were no effects on number of pregnancies, length of gestation or number of implantation sites. Post-implantation loss (↓8.4%) and number of live pups at birth (↓2.9 pups) were slightly decreased at high dose compared to controls. While there was an increase in enlarged thyroids in males at all doses and in females at 3000 ppm, no changes were noted at gross necropsy. Pup body weights were decreased at 3000 ppm along with post-weaning feed intake (Pössnecker, 2008).

In a two-generation oral reproductive toxicity study, fenpyrazamine (purity 94.7%) was administered in the diet at doses of 0, 400, 1000 or 3000 ppm (equal to 0, 27, 69 and 213 mg/kg bw per day for males and 0, 32, 80 and 237 mg/kg bw per day for females, respectively). Treated diet was administered for 10 weeks pre-pairing until 43 days after pairing in males and the end of the lactation period in females. From these rats, 24 males and 24 females selected to become the F_1 parental animals were given treated diet for 70 days pre-mating starting at approximately 28 days of age, through gestation and lactation until postnatal day 21. Parameters evaluated included clinical observations, body weight, body weight gain, feed consumption, feed efficiency, gestation length, organ weight and histopathology, implantation site numbers, post-implantation loss (%), implantation efficiency, mean number of pups per litter, per cent born alive, 0–4 day viability, viability index, lactation index, precoital interval, vaginal patency, preputial separation, oestrous cycle parameters, sperm parameters, ovarian follicle counts, sex ratio, mean pup weights, anogenital distance, mating index, fertility index, gestation index and litter survival.

There were no treatment-related paternal mortalities or clinical signs of toxicity. There were no effects on body weight gain, sperm parameters or ovarian histopathology. At 1000 ppm, liver and thyroid weights were increased in F_1 and F_2 males and females, body weights were decreased in F_1 males and thyroids were enlarged macroscopically in F_1 females (Table 13). At the high dose of 3000 ppm, body weights were decreased in males and females of both generations. There was an increase in centrilobular hepatocellular hypertrophy and thyroid follicular hypertrophy/hyperplasia in F_0 generation males and females. Feed intake was decreased in F_1 males and females along with enlarged thyroids in F_1 males and brownish foreign pigment within the bile ducts and peribiliary chronic inflammation in F_1males and females. In F_0 generation females, feed intake was decreased and there was an increase in enlarged livers.

Table 13. Effects of fenpyrazamine on parental rats in two-generation reproductive toxicity study

Generation / time period	Measure per dose level[a]							
	Males				Females			
	0 ppm	400 ppm	1000 ppm	3000 ppm	0 ppm	400 ppm	1000 ppm	3000 ppm
F_0 generation								
Body weight gain (g)[b]								
Pre-mating period	189	198 (+4.8%)	185 (−2.1%)	171 (−9.5%)	98	95 (−3.1%)	99 (+1%)	69 (−29.6%)
Gestation	36	41 (+13.9%)	37 (+2.8%)	42 (+16.7%)	117	119 (+1.7%)	124 (+6.0%)	96 (−17.9%)
Lactation	–	–	–	–	36	38 (+5.6%)	36 (0.0%)	47 (+30%)
Mean feed consumption (g/rat per day)								
Pre-mating period	23.0	23.4	22.7	22.9	17.5	17.6	17.6	15.8**
Gestation	23.1	23.6	23.1	23.4	22.6	23.1	22.9	20.1*
Lactation	–	–	–	–	46.0	44.4	45.7	39.8
Organ weights[b]								

Generation / time period	Measure per dose level [a]							
	Males				Females			
	0 ppm	400 ppm	1000 ppm	3000 ppm	0 ppm	400 ppm	1000 ppm	3000 ppm
Necropsy body weight (g)	430 ± 26.8	443 ± 35.4	424 ± 32.9	413 ± 28.8	297 ± 27.8	287 ± 23.0	289 ± 22.1	267** ± 18.8
Absolute liver weight (g)	11.7 ± 1.11	13.0** ± 1.52	13.2** ± 1.28	15.3** ± 1.38	14.5 ± 1.8	13.6 ± 2.2	15.3 ± 1.47	16.3* ± 2.35
Relative liver weight (%)	2.7 ± 0.18	2.9** ± 0.17	3.1** ± 0.18	3.7** ± 0.22	4.9 ± 0.43	4.7 ± 0.55	5.3* ± 0.37	6.1** ± 0.58
Absolute thyroid weight (mg) R / L [c]	11 ± 3 / 10 ± 2	11 ± 2 / 10 ± 2	11 ± 2 / 10 ±1	14** ± 3 / 13** ± 3	9 ± 2 / 8 ± 2	9 ± 2 / 10 ± 3	12* ± 3 / 11* ± 3	18** ± 6/ 16** ± 5
Relative thyroid weight (%) R / L [c]	0.002 ± 0.001/ 0.002 ± 0	0.002 ± 0.001/ 0.002 ± 0	0.003 ± 0.001/ 0.002 ± 0	0.003** ± 0.001/ 0.003** ± 0.001	0.003 ± 0.001/ 0.003 ± 0.001	0.003 ± 0.001/ 0.003 ± 0.001	0.004* ± 0.001/ 0.004* ± 0.001	0.007** ± 0.002/ 0.006** ± 0.002
Histopathological findings (incidence) [c,d]								
Liver, hepatocellular hypertrophy	2/24	0/24	2/24	11/24	12/24	13/24	19/24	24/24
Liver, fatty change	10/24	15/24	18/24	14/24	10/24	6/24	1/24	2/24
Thyroid, follicular cell hypertrophy	1/24	2/24	5/24	20/24	0/24	0/24	14/24	22/24
Thyroid, follicular cell hyperplasia	0/24	0/24	0/24	3/24	0/24	0/24	0/24	5/24
F_1 generation								
Body weight gain (g) [b]								
Pre-mating period	300	292 (−2.7%)	281 (−6.3%)	249 (−17.0%)	133	138 (+3.8%)	128 (−3.8%)	116 (−12.8%)
Gestation	55	57	62	48	104	114 (+9.6%)	114 (+9.6%)	78 (−25%)
Lactation	–	–	–	–	39	40 (+2.6%)	40 (+2.6%)	42 (+7.7%)
Mean feed consumption (g/rat per day)								
Pre-mating period	23.8	23.6	23.0	20.2**	16.8	17.1	16.3	14.0**
Gestation	23.8	23.6	23.6	21.6**	20.4	21.8	21.8	17.3*
Lactation	–	–	–	–	43.5	43.8	43.1	33.7**
F_1 parental animals								
Organ weights								
Necropsy body weight (g)	489 ± 56.6	481 ± 37.8	465 ± 45.3	392** ± 31.9	279 ± 27.2	289 ± 24.4	280 ± 21.3	228** ± 15.5
Absolute liver weight (g)	13.4 ± 1.71	14.2 ± 1.51	14.6 ± 2.1	14.8* ± 1.6	13.6 ± 2.06	14.7 ± 2.0	14.2 ± 1.44	13.1 ± 1.43
Relative liver weight (%)	2.8 ± 0.17	2.9** ± 0.23	3.1** ± 0.29	3.8** ± 0.17	4.9 ± 0.47	5.1 ± 0.46	5.1 ± 0.35	5.8** ± 0.51
Absolute thyroid weight (mg) R / L [c]	14 ± 3/ 13 ± 3	14 ± 3/ 14 ± 3	15 ± 3/ 14 ± 3	14 ± 3/ 14 ± 3	19 ± 4/ 19 ± 6	18 ± 5/ 18 ± 5	24* ± 6/ 22 ± 5	28** ± 8/ 26** ± 6
Relative thyroid weight (%) R / L [c]	0.003 ± 0.001/ 0.003 ± 0.001	0.003 ± 0.001/ 0.003 ± 0.001	0.003 ±0/ 0.003 ± 0.001	0.004** ± 0.001/ 0.004** ± 0.001	0.007 ± 0.002/ 0.007 ± 0.002	0.006 ± 0.002/ 0.006 ± 0.002	0.008 ± 0.002/ 0.008 ± 0.002	0.012** ± 0.004/ 0.012** ± 0.003

Generation / time period	Measure per dose level [a]							
	Males				Females			
	0 ppm	400 ppm	1000 ppm	3000 ppm	0 ppm	400 ppm	1000 ppm	3000 ppm
Histopathology (incidence) – F_1 parental animals [d]								
Liver, hepatocellular hypertrophy	0/24	0/24	5/24	10/24	0/24	0/24	6/24	20/24
Liver, fatty change	9/24	11/24	16/24	12/24	2/24	0/24	2/24	2/24
Liver, bile duct pigmentation	0/24	0/24	0/24	16/24	0/24	0/24	0/24	14/24
Liver, peribiliary inflammation	0/24	0/24	0/24	7/24	0/24	0/24	0/24	3/24
Thyroid, follicular cell hypertrophy	1/24	0/24	1/24	7/24	3/24	0/24	13/24	22/24
Thyroid, follicular cell hyperplasia	0/24	0/24	0/24	6/24	0/24	0/24	0/24	13/24

(*Continued on next page*)

F_1: first filial; F_0: parental; L: left; R: right; no.: number; ppm: parts per million;
*: $P < 0.05$; **: $P < 0.01$

[a] Standard deviations as given in study report for body weight gain and feed consumption.

[b] Results shown in units and, in parentheses, as a percentage of control value.

[c] Results shown in relevant units for organs on the right (R) and left (L) sides.

[d] Results shown as no. of animals with the findings / no. of animals examined.

Source: Gerspach, Weber & Flade (2009)

Changes to the reproductive parameters consisted of decreased birth weights at postnatal day 1 at 1000 ppm and decreased implantations and litter size and increased post-implantation loss at 3000 ppm (Table 14).

Table 14. Effects in female rats in two-generation reproductive toxicity study

	Measure per dose level			
	0 ppm	400 ppm	1000 ppm	3000 ppm
F_0 generation				
No. of pregnancies	22	23	24	23
Gestation index (%)	100	100	100	100
Gestation duration (days)	21.8	21.6	21.4	21.5
Mean number of implantations/dam	13.1	13.7	14.3	12.6
Total number of postimplantation loss (number of litters affected / no. of implantations)	15/35	16/31	16/31	18/30
Postimplantation loss (%)	12.2	9.9	9.1	10.8
Mean litter size (*n*)	11.5	12.3	13.0	11.2
Total number of postnatal loss (no. of litters affected / no. of pups)	4/4	3/11	5/6	8/12*
Postnatal loss (%)				
Days 0–4 postpartum	1.6	3.9	1.9	4.9 [a]
Days 0 – scheduled kill		0.7–4.8		
Viability index (%)				
Days 0–4 (%)	98.1			
Days 0 – scheduled kill [b]		95.2–99.6		

	Measure per dose level			
	0 ppm	400 ppm	1000 ppm	3000 ppm
				(*Continued on next page*)
F_1 generation				
Number of pregnancies	24	23	23	24
Gestation index (%)	100	100	95.7	100
Gestation duration (days)	21.7	21.7	21.6	21.5
Mean implantation/dam	12.6	13.3	12.7	10.6*
Total number of postimplantantion loss (no. litters affected / no. of implantations)	11/30	13/23	14/39	18*/49**
Postimplantation loss (%)	9.9	7.5	13.4	19.3
Mean litter size	11.4	12.3	11.0	8.5*
Total no. of postnatal loss (no. litters affected / no. pups)	0/0	4*/19**	6**/15**	10**/13**
Postnatal loss (%)				
Days 0–4 postpartum	0.0	6.7	6.0	6.3
HC: Days 0 – scheduled kill [b]			– 8.5	
Viability index (%)				
Days 0–4	100	93.3** [a]	94.0** [a]	93.7** [a]
HC: Days 0 – scheduled kill [b]			91.5–100	

HC: historical control data; *: $P < 0.05$ in comparison to controls; **: $P < 0.01$ in comparison to controls; no. number

[a] Outside of historical control data range.

[b] Historical control data, May 2002–December 2007. Study conducted from 2006 to 2007; study report finalized in 2009.

Source: Gerspach, Weber & Flade (2009)

Effects on offspring toxicity consisted of decreased pup weights in the F_1 and F_2 generations at 1000 ppm and delayed sexual maturation, hepatocellular hypertrophy and brownish foreign pigment in the bile ducts in the F_2 pups and increased postnatal loss in the F_1 pups (Table 15).

The parental NOAEL was 400 ppm (equal to 27 mg/kg bw per day for males and 32 mg/kg bw per day for females) based on increased liver and thyroid weights in both generations, decreased body weights in F_1 males and enlarged thyroid in F_1 females at the LOAEL of 1000 ppm (equal to 69 mg/kg bw per day in males and 80 mg/kg bw per day in females).

The reproductive NOAEL was 400 ppm (equal to 32 mg/kg bw per day) based on decreased birth weight on postnatal day 1 at the LOAEL of 1000 ppm (equal to 80 mg/kg bw per day).

The offspring NOAEL was 400 ppm (equal to 32 mg/kg bw per day in females) based on decreased pup weight in the F_1 and F_2 pups at the LOAEL of 1000 ppm (equal to 80 mg/kg bw per day in females) (Gerspach, Weber & Flade, 2009).

Table 15. Effects of fenpyrazamine on offspring in two-generation reproductive toxicity study

Parameter / generation	Measure per dose level (ppm)							
	Males				Females			
	0	400	1000	3000	0	400	1000	3000
F_1 pups								
Body weight (g) pp day 1	6.2 ± 0.16	6.0 ± 0.49	6.0 ± 0.58	5.3** ± 0.62	6.0 ± 0.57	5.7 ± 0.48	5.7 ± 0.67	5.1** ± 0.68
Body weight (g) pp day 21	50.5 ± 4.01	48.3 ± 7.32	47.6 ± 4.32	35.8** ± 3.50	49.1 ± 3.89	48.0 ± 2.43	46.1* ± 3.83	35.1** ± 3.40
At preputial separation/vaginal patency								
Age (days)	26.5 ± 1.5	26.0 ± 1.5	26.4 ± 1.1	27.7* ± 1.4	32.4 ± 1.1	32.9 ± 1.2	33.3 ± 1.2	35.9* ± 2.3

Parameter / generation	Measure per dose level (ppm)							
	Males				Females			
	0	400	1000	3000	0	400	1000	3000
Body weight (g)	78.9 ± 8.83	77.2 ± 7.62	73.0* ± 9.18	61.0** ± 6.55	101.9 ± 10.43	106.7 ± 8.53	106.0 ± 10.52	94.4* ± 10.33
F2 pups								
Histopathology (incidence)								
Liver, hepatocellular hypertrophy	0/24	0/22	0/21	2/23	0/24	0/22	0/21	5/22
Liver, bile duct pigmentation	0/24	0/22	0/21	8/23	0/24	0/22	0/21	7/22
Body weight (g) pp day 1	6.0 ± 0.59	5.9 ± 0.60	5.9 ± 0.54	5.1** ± 0.79	5.7 ± 0.54	5.6 ± 0.54	5.6 ± 0.60	4.7** ± 0.64
Body weight (g) pp day 14	30.5 ± 2.57	30.4 ± 2.11	29.4 ± 2.19	22.2** ± 2.98	29.7 ± 2.30	29.5 ± 2.06	27.9* ± 2.18	21.3** ± 2.74
Body weight (g) pp day 21	50.0 ± 4.13	50.0 ± 3.95	47.0 ± 3.68	34.1** ± 5.30	48.2 ± 3.49	48.0 ± 3.61	44.8* ± 3.54	32.3** ± 4.70

F_1: first filial generation; F_2: second filial generation; pp: postpartum; ppm: parts per million;
*: $P < 0.05$ in comparison to controls; **: $P < 0.01$ in comparison to controls

Source: Gerspach, Weber & Flade (2009)

(b) Developmental toxicity

Rat

In a range-finding oral developmental toxicity study, fenpyrazamine (purity 94.7%) in 1% w/v aqueous carboxymethylcellulose (CMC) was administered via gavage to pregnant and fasted young adult Wistar HanRcc:WIST(SPF) rats (n = 5/group) at 0, 150, 300 or 500 mg/kg bw per day from gestation days 6 to 20. The dams were monitored for mortality and clinical signs of toxicity twice daily. Body weights were measured daily from day 0 to 21, and feed consumption was measured at three-day intervals (days 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–21) throughout pregnancy. The dams were killed on gestation day 21 and the fetuses removed by caesarean section. The dams underwent gross necropsy including of the uteri and ovaries. Placental weights were measured separately. Fetuses were sexed, weighed and examined for gross external abnormalities. One fetus with an obvious skeletal defect was stained for skeletal examination.

One dam in the 500 mg/kg bw per day dose group died after being observed as having discharge from the snout; gas-filled stomach with reddish contents and partly emphysematous and reddish coloured lungs were noted at gross necropsy. Body weight gain was decreased at 300 and 500 mg/kg bw per day and feed consumption was decreased at 500 mg/kg bw per day. Fetal body weights were decreased and placental weights were increased at 500 mg/kg bw per day (Pössnecker & Flade, 2006).

In an oral developmental toxicity study, fenpyrazamine (purity 94.7%) was administered via gavage to young adult, pregnant fasted Wistar HanRcc:WIST(SPF) rats (n = 22/group) at 0, 30, 125 or 500 mg/kg per day from gestation day 6 to 20. The dams were monitored for mortality and clinical signs of toxicity twice daily. Body weights were measured daily from day 0 to 21 and feed consumption was measured at three-day intervals (days 0–3, 3–6, 6–9, 9–12, 12–15, 15–18 and 18–21) throughout pregnancy. The dams were killed on gestation day 21, and fetuses removed by caesarean section. Dams underwent gross necropsy including of the uteri and ovaries. Placental weights were measured separately. The fetuses were sexed, weighed and examined for gross external abnormalities; half were allocated for microdissection for visceral abnormalities and the other half for skeletal examination.

No maternal deaths, treatment-related clinical signs of toxicity or effects on reproductive parameters or gross pathology were noted. At 125 and 500 mg/kg bw per day, maternal body weight and body weight gain were decreased compared to controls. Feed consumption was decreased and placental weights were increased at 500 mg/kg bw per day.

In the fetuses, there were no effects at or below 125 mg/kg bw per day. At 500 mg/kg bw per day, fetal body weights were decreased and there was an increase in visceral and skeletal variations including supernumerary liver lobes, dilated ureters, dilated urinary bladder and pelvis, zygomatic arch fusions, offset sternebrae, sternebrae with misshapen ossification sites, incomplete ossification of the frontal bone and/or asymmetrically aligned cartilages at sternum (Table 16). These changes were considered to be as a result of maternal toxicity.

Table 16. Fetal visceral and skeletal changes in a developmental toxicity study of fenpyrazamine in rats

Findings	Measure per dose level (mg/kg bw per day)			
	0	**30**	**125**	**500**
Visceral fetal findings				
Liver abnormal lobation				
Total fetuses/total litter	4/4	6/4	8/8	14**/10*
(% litter)	(18)	(19)	(36)	(50) [a]
Historical control data (2002–2007) (% litter)		5–41		
Renal pelvis dilated				
Total fetuses/total litter	1/1	0/0	2/2	7*/5
(% litter)	(5)	(0)	(9)	(25) [a]
Historical control data (2002–2007) (% litter)		5–14		
Ureter dilated				
Total fetuses/total litter	0/0	0/0	0/0	3/3
(% litter)	–	–	–	(15%) [a]
Historical control data (2002–2007) (% litter)		0		
Urinary bladder distended				
Total fetuses/total litter	0/0	0/0	0/0	3/3
(% litter)				(15%)[a]
Historical control data (2002–2007) (% litter)		0–5		
Skeletal fetal findings				
Skull: zygomatic arch fusion				
Total fetuses/total litter	6/5	3/2	13/7	29**/17**
(% litter)	(23) [a]	(10)	(32) [a]	(85) [a]
Historical control data (2002–2007) (% litter)		5–18		
Sternebrae offset/misshapen ossification site				
Total fetuses/total litter	2/1	0/0	3/3	7/7*
(% litter)	(5)	(0)	(14)	(35) [a]
Historical control data (2002–2007) (% litter)		0–14		
Costal cartilage asymmetrically aligned at sternum				
Total fetuses/total litter	1/1	2/2	4/4	8*/7*
(% litter)	(5)	(10)	(18)	(35)[a]
Historical control data (2002–2007) (% litter)		0–23		
Os frontal (L), incompletely ossified (% litter)	0	0	0	25** [a]
Historical control data (2002–2007) (% litter)	0			
Os frontal (R), incompletely ossified (% litter)	0	0	0	25** [a]
Historical control data (2002–2007) (% litter)		0		

bw: body weight; L: left; R: right; *: $P < 0.05$ (Fisher exact test); **: $P < 0.01$ (Fisher exact test)

[a] Above historical control data

Source: Gerspach & Flade (2009)

The maternal NOAEL was 30 mg/kg bw per day based on decreased body weight and body weight gain at the maternal LOAEL of 125 mg/kg bw per day.

The developmental NOAEL was 125 mg/kg based on decreased fetal weight and increased visceral and skeletal variations and delayed ossification at the developmental LOAEL of 500 mg/kg bw per day. There was no evidence of teratogenicity or increased sensitivity of the young (Gerspach & Flade, 2009)

Rabbit

In a range-finding oral developmental toxicity study, fenpyrazamine (purity 94.7%) in 1% CMC was administered by gavage to artificially inseminated young adult Kbl:NZW SPF New Zealand White rabbits (n = 5/group) at 0, 60, 90, 120 or 150 mg/kg bw per day from gestation day 6 to 27. The does were monitored for mortality and clinical signs of toxicity twice daily during dosing and once daily outside the dosing period. Body weights were measured on gestation days 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28, and feed consumption at three-day intervals (gestation days 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24, 24–27 and 28) throughout pregnancy. The surviving dams were killed on gestation day 28, and the fetuses were removed by caesarean section. Dams underwent gross necropsy including of the uteri and ovaries. Placental weights were measured separately. Fetuses were sexed, weighed and examined for gross external abnormalities.

There were no effects on maternal body weight or body weight gain. At 120 mg/kg bw per day, one doe died. At 120 and 150 mg/kg bw per day, does had clinical signs of orange urine, red fluid under the grid floor of cage and/or red fluid around the vagina. At 150 mg/kg bw per day, there was a decrease in feed intake that was not reflected in the body weight parameters. There was a treatment-related increase in abortions starting with one abortion at 90 mg/kg bw per day. There were no effects on the fetus; however, only 5, 5, 3, 2 and 1 dams at 0, 60, 90, 120 and 150 mg/kg bw per day, respectively, were available for assessment of developmental toxicity (Inawaka, 2008a).

In an oral developmental toxicity study, fenpyrazamine (purity 94.7%) in 1% CMC was administered by gavage to artificially inseminated young adult Kbl:NZW SPF New Zealand White rabbits (n = 24/group) at 0, 30, 50 or 90 mg/kg bw per day from gestation days 6 to 27. Does were monitored for mortality and clinical signs of toxicity twice daily during dosing and once daily outside the dosing period. Body weights were measured on gestation days 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28, and feed consumption was measured at 3-day intervals (gestation days 3–6, 6–9, 9–12, 12–15, 15–18, 18–21, 21–24, 24–27 and 28) throughout pregnancy. Surviving dams were killed on gestation day 28 and fetuses removed by caesarean section. Dams underwent gross necropsy including of the uteri and ovaries. Placental weights were measured separately. The fetuses were sexed, weighed and examined for gross external abnormalities. Fetuses with external abnormalities did not undergo visceral or skeletal examination. The cervical, thoracic and abdominal viscera of all fetuses were examined for visceral alterations. The heads of approximately half of the live fetuses were examined by cross-section of the coronal suture. The heads of the remaining fetuses and hearts of all fetuses were fixed in Bouin's solution and examined. The remaining fetuses were allocated for skeletal examination.

There were no effects on maternal mortality and there was no treatment-related effect on the number of pregnancies. There was a treatment-related increase in abortions and premature delivery with one abortion at 50 mg/kg bw per day and seven at 90 mg/kg bw per day, all occurring between gestation day 24 and 27. Clinical signs of toxicity consisted of orange urine and red fluid found in the tray pan under the grid floor. Clinical signs of toxicity were limited to the animals that subsequently aborted or delivered prematurely. Feed intake was severely suppressed in two dams at 50 mg/kg bw per day and six at 90 mg/kg bw per day. Increases in distention of the gallbladder, pale liver, white foci in the liver, muddy and dark brown content in the caecum (at 50 mg/kg bw per day only) were noted at gross necropsy. In addition, pale hearts were seen in the does that either aborted or littered prematurely. There was no evidence of teratogenicity in the fetuses.

The maternal NOAEL was 30 mg/kg bw per day based on increased abortions, orange urine and red fluid in the tray pan, decreased feed intake, distention of the gallbladder, pale liver with white foci, muddy and dark brown content in the caecum and pale hearts at the maternal LOAEL of 50 mg/kg bw per day.

The developmental NOAEL was 30 mg/kg bw per day based on abortion at 50 mg/kg bw per day (Inawaka, 2008b).

2.6 Special studies

(a) Neurotoxicity

In an acute time-to-peak-effect oral neurotoxicity study, fenpyrazamine (purity 94.8%) in 1% CMC was administered by gavage to unfasted, young adult HanRcc:WIST Wistar rats (n = 3/sex per dose) at 0, 100, 1000 or 2000 mg/kg bw. The animals were observed at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours on day 1 and once daily thereafter for up to day 8. Mortality/viability was recorded twice daily during days 1–8. Body weights were recorded at acclimatization, on day 1 (prior to administration) and on day 8. All animals were necropsied on day 8 and examined macroscopically.

There were no effects on mortality, clinical signs of toxicity or gross pathology. Body weights were decreased at the end of the observation period at 2000 mg/kg bw in males only. There were no effects on body weight in females.

Based on the absence of relevant treatment-related clinical signs, it was concluded that no peak effect was established after oral administration of fenpyrazamine at up to 2000 mg/kg bw in rats (Sommer, 2007).

In an acute oral neurotoxicity study, fenpyrazamine (purity 94.7%) was administered by gavage in 1% aqueous CMC to young adult, fasted HanRcc:WIST Wistar rats (n = 10/sex per dose) at 0, 80, 400 or 2000 mg/kg bw. The animals were observed for up to 14 days after dosing. Feed consumption and body weight was measured on days 1, 8 and 14. A detailed clinical observation and FOB were performed pretest and on days 1, 7 and 14. At termination, five animals per sex per group were selected for neuropathological examination. The animals were killed, the brains with olfactory bulb weighed and the brain, spinal cord, eyes, tibial and sciatic nerves, gastrocnemius muscle and all gross lesions preserved for histopathological examination.

There were no effects on mortality or clinical signs of toxicity. Body weight and feed consumption were decreased initially in males at 2000 mg/kg bw, but there was no effect from day 7 onwards and no effects in females. On day 1, there was a decrease in locomotor activity in males at 400 mg/kg bw and in females at 2000 mg/kg bw, but no effects on locomotor activity on days 7 or 14. No effects were seen in the neurohistopathological examination. The changes in locomotor activity were attributed to transient systemic toxicity and, based on the lack of histopathological changes, considered not due to specific neurotoxicity.

The NOAEL was 80 mg/kg bw based on decreased locomotor activity at the LOAEL of 400 mg/kg bw in males and 2000 mg/kg bw in females. There was no evidence of specific neurotoxicity (Sommer, 2008a).

In a 90-day oral neurotoxicity study, fenpyrazamine (purity 94.7%) was administered in the diet to HanRcc:WIST Wistar rats (n = 10/sex per dose) at 0, 500, 1200 or 3000 ppm (equal to 0, 37, 88 and 224 mg/kg bw per day for males and 0, 42, 100 and 248 mg/kg bw per day for females, respectively). Animals were monitored for viability/mortality twice daily and for clinical signs of toxicity once daily; detailed clinical observations, including a pretest observation, were performed once weekly. An FOB including locomotor activity was performed during pretest and at weeks 2, 5, 9 and 13. Feed consumption and body weights were recorded once weekly during acclimatization, twice weekly during weeks 1–4 and weekly during weeks 5–13. Ophthalmoscopic observations were performed during acclimatization and week 13. At scheduled kill, five animals per sex per group were selected for neuropathological examination. Animals were killed, the brain with olfactory bulb was weighed and the brain, spinal cord, eyes, tibial and sciatic nerves, gastrocnemius muscle and all gross lesions were preserved for histopathological examination.

There were no effects on mortality, clinical signs of toxicity or feed consumption. Ophthalmoscopic evaluation, FOB including locomotor activity and neurohistopathological examination found no changes. Body weights were decreased by 11.5% in males and 8.7% in females by the end of the study, with body weight gains decreased 13% and 21.6%, respectively.

The NOAEL was 1200 ppm (equal to 88 mg/kg bw per day in males and 100 mg/kg bw per day in females) based on decreased body weight and body weight gain at the LOAEL of 3000 ppm (equal to 224 mg/kg bw per day in males and 248 mg/kg bw per day in females) (Sommer, 2008b).

(b) Immunotoxicity

In a four-week oral immunotoxicity study, fenpyrazamine (purity 94.7%) was administered in the diet to female Han Wistar rats (n = 10/dose) at 0, 500, 1500 or 4000 ppm (equal to 0, 36, 123 and 392 mg/kg bw per day, respectively) for 28 days. On day 25, all animals were immunized with a suspension of sheep red blood cells (2×10^8 SRBC) by intravenous injection (1 mL/animal). A positive control group of female rats (n = 8) was given an intraperitoneal injection of cyclophosphamide at 50 mg/kg bw on day 27 (two days prior to scheduled kill). On day 29, all animals were killed and their spleens were harvested for evaluation of T-cell dependent antibody response (TDAR) with an anti-SRBC plaque-forming cell (PFC) assay. Other parameters evaluated were mortality, clinical signs, body weight changes, feed and water intake, gross pathology and liver, spleen and thymus weights.

There were no deaths, clinical signs of toxicity or effects on feed or water consumption. No changes were noted at gross necropsy. Body weights and body weight gains were decreased in the high-dose group. There was a nonstatistically significant increase in absolute liver and spleen weights and a statistically significant increase in relative liver and spleen weights at the high dose. Absolute thymus weights were nonstatistically significantly decreased by 17%. While no changes were observed at gross necropsy, in the absence of histopathological examination, the changes in organ weight were considered adverse. There was no evidence of immunotoxicity in the adequately validated plaque-forming-cell assay.

The NOAEL was 1500 ppm (equal to 123 mg/kg bw per day) based on decreased body weight and body weight gain, increased liver and spleen weights and decreased thymus weights at the LOAEL of 4000 ppm (equal to 392 mg/kg bw per day) (Saunders, 2010).

(c) Studies on metabolites

In an acute oral toxicity study, the metabolite S-2188-DC (purity 100%) was considered to be of moderate acute oral toxicity based on a lack of mortality in female rats (BrlHan:WIST@Jcl (GALAS)) at 500 mg/kg bw, the highest dose tested (Asano, 2008).

Negative results were obtained in an in vitro genotoxicity study of S-2188-DC (Table 17) (Kitamoto, 2008).

Table 17. Genotoxicity study on a metabolite of fenpyrazamine

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Ref
In vitro						
S-2188-DC	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 156, 313, 625, 1250, 2500 and 5000 µg/plate (± S9)	100	Negative	Kitamoto (2008)

S9: 9000 × *g* supernatant fraction from liver homogenate (metabolic activation)

(d) Mode-of-action studies

A number of studies have investigated the effect of fenpyrazamine on the liver and thyroid tumours in male rats.

In a time-course oral study, fenpyrazamine (94.7%) was administered in the diet to male Wistar (BrlHan:WIST@Jcl(GALAS) rats (n =v10/group) for 3, 7 or 14 days at 0 or 2400 ppm (equal to 0 and 216.5, 0 and 222.6 and 0 and 216.6 mg/kg bw per day in the 3-, 7- and 14-day groups, respectively). Animals were observed for mortality and clinical signs once daily; feed consumption and body weight were monitored. Unfasted, sedated animals were killed via decapitation, and blood was collected for hormone analysis. Serum hormone concentrations were determined by immunoassay for total triiodothyronine (T_3), tetraiodothyronine (T_4; thyroxine) and thyroid-stimulating hormone (TSH). Animals underwent a detailed necropsy; livers and thyroids were weighed and histopathologically examined. Liver samples were collected to determine 5-bromo-2′-deoxyuridine (BrdU) indices and duodenum samples were collected as an immunohistochemical staining control.

There were no unscheduled deaths and no effects on clinical signs of toxicity, body weight or feed consumption. Changes to the liver consisted of a gross enlargement, increased organ weights and centrilobular hepatocellular hypertrophy. In addition, there was a pronounced increase in hepatic

cytochrome P450 (CYP) 2B activity, determined as 7-pentoxyresorufin *O*-dependentylase (PROD) activity, and an increase of hepatic thyroxine-uridine 5′-diphospho-glucuronosyltransferase (T_4-UGT) activity toward thyroxine. BrdU indices were increased after the three-day treatment, indicating an increase in replicative DNA synthesis (not seen in the 7- and 14-day groups) and that a peak in hepatocellular proliferation occurred in less than one week. S9 protein content was increased in the 7-day group only, while PROD and T_4-UGT activity were increased in all time groups.

Thyroid weights were increased in the 7- and 14-day groups, and follicular cell hypertrophy was increased in all treated groups. There was a time-related increase in TSH starting at 40% in the three-day group to 75% in the 14-day group. T_3 was decreased by 38% in the three-day group and 29% in the seven-day group, and was unchanged in the 14-day group. T_4 was decreased by 31% in the three-day group, 14% in the seven-day group and 2% in the 14-day group (Kondo, 2010)

Appendix 1 contains further summaries of the MOA studies within the International Programme on Chemical Safety (IPCS) framework.

In an in vitro study to determine the role of the constitutive androstene receptor (CAR) nuclear receptor in fenpyrazamine-induced messenger ribonucleic acid (mRNA) expression of CYP2B1, UGT1A and UGT2B1, rat hepatocytes were transfected with short-interfering ribonucleic acid (siRNA) to impair CAR synthesis. The hepatocytes were then exposed to fenpyrazamine (purity 97.4%) at concentrations of 0 or 50 µmol/L. The concentration of fenpyrazamine was selected as the highest concentration without cytotoxicity in a range-finding assay that tested concentrations of 10, 25, 50, 75, 100, 150, 300 and 450 µmol/L. Hepatocyte cultures of untransfected cells and of cultures transfected either with short-interfering ribonucleic acid for constitutive androstene receptor siRNA(CAR) or with short-interfering ribonucleic acid for negative control (siRNA(NC)) were incubated in a medium containing fenpyrazamine.

At a concentration of fenpyrazamine of 50 µmol/L, a peak effect on mRNA expression levels of CYP2B1 and UGT2B1 was observed. An additional culture of untransfected cells, not exposed to S-2188, served as an untransfected control. Four wells per culture were assayed. Cultures of transfected or untransfected cells were incubated under test conditions for three days. On day 3, cultures were terminated and total RNA extracted. The RNA was purified to remove DNA contamination. From the RNA, complementary DNA (cDNA) was prepared by reverse transcription. The cDNA was then assayed by real-time polymerase chain reaction (PCR) using specific primers for rat CAR, CYP2B1, UGT1A and UGT2B1. Quantitative real-time PCR assays for rat CAR, CYP2B1, UGT1A and UGT2B1 were performed.

While untreated hepatocytes exposed to fenpyrazamine exhibited increased CAR enzyme induction, CAR knockdown hepatocytes exhibited decreased enzyme induction (Table 18). This would indicate that increased mRNA production is mediated by CAR (Yamada, 2010a).

Table 18. mRNA levels for CAR enzyme induction in rat hepatocytes

Gene[a]	Untreated control	Fenpyrazamine	Fenpyrazamine + negative control siRNA	Fenpyrazamine + siRNA(CAR) (CAR knockdown)
CAR/GAPDH	0.232	1.025**	0.906	0.126##
CYP2B1/GAPDH	0.241	0.865**	0.657	0.266##
UGT1A/GAPDH	0.785	1.024**	1.031	0.382##
UGT2B1/GAPDH	0.049	1.495**	1.061	0.330##

CAR: constitutive androstene receptor; cDNA: complementary deoxyribonucleic acid; CYP: cytochrome P450; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mRNA: messenger ribonucleic acid; siRNA: short-interfering RNA; siRNA(CAR): short-interfering ribonucleic acid for constitutive androstene receptor; UGT: uridine-5'-diphospho-glucuronosyltransferase; **: $P < 0.01$ in comparison to untreated control; ## : $P < 0.01$ in comparison to transfected short-interfering RNA for negative control (siRNA(NC))

[a] Quantity of each cDNA is normalized to GAPDH, so it is expressed as a ratio to GADPH; $N = 4$ wells/culture

Source: Yamada (2010a)

3. Observations in humans

The sponsor submitted a report on the health status of factory workers engaged in the production of fenpyrazamine technical. The report stated that no exposure-related effects were observed during extensive medical examinations. The sponsor attributed this to the low toxicity of the compound in conjunction with adequate personal protective equipment (Nishimoto, 2016).

Comments

Biochemical aspects

In metabolism studies conducted in rats using fenpyrazamine labelled with ^{14}C at the 5-pyrazolyl position, the absorption of fenpyrazamine was rapid and extensive, with almost complete metabolism at low doses (3 mg/kw bw). Approximately 4% was eliminated unmetabolized in the faeces in the high-dose groups (300 mg/kg bw). There were no significant sex differences in pharmacokinetics. Maximum blood and plasma ^{14}C concentrations (C_{max}) were observed at 1 and 6 hours (T_{max}) after administration in the low- (3 mg/kg bw) and high-dose (300 mg/kg bw) groups, respectively, and C_{max} values ranged between 1.5 and 2.0 µg/g at low doses and 45 and 68 µg/g at high doses. The half-lives were 2–3 hfor the low dose and 14–17 h for the high dose. The AUC for the 300 mg/kg bw dose was 150- to 170-fold greater than that for the 3 mg/kg bw dose, indicating some saturation in the elimination processes at the higher dose (Dohn, 2007).

Less than 0.5% of the administered dose was retained in the tissues 12 hours following a single oral low dose. Peak radioactive residues were generally detected in the plasma, liver, kidneys and stomach within the first few hours after dosing. After repeated-dose administration, the radiolabel reached steady state from days 7 to 15 and then declined from the cessation of dosing until 97.2% had been excreted by day 25. The stomach (including contents) and the liver had the highest levels of radioactivity in the repeated-dose study (Quistad & Kovatchev 2007a,b).

Following administration of a single low and single high dose, the main route of elimination was urinary (80–87% administered dose). Faecal elimination of fenpyrazamine was minor (8–12% administered dose). Elimination was rapid, with about 90% of the radiolabel eliminated within 24 hours for the low-dose group and 48 hours for the high-dose group. There was no significant difference in route or rate of elimination between males and females. Elimination via expired air was negligible. With repeat dosing for 14 days, the excretion patterns of orally administered [^{14}C]fenpyrazamine were similar to those after single doses (Dohn, Kavatchev & Estigoy 2007, Quistad & Kovatchev 2007b).

The principal routes of metabolism of fenpyrazamine involved hydrolysis to remove the allylsulfanylcarbonyl group to produce the main metabolite, S-2188-DC, followed by dealkylation of S-2188-DC to MPPZ and hydroxylation to S-2188-CH_2OH-DC and S-2188-OH. MPPZ was then conjugated with sulfate and with glucuronide prior to elimination. The metabolite profile was qualitatively similar in males and females at both dose levels as well as after single and repeat 14-day dosing (Dohn, Kavatchev & Estigoy, 2007; Quistad & Kovatchev, 2007b).

Toxicological data

In rats, fenpyrazamine had an oral median lethal dose (LD_{50}) greater than 2000 mg/kg bw, a dermal LD_{50} greater than 2000 mg/kg and an inhalation median lethal concentration (LC_{50}) greater than 4.84 mg/L. Fenpyrazamine was non-irritating to the skin and eyes of rabbits. It did not induce dermal sensitization in guinea pigs (Deguchi, 2007a,b,c; Odawara, 2007a,b,c).

The main toxic effects of fenpyrazamine in short- and long-term toxicity studies in mice, rats and dogs were decreased body weight and body weight gain; additionally, liver effects were seen in the mouse and liver and thyroid effects in the rat.

In a 13-week oral toxicity study in which mice received dietary concentrations of fenpyrazamine of 0, 200, 2000, 4000 or 6000 ppm (equal to 0, 28, 296, 640 and 1023 mg/kg bw per day in males and 0, 33, 363, 720 and 1098 mg/kg bw per day in females, respectively), the NOAEL was 2000 ppm (equal to 296 mg/kg bw per day) based on decreased body weight and haematocrit at 4000 ppm (equal to 640 mg/kg bw per day) (Sommer et al., 2007).

In another 13-week oral toxicity study in which rats received dietary concentrations of fenpyrazamine of 0, 300, 600, 1000 or 3000 ppm (equal to 0, 19, 38, 64 and 196 mg/kg bw per day in males and 0, 21, 42, 69 and 207 mg/kg bw per day in females, respectively), the NOAEL was 1000 ppm (equal to 64 mg/kg bw per day) based on decreased body weight and body weight gain in males and females and increased thyroid follicular cell hypertrophy in males at the LOAEL of 3000 ppm (equal to 196 mg/kg bw per day) (Sommer et al., 2006).

In a 90-day oral capsule toxicity study, dogs received fenpyrazamine at doses of 0, 25, 50 or 150 mg/kg bw per day. The NOAEL was 25 mg/kg bw per day based on body weight loss in one male at the LOAEL of 50 mg/kg bw per day (Sato, 2008).

In a one-year oral capsule toxicity study, dogs received fenpyrazamine at doses of 0, 5, 25 or 100 mg/kg bw per day. The NOAEL was 25 mg/kg bw per day based on increased MCV and ALP activity in males and increased platelets in females at the LOAEL of 100 mg/kg bw per day (Sato 2009).

The Meeting concluded that the overall NOAEL for oral toxicity in dogs was 25 mg/kg bw per day.

In a 78-week dietary toxicity study in which mice received dietary concentrations of fenpyrazamine of 0, 100, 1500 or 3000 ppm in males (equal to 0, 11, 176 and 349 mg/kg bw per day) and 0, 100, 2000 or 4000 ppm in females (equal to 0, 14, 283 and 552 mg/kg bw per day), the NOAEL was 1500 ppm (equal to 176 mg/kg bw per day) based on decreased body weight and RBC counts and increased MCV and MCH. The LOAEL was 3000 ppm (equal to 349 mg/kg bw per day). Findings suggestive of hepatocarcinogenicity were observed at the mid and high dose in male mice (Sommer, 2009a).

In a two-year dietary toxicity study in which rats received dietary concentrations of fenpyrazamine of 0, 100, 300, 1200 or 2400 ppm (equal to 0, 4.3, 12.7, 52 and 107 mg/kg bw per day in males and 0, 5.3, 15.6, 64 and 130 mg/kg bw per day in females, respectively), the NOAEL was 1200 ppm (equal to 52 mg/kg bw per day) based on decreased body weights compared to controls. The LOAEL was 2400 ppm (equal to 107 mg/kg bw per day). Findings suggestive of thyroid carcinogenicity were observed at the high dose in male rats (Sommer, 2009b).

In an oral study investigating the time-course of changes in the liver and thyroid in which male rats received fenpyrazamine at dietary concentrations of 0 or 2400 ppm for 3, 7 or 14 days (equal to 0 and 216.5 in the three-day group, 0 and 222.6 in the seven-day group and 0 and 216.6 mg/kg bw per day in the 14-day group, respectively), there were increases in cell proliferation seen following the three-day treatment that were not seen following the 7- and 14-day treatments. However, the protein content in the rat liver S9 fraction was increased following the seven-day treatment only, and 7-pentoxyresorufin-*O*-dependentylase and T_4-UGT activities were increased in all treatment groups. Thyroid weights were increased in the 7- and 14-day treatment groups and follicular cell hypertrophy was increased in all treatment groups. TSH levels increased and T_3 and T_4 levels decreased with treatment time. Changes were considered consistent with phenobarbital-like mode of action (Kondo, 2010).

In a related study, the role of CAR was investigated in fenpyrazamine-treated rat hepatocytes. Increased activity of CYP2B1, UGT1A and UGT2B1 mRNA expression were observed in fenpyrazamine-treated hepatocytes. Following knockdown of CAR using siRNA, the induction of mRNA levels for CYP2B1, UGT1A and UGT2B1 was significantly reduced consistent with CAR-mediated induction of the hepatocytes (Yamada, 2010a).

The pattern of effects in the liver and thyroid is consistent with a CAR-mediated mode of action. Tumours in rodents induced by this mode of action are not relevant to humans.

Fenpyrazamine has been tested in an adequate range of genotoxicity studies, both in vitro and in vivo. No evidence of genotoxicity was found.

In view of the lack of genotoxicity and lack of human-relevant carcinogenicity in mice and rats, the Meeting concluded that fenpyrazamine is unlikely to pose a carcinogenic risk to humans.

In a two-generation dietary reproductive toxicity study in which rats received concentrations of fenpyrazamine at 0, 400, 1000 or 3000 ppm (equal to 0, 27, 69 and 213 mg/kg bw per day in males and 0, 32, 80, 237 mg/kg bw per day in females, respectively), the parental NOAEL was 400 ppm (equal to 27 mg/kg bw per day) based on increased liver and thyroid weights in both generations, decreased body weights in F_1 males and enlarged thyroids in F_1 females at the LOAEL

of 1000 ppm (equal to 69 mg/kg bw per day). The NOAEL for reproductive toxicity was 1000 ppm (equal to 80 mg/kg bw per day) based on decreased birth weight, increased postimplantation loss and decreased implantations and litter size at the LOAEL of 3000 ppm (equal to 237 mg/kg bw per day). The NOAEL for offspring toxicity was 400 ppm (equal to 32 mg/kg bw per day) based on decreased pup weight in the F_1 and F_2 pups at the LOAEL of 1000 ppm (equal to 80 mg/kg bw per day) (Gerspach, Weber & Flade, 2009).

In a developmental toxicity study in which rats received fenpyrazamine by gavage at doses of 0, 30, 125 or 500 mg/kg per day, the NOAEL for maternal toxicity was 30 mg/kg bw per day based on decreased body weight and body weight gain at the maternal LOAEL of 125 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 125 mg/kg based on decreased fetal weight and increased visceral and skeletal variations and delayed ossification at the LOAEL for embryo/fetal toxicity of 500 mg/kg bw per day. There was no evidence of teratogenicity (Gerspach & Flade, 2009).

In a developmental toxicity study in which rabbits received fenpyrazamine by gavage at doses of 0, 30, 50 or 90 mg/kg bw per day, the NOAEL for maternal toxicity was 30 mg/kg bw per day based on increased late abortions accompanied by decreased feed intake and increased gross pathological findings in the aborting animals at the maternal LOAEL of 50 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day based on abortion at 50 mg/kg bw per day (Inawaka, 2008b).

The Meeting concluded that fenpyrazamine is not teratogenic.

In an acute neurotoxicity study in which rats received fenpyrazamine by gavage at doses of 0, 80, 400 or 2000 mg/kg bw, the NOAEL was 80 mg/kg bw based on decreased locomotor activity as a result of general toxicity at the LOAEL of 400 mg/kg bw. There was no evidence of specific neurotoxicity (Sommer, 2008a).

In a 90-day neurotoxicity study in which rats received fenpyrazamine in the diet at concentrations of 0, 500, 1200 or 3000 ppm (equal to 0, 37, 88 and 224 mg/kg bw per day in males and 0, 42, 100 and 248 mg/kg bw per day in females, respectively), the NOAEL was 1200 ppm (equal to 88 mg/kg bw per day) based on decreased body weight and body weight gain. The LOAEL was 3000 ppm (equal to 224 mg/kg bw per day). There was no evidence of specific neurotoxicity (Sommer, 2008b).

The Meeting concluded that fenpyrazamine is not neurotoxic.

In a four-week immunotoxicity study in which female rats received fenpyrazamine in the diet at concentrations of 0, 500, 1500 or 4000 ppm (equal to 0, 36, 123 and 392 mg/kg bw per day), the NOAEL was 1500 ppm (equal to 123 mg/kg bw per day) based on decreased body weight and body weight gain and increased liver and spleen weights. The LOAEL was 4000 ppm (equal to 392 mg/kg bw per day) (Saunders, 2010).

Toxicological data on metabolites and/or degradates

The acute oral LD_{50} of S-2188-DC, a plant and rat metabolite, was greater than 500 mg/kg bw. It was negative in the Ames test. As S-2188-DC was a major rat metabolite, the toxicity of the metabolite would be covered by that of the parent (Asano, 2008).

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisoning in humans was available (Nishimoto, 2016).

The Meeting concluded that the existing database on fenpyrazamine 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) of 0–0.3 mg/kg bw on the basis of the overall NOAEL of 25 mg/kg bw per day from the dog studies for body weight loss at 50 mg/kg bw per day. A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) of 0.8 mg/kg bw on the basis of the NOAEL of 80 mg/kg bw in the acute neurotoxicity study for reduced locomotor activity at 400 mg/kg bw. A safety factor of 100 was applied.

The reference doses also cover the metabolite, S-2188-DC.

Levels relevant to risk assessment of fenpyrazamine

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity [a]	Toxicity	1500 ppm, equal to 176 mg/kg bw per day	3000 ppm, equal to 349 mg/kg bw per day
		Carcinogenicity	100 ppm, equal to 11 mg/kg bw per day [b]	1 500 ppm, equal to 176 mg/kg bw per day [b]
Rat	Acute neurotoxicity study [d]	Toxicity	80 mg/kg bw	400 mg/kg bw
		Neurotoxicity	400 mg/kg bw [e]	–
	Subchronic neurotoxicity study [a]	Toxicity	1200 ppm, equal to 88 mg/kg bw per day	3000 ppm, equal to 224 mg/kg bw per day [b]
		Neurotoxicity	3 000 ppm, equal to 224 mg/kg bw per day [c]	–
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	1200 ppm, equal to 52 mg/kg bw per day	2400 ppm, equal to 107 mg/kg bw per day [b]
		Carcinogenicity	1200 ppm, equal to 52 mg/kg bw per day	2400 ppm, equal to 107 mg/kg bw per day [b]
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	1000 ppm, equal to 69 mg/kg bw per day [c]	3000 ppm, equal to 237 mg/kg bw per day
		Parental toxicity	400 ppm, equal to 27 mg/kg bw per day	1000 ppm, equal to 80 mg/kg bw per day
		Offspring toxicity	400 ppm, equal to 32 mg/kg bw per day	1000 ppm, equal to 80 mg/kg bw per day
	Developmental toxicity study [d]	Maternal toxicity	30 mg/kg bw per day	125 mg/kg bw per day
		Embryo/fetal toxicity	125 mg/kg bw per day	500 mg/kg bw per day
	Immunotoxicity [a]	Toxicity	1500 ppm, equal to 123 mg/kg bw per day	4000 ppm, equal to 392 mg/kg bw per day
		Immunotoxicity	4000 ppm, equal to 392 mg/kg bw per day [c]	–
Rabbit	Developmental toxicity study [d]	Maternal toxicity	30 mg/kg bw per day	50 mg/kg bw per day
		Embryo/fetal toxicity	30 mg/kg bw per day	50 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity [e,f]	Toxicity	25 mg/kg bw per day	50 mg/kg bw per day

[a] Dietary administration. [b] Not applicable to human toxicity. [c] Highest dose tested [d] Gavage administration.
[e] Two or more studies combined. [f] Capsule administration.

Estimate of acceptable daily intake (ADI)

0–0.13 mg/kg bw

Estimate of acute reference dose (ARfD)

0–0.30 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 fenpyrazamine

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and extensive with some saturation of excretion at high doses
Dermal absorption	No data
Distribution	Widely distributed; higher concentrations in plasma, liver, kidneys and stomach
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Primarily via the urine, about 90% of radiolabel eliminated within 24/48 hours in low and high doses, respectively
Metabolism in animals	Extensively metabolized
Toxicologically significant compounds in animals and plants	Parent, S-2188-DC
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kw bw
Rat, LC_{50}, inhalation	> 4.84 mg/kg bw
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Guinea pig, dermal sensitization	Non-sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Body weight loss
Lowest relevant oral NOAEL	25 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Body weight.
Lowest relevant NOAEL	52 mg/kg bw per day (rat)
Carcinogenicity	Evidence suggestive of carcinogenicity in male mice and rats; however, not relevant to humans [a]
Genotoxicity	No evidence of genotoxicity [a]
Reproductive toxicity	
Target/critical effect	Decreased birth weight (rat)
Lowest relevant parental NOAEL	27 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	80 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	32 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Abortion (rabbit)
Lowest relevant maternal NOAEL	30 mg/kg bw per day, highest dose tested (rabbit)
Lowest relevant embryo/fetal NOAEL	30 mg/kg bw per day, highest dose tested (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	80 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	88 mg/kg bw per day (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	123 mg/kg bw per day (rat)
Studies on toxicologically relevant metabolites	
S-2188-DC	LD_{50} > 500 mg/kg bw No evidence of genotoxicity
Human data	No evidence of adverse effects from reports on health status of factory workers

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI	0–0.13 mg/kg bw	Two-year toxicity (rat)	100
ARfD	0–0.3 mg/kg bw	Developmental toxicity (rabbit)	100

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Appendix 1: Mode of action

Rationale for the proposed mode of action (MOA)

Exposure to fenpyrazamine over a period of two years produced increases in hepatocellular carcinomas and thyroid follicular cell carcinomas in male rats. While the changes were not statistically significant, the incidences in high-dose animals were above historical controls. Liver carcinoma incidence was 0, 0, 0, 0 and 2 at 0, 4.3, 12, 7, 52 and 107 mg/kg bw per day, respectively, and C-cell carcinomas and follicular cell carcinomas of the thyroid were 0, 1, 1, 0 and 3 and 2, 4, 2, 3 and 3, respectively, at the same doses. The sponsor did not consider the increased tumour incidences to be treatment related; however, they have submitted the following MOA in the spirit of an abundance of caution.

A. Postulated MOA for liver tumours

A brief description of the sequence of measured effects, starting with chemical administration, to cancer formation at a given site (International Life Sciences Institute Risk Science Institute Human Cancer Relevance Framework [ILSI/RSI HRF]) was supplied in the working document provided by the sponsor to support a possible induction of liver tumours through the activation of the constitutive androstane receptor (CAR), which results in a pleiotropic response including the stimulation of cytochrome P450 (CYP) 2B isoforms and increased cell proliferation (Yamada, 2010a,b). This MOA is similar to that of other non-genotoxic liver CYP2B form inducers/constitutive androstene receptor (CAR) activators, such as phenobarbital (Whysner, Ross & Williams, 1996; Holsapple et al., 2006).

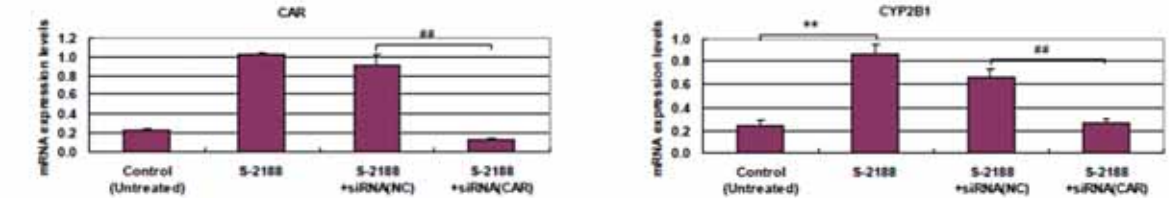
B. Key events in experimental animals

The key events for fenpyrazamine-induced liver effects include induction of CYP2B isoforms through CAR, increased hepatocellular proliferation and eventually liver carcinomas. To test each of these postulated key events, several parameters were evaluated in male rats in short-term and subchronic studies and at interim and terminal kills in a chronic study (Table A3).

i. Activation of the nuclear CAR

Phenobarbital has been shown to produce liver tumours in rodents by an MOA that involves activation of CAR (Holsapple et al., 2006). Indeed, phenobarbital does not produce liver tumours in CAR knockout mice (Yamamoto et al., 2004). Since a CAR knockout rat has, to date, not been developed, the role of CAR in the MOA for fenpyrazamine CYP2B induction was determined in rat hepatocytes using the recently developed RNA interference (RNAi) technique. The RNAi technique enables sequence-specific gene silencing using short-interfering RNA (siRNA) (Fire et al., 1998; Caplen et al., 2001; Elbashir. et. al., 2001). As shown in Fig. A1, the treatment of rat hepatocytes with CAR-siRNA significantly reduced CAR messenger ribonucleic acid (mRNA) in the presence of fenpyrazamine, resulting in a significant reduction in the magnitude of induction of CYP2B1 mRNA levels by fenpyrazamine (Yamada, 2010a,b). This finding was consistent with that of phenobarbital (Deguchi et al., 2009). These findings demonstrate that fenpyrazamine induces CYP2B1 through CAR, fenpyrazamine thus having activating effect on CAR in rat hepatocytes, similar to phenobarbital.

Figure A1. mRNA levels [a] of CAR and CYP2B1 induced by fenpyrazamine [b] in hepatocytes with or without CAR knockdown



CAR: constitutive androstene receptor; Control (Untreated): without fenpyrazamine or siRNA; CYP: cytochrome P450; mRNA: messenger ribonucleic acid; S-2188: fenpyrazamine; SD: standard deviation; siRNA(CAR): short-interfering ribonucleic acid for constitutive androstene receptor; siRNA(NC): short-interfering ribonucleic acid for negative control; **: statistically different from Control (Untreated) group ($P < 0.01$); ##: statistically different from fenpyrazamine + siRNA(NC) group ($P < 0.01$).

[a] Columns present expression level of mRNA. Mean ± SD, $N = 4$.

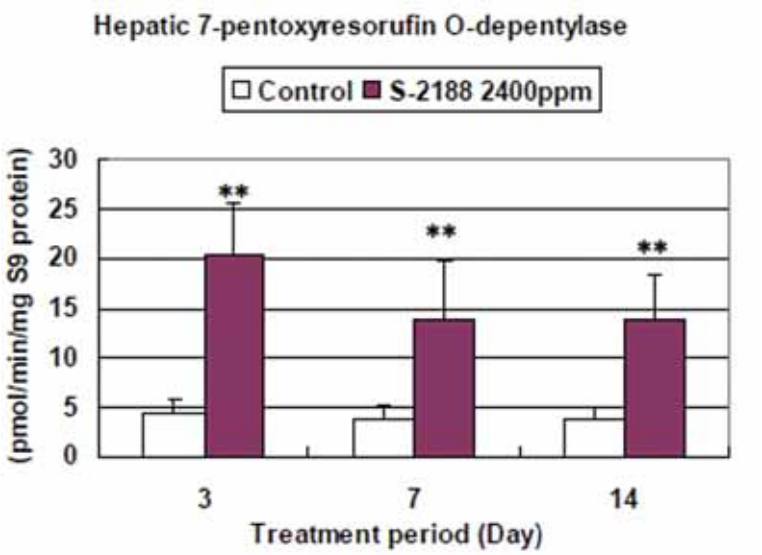
[b] Concentration of fenpyrazamine was 50 µmol/L.

ii. Enzyme induction

CYP2B isoform induction has been shown to be associated with liver tumour formation in rodents exposed to phenobarbital and related compounds (Whysner, Ross & Williams, 1996; Holsapple et al., 2006; Yamada et al., 2009). It involves activation of nuclear receptors, particularly the CAR (Wei et al., 2000; Yoshinari et al., 2001; Ueda et al., 2002; Yamamoto, Kawamoto & Negishi, 2003; Deguchi et al., 2009; Yamada et al., 2009). Although the magnitude of induction of CYP2B isoforms in rodent liver is not by itself a predictor of carcinogenic potential, in the rat studies with a series of barbiturates, some correlation was observed between the magnitude of CYP2B form induction and tumour-promoting activity (Lubet et al., 1989; Rice et al., 1994). The MOA study (Kondo, 2010) utilized a dose of 2400 ppm, which is the highest dose level in rat 2-year bioassay. Significant increases of hepatic 7-pentoxyresorufin *O*-dependentylase (PROD; a CYP2B marker) were observed in rats treated with 2400 ppm of fenpyrazamine for 3, 7 and 14 days (Fig. A2).

Additional evidence that CYP induction is occurring at biologically relevant levels is supported by the observation of hepatocellular hypertrophy, particularly in the centrilobular region of the liver lobule (Kondo, 2010). This is a characteristic change of CYP inducers, such as phenobarbital and related compounds. Thus, there is both direct and indirect evidence for CYP induction by fenpyrazamine administration with biologically important consequences.

Figure A2. Hepatic activities of PROD [a]



CYP: cytochrome P450; ppm: parts per million; PROD: 7-pentoxyresorufin O-dependentylase; S-2188: fenpyrazamine; SD: standard deviation; **: $P < 0.01$ (statistically significant difference from the control)

[a] 7-Pentoxyresorufin *O*-dependentylase (PROD) is a CYP2B marker PROD activities were determined in the liver from rats treated with fenpyrazamine for 3, 7 or 14 days. Values represent group mean ± SD, $N = 10$.

Source: Kondo (2010)

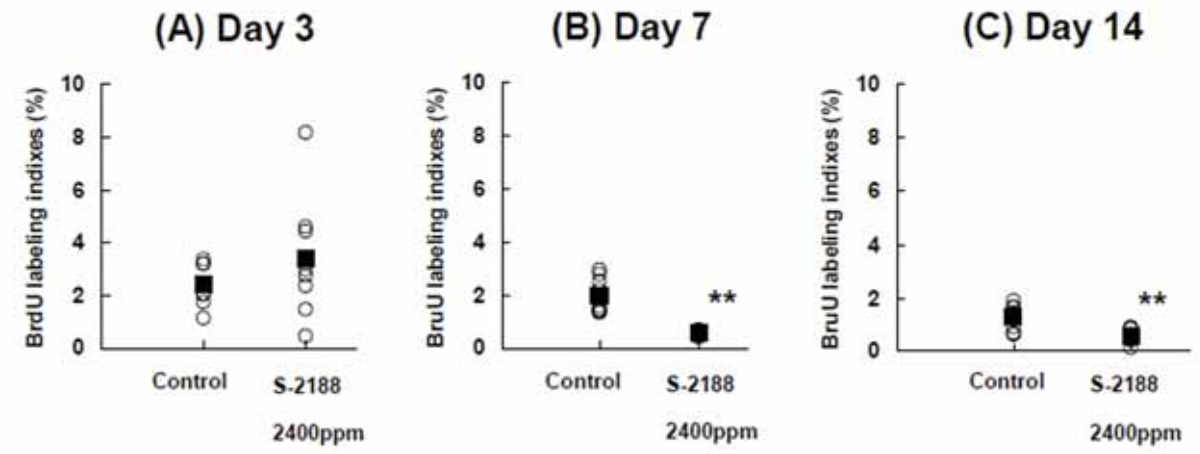
iii. Cell proliferation

The support of the key event of increased cell proliferation is the observation of increased liver weight, particularly relative weight, which was observed after 3-day treatment with fenpyrazamine (Kondo, 2010). Previous studies with phenobarbital in the rat and mouse demonstrated that the increase in relative liver weight after short-term treatment is due to both hepatocyte hypertrophy and hyperplasia (Whysner, Ross & Williams, 1996; IARC, 2001). A sensitive measure of cell proliferation is to assess the rate of S-phase activity of the cell cycle using 5-bromo-2′-deoxyuridine (BrdU) incorporation as a marker of DNA synthesis. Marginally increased proliferation was demonstrated by an increased BrdU-labelling index at 2400 ppm in male rats (30%; 3/10 animals) after 3 days of treatment. However, the BrdU-labelling index values returned to control levels when fenpyrazamine treatment was continued for 7 and 14 days. Since hepatocyte cell number is increased by 14 days as evidenced by increased liver weight, the actual number of DNA replications remains greater than

controls despite the labelling index being similar to controls. Since it is the number of DNA replications that is critical for carcinogenesis, not necessarily the rate of proliferation, there clearly is increased cell proliferation (Cohen, 2010). Previous studies have demonstrated that the initial stimulation of hepatocyte-labelling index values in rodent liver by phenobarbital is not sustained by chronic administration of this compound (Jones & Clarke, 1993; Jones, Clarke & Barrass, 1993; Kolaja et al., 1996; Whysner, Ross & Williams, 1996; Furukawa et al., 2000; IARC, 2001; Jones, Orton & Lake, 2009). Thus, there is direct and strong evidence in support of the key events of increased cell proliferation by fenpyrazamine. Especially, time-course alteration such as the tangent increase and suppression after peak of the index is similar to the CYP enzyme inducer, phenobarbital.

It should be noted that the response to fenpyrazamine was relatively weak in comparison to those usually seen with a strong inducing agent such as phenobarbital, but the tumorigenic effect (if it exists) was also quite weak even at doses of the MTD (2400 ppm) (Fig. A3).

Figure A3. Cell proliferation of hepatocytes[a]



BrdU: 5-bromo-2′-deoxyuridine; DNA: deoxyribonucleic acid; ppm: parts per million; S-2188: fenpyrazamine; **: indicates statistically significant difference from the control ($P < 0.01$)

[a] Hepatocyte cell replicative DNA synthesis determined as BrdU-labelling indices in the liver of rats treated with fenpyrazamine for (A) 3, (B) 7 or (C) 14 days. Open circles represent data from individual rats; closed square represent group mean, $N = 8$~10. At day 3, although statistically significant difference was not observed for group mean values, 30% of animals (3/10 animals) in the fenpyrazamine showed higher values of BrdU-labelling indices than the highest value of controls (3.4%).

C. Dose–response relationship

The parameters described above were not evaluated with respect to dose in the Kondo (2010) MOA study. However, some of the parameters can be evaluated using data from the 90-day study (0, 300, 600, 1000 and 3000 ppm) and the 2-year study (0, 100, 300, 1200 and 2400 ppm) (Table A1).

i. Enzyme induction

Increased CYP2B activity with hepatocellular hypertrophy was observed in males at 2400 ppm at which hepatocellular carcinoma was observed in the 2-year study. Although CYP2B activity was not examined, hepatocellular hypertrophy, which indicated occurrence of enzyme induction (Cohen, 2010), was observed at dose levels of 3000 ppm in the 90-day study; and at 1200 and 2400 ppm on weeks 52 and 104 in the 2-year study.

ii. Cell proliferation

Increased BrdU-labelling indices with increased relative liver weight was confirmed at the 2400 ppm dose at which hepatocellular carcinoma was observed in the 2-year study. Although BrdU-labelling indices were not examined, increased relative liver weight, which indicate possible occurrence of enhanced cell proliferation in case of enzyme inducers (Cohen, 2010), was observed at 3000 ppm in the 90-day study, and at 1200 ppm on week 52 and at 2400 ppm on weeks 52 and 104 in the 2-year study.

iii. Tumour induction

Hepatocellular carcinoma was observed in male rats given fenpyrazamine at 2400 ppm but not in other groups, including control males, or in females. The various parameters described above that are related to the key events in the induction of liver tumours were observed at or below the dose levels at which these tumours occurred in males in the 2-year study of fenpyrazamine (Table A1).

Taken together, there are strong parallels in the dose response for the key events and the tumour appearance. Again, it is noteworthy that the degree of changes observed in each of the parameters was relatively mild, consistent with the marginal increase in tumour incidences, particularly when compared not only to the current controls but to the range of historical controls.

Table A1. Dose–response relationship of alteration in liver of male rats treated with fenpyrazamine

Parameter	Measure per dose level							
	0 ppm	**100 ppm**	**300 ppm**	**600 ppm**	**1000 ppm**	**1200 ppm**	**2400 ppm** [a]	**3000 ppm**
Liver tumours in 2-year study [b]								
Hepatocellular carcinoma	0/50	0/50	0/50	ND	ND	0/50	2/50	ND
Hepatocellular adenoma	1/50	1/50	1/50	ND	ND	1/50	1/50	ND
CYP2B (pmol/min per mg S9 protein)								
Day 3 in 14-day MOA study	4.3	ND	ND	ND	ND	ND	20.3** (472%)	ND
Day 7 in 14-day MOA study	3.8	ND	ND	ND	ND	ND	13.9** (365%)	ND
Day 14 in 14-day MOA study	3.7	ND	ND	ND	ND	ND	13.9** (376%)	ND
Hepatocellular hypertrophy [b]								
Day 3 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	5/10* (50%)	ND
Day 7 in 14-day MOA study	1/10	ND	ND	ND	ND	ND	6/10* (60%)	ND
Day 14 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	9/10** (90%)	ND
90-day study	1/12	ND	1/12	0/12	1/12	ND	ND	4/12* (33%)
Week 52 in 2-year study	1/20	3/20	7/20* (35%) [c]	ND	ND	10/20** (50%)	8/20** (40%)	ND
Week 104 in 2-year study	0/50	2/50	2/50	ND	ND	7/50* (14%)	6/50* (12%)	ND
Cell proliferation (BrdU-labelling indices, %)								
Day 3 in 14-day MOA study	2.38	ND	ND	ND	ND	ND	3.36 (141%)	ND
Day 7 in 14-day MOA sstudy	1.93	ND	ND	ND	ND	ND	0.57** (30%)	ND
Day 14 in 14-day MOA study	1.25	ND	ND	ND	ND	ND	0.57** (46%)	ND
Relative liver weight (g/100 g bw)								
Day 3 in 14-day MOA study	4.60	ND	ND	ND	ND	ND	5.49** (119%)	ND
Day 7 in 14-day MOA study	4.45	ND	ND	ND	ND	ND	5.38** (121%)	ND

Parameter	Measure per dose level							
	0 ppm	100 ppm	300 ppm	600 ppm	1000 ppm	1200 ppm	2400 ppm [a]	3000 ppm
Day 14 in 14-day MOA study	4.07	ND	ND	ND	ND	ND	5.20** (128%)	ND
90-day study	2.58	ND	2.54	2.52	2.63	ND	ND	3.15** (112%)
Week 52 in 2-year study	2.49	2.43	2.38	ND	ND	2.69* (108%)	2.98** (120%)	ND
Week 104 in 2-year study	2.34	2.38	2.38	ND	ND	2.44	2.85** (122%)	ND

BrdU: 5-bromo-2′-deoxyuridine; bw: body weight; CYP: cytochrome P450; MOA: mode of action; ND: not determined; ppm: parts per million; *: $0.01 < P < 0.05$, **: $P < 0.01$, in comparison to control

[a] Highest dose of the 2-year study.

[b] Results presented as number of animals with finding / number of animals examined.

[c] This change is statistically significant but not toxicologically significant because no change in liver weight was observed.

Sources: Kondo (2010); Sommer (2009b)

D. Temporal association

It is critical in the evaluation of MOA that early key events occur before the appearance of tumours, and this is clearly in the case with fenpyrazamine. Increased enzyme induction and BrdU-labelling index were observed after as little as 3 days of dosing (Kondo, 2010). In addition, the increased BrdU-labelling indices returned to control values within 7 days of dosing (Kondo, 2010). Increased liver weight and hepatocellular hypertrophy were shown to appear in short-term (3, 7 and 14 days), subacute (90 days) and chronic (52 and 104 weeks) studies. Like phenobarbital, while increased liver weight with enzyme induction and enhanced cell proliferation was observed at early stages of administration, the appearance of liver carcinomas occurred only after chronic administration of fenpyrazamine. Thus, there is a logical temporal response for the key events of hepatocellular tumour formation in which all key events precede tumour development.

E. Strength, consistency and specificity of association of tumour response with key events

Strength, consistency and specificity of the association can be established from the studies described earlier. The quantifiable precursor events, fundamental to the proposed MOA, are relatively consistent with the emergence of liver tumours.

While some species differences in CYP2B induction are known (Pustylnyak et al., 2007), enzyme inducers such as phenobarbital normally induce CYP2B isoforms in both the rat and mouse. It is consistent with existing data, that is, increased liver weight and centrilobular hepatocellular hypertrophy observed in mice treated with fenpyrazamine for 3 months and 1.5 years. However, bioassay dose levels of fenpyrazamine (up to 3000 ppm) induced no hepatic tumours in mice, which is consistent with other CYP2B inducers such as pyrethrins (Osimitz & Lake, 2009) and metofluthrin (Yamada et al., 2009).

F. Biological plausibility and coherence

The liver is by far the most commonly affected target tissue in cancer bioassays (Gold et al., 2001). This may be because the liver is the major site of metabolic activation of chemicals. Furthermore, the liver is the first organ exposed to an orally administered chemical following its absorption from the gastrointestinal tract (as in the case with the bioassays for fenpyrazamine).

Several MOAs have been identified for the induction of liver tumours in rodents (Holsapple et al., 2006; Cohen, 2010), including DNA reactivity, cytotoxicity and consequent regenerative proliferation, peroxisome proliferation, estrogen stimulation and CYP induction. The MOA for liver tumour formation by phenobarbital, which involves activation of CAR and CYP2B induction, is well established (Whysner, Ross & Williams, 1996; Holsapple et al., 2006;

Yamada et al., 2009; Cohen, 2010). This is associated with increased liver weight, hepatocellular hypertrophy (especially in the centrilobular region), proliferation of smooth endoplasmic reticulum and stimulation of cell replication as evidenced by labelling indices such as BrdU (Deguchi et al., 2009; Yamada et al., 2009). Phenobarbital and related compounds are also known promoters of liver cell foci once formed. CYP2B induction has been shown to be associated with liver tumour formation in rodents for phenobarbital and related compounds (Whysner, Ross & Williams, 1996; Holsapple et al., 2006; Deguchi et al., 2009; Yamada et al., 2009) and involves activation of nuclear receptors, particularly CAR (Wei et al., 2000; Yoshinari et al., 2001; Ueda et al., 2002; Yamamoto et al., 2004; Deguchi et al., 2009; Yamada et al., 2009). While it is uncertain if CYP2B induction is a surrogate for a wider pleiotropic response (Ueda et al., 2002) or if CYP2B isoforms themselves play a role, for example, by generating reactive oxygen species (Holsapple et al., 2006), there is clear evidence from studies in knockout mice that CAR plays an essential role in phenobarbital-induced liver tumour formation (Yamamoto et al., 2004). As described above, CAR-dependency of fenpyrazamine-induced CYP2B induction was confirmed in rat hepatocytes (Yamada, 2010a,b). The fenpyrazamine-induced key events summarized above are similar to those seen with phenobarbital, and we can therefore conclude that fenpyrazamine has a similar MOA to that of phenobarbital for rat liver tumour formation.

G. Other MOAs

As described above, there is strong evidence in support of CYP2B enzyme induction as the critical MOA for fenpyrazamine. Other known MOAs for rodent liver carcinogenesis can be assessed from the data available from the various studies performed with fenpyrazamine.

Fenpyrazamine was tested in a standard battery of genotoxicity and mutagenicity tests in vitro and in vivo. There was no indication of gene mutation either in the presence or absence of metabolic activation in both the bacterial reverse mutation and mammalian gene mutation tests (HPRT). A negative response for chromosomal aberrations was observed in vitro in the Chinese hamster lung cells (CHL/IU). In mouse micronucleus test in vivo, the results were clearly negative. It can be concluded that fenpyrazamine has no genotoxic potential. In the general toxicity studies, utilizing histopathology or blood biochemistry, there was no evidence of hepatocellular cytotoxicity (necrosis), peroxisome proliferation or sex hormone perturbations. Thus, it is highly likely that the MOA for fenpyrazamine-induced liver tumours in rats is through a CYP induction, similar to that seen for phenobarbital, and not via some other MOA.

H. Uncertainties, inconsistencies and data gaps

For the most part, the data are quite consistent with key events at several time points and at several doses, as noted in Table A2. However, there are some apparent inconsistencies. The development of altered hepatic foci is also a key event in the MOA for phenobarbital-induced liver tumours (Holsapple et al., 2006). However, such foci were not observed in any studies with fenpyrazamine in rats. Since carcinogenic activity of fenpyrazamine is very weak (if indeed real), evidenced by the incidence of hepatocellular carcinoma at the highest dose of fenpyrazamine being only marginally above comparable historical control data, the development of altered hepatic foci may not be detectable under the experimental conditions.

CYP inducers significantly increased smooth endoplasmic reticulum observed by transmission electron microscopy (Ghadially, 1997). Therefore, this finding is considered as indirect evidence for CYP induction. Although transmission electron microscopy was not evaluated in liver of rats treated with fenpyrazamine, the Meeting did not believe that this evaluation is essential because the direct evidence for CYP induction by fenpyrazamine administration was obtained. Although increased liver weight with hepatocellular hypertrophy was observed in both rats and mice, liver carcinomas were only observed in male rats in the 2-year study with fenpyrazamine. The reason is unknown, but it was also observed with other CYP2B inducers including pyrethrins (Osimitz & Lake, 2009) and metofluthrin (Yamada et al., 2009), which induced liver tumours in rats but not in mice.

I. Assessment of postulated MOA

As described above, the key events in the MOA for fenpyrazamine have been well documented, with a strong dose and temporal consistency. In addition, this is an established MOA, and the various parameters essential for documenting this MOA have been presented for fenpyrazamine. Thus, we consider that the level of confidence in the postulated MOA is high.

J. Human applicability of the proposed MOA

The International Programme on Chemical Safety (IPCS) Human Relevance Framework, which was developed from the Risk Sciences Institute/International Life Sciences Institute "Human Relevance Framework" (Meek et al., 2003) and modified based on discussions by the IPCS Cancer Working Group (Boobis et al., 2006), presents a four-part approach to addressing a series of three questions leading to a documented, logical conclusion regarding the human relevance of the MOA in animal tumours.

1. *Is the weight of evidence sufficient to establish an MOA in animals?*

As described in detail earlier, there is clear evidence that fenpyrazamine activates the CAR that results in a pleiotropic response including the stimulation of CYP2B isoforms and increased hepatocellular proliferation.

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?*

Examination of the relevance of the MOA to humans requires not only information regarding the chemical of concern, but also an evaluation of the MOA for similarly acting compounds (Meek et al., 2003; Cohen et al., 2004). The first chemicals evaluated for a specific MOA will have a much higher requirement of data to prove the relevance or non-relevance of the MOA than will subsequent chemicals. Thus, in this examination of fenpyrazamine with regard to human relevance of the animal data, reliance can also be made on the evaluation of the MOA for similarly acting compounds, and in this case, the best example is phenobarbital. Phenobarbital has been identified as having an MOA involving CAR activation that results in the induction of CYP2B isoforms, an increase in cell proliferation and, ultimately, the development of liver tumours in rodents (Whysner, Ross & Williams, 1996; Wei et al., 2000; Yoshinari et al., 2001; Ueda et al., 2002; Holsapple et al., 2006; Hirose et al., 2009; Yamada et al., 2009). As described above, the data strongly support a similar MOA for fenpyrazamine..

In analysing the relevance of the animal MOA data to humans, a concordance table has been suggested as being of considerable value (Meek et al., 2003). This includes not only the data from fenpyrazamine in the rat, but also a similar listing for the likely findings in human (Table A2).

Table A2. A comparison of key fenpyrazamine events in rats and humans

Key events	Evidence in rats	Evidence in humans
Increased hepatic CYP2B	Direct experimental evidence. It was also proven that CYP2B activity was enhanced through CAR activation, evidenced by RNAi experiment. Enhanced CYP2B activity was also supported by the observation of centrilobular hepatocellular hypertrophy.	No data available for fenpyrazamine, but microsomal enzyme induction may be plausible.
Increased hepatocellular proliferation	Direct experimental evidence. Increase of BrdU-labelling index, and supported by observation of increased liver weight.	No data available for fenpyrazamine, but not plausible given that other microsomal enzyme inducers such as phenobarbital and methfluthrin have not been shown to increase hepatocellular proliferation in vitro and/or in vivo.
Appearance of hepatocellular tumours	Equivocal increase of hepatocellular carcinoma; its incidence was marginally above comparable historical control data.	Not likely based on data available for other microsomal enzyme inducers.

BrdU: 5-bromo-2′-deoxyuridine; CAR: constitutive androstane receptor; CYP: cytochrome P450; RNAi: RNA interference

As described above, the sequence of key events in the rat is the induction of CYP2B enzyme, an increase in hepatocellular proliferation and, ultimately, the formation of a low incidence of hepatocellular adenomas and/or carcinomas. This occurs in both the male and female rat, with the male rat being slightly more sensitive. In humans, there is no direct evidence for the effects of fenpyrazamine. It is unlikely that such data will become available, but data derived from in vitro studies on human hepatocytes

can be contributory and of value. Such data has been obtained using an in vitro comparison of human hepatocytes to rat hepatocytes treated with phenobarbital and another CYP2B inducer metofluthrin (Hirose et al., 2009). Metofluthrin slightly induced CYP2B enzyme in rat and human hepatocytes as well as phenobarbital (but phenobarbital appeared to have a more marked effect) (Hirose et al., 2009). The CYP2B induction with metofluthrin in rat and human are similar to those observed with pyrethrins (Price et al., 2008). Fenpyrazamine also induced CYP2B activity through CAR activation in rat (Yamada, 2010a,b), consistent with phenobarbital and metofluthrin (Deguchi et al., 2009; Yamada et al., 2009). Although CAR is expressed in humans, and phenobarbital induces CYP enzymes in human liver, phenobarbital apparently can act through other receptors as well, such as pregnane X receptor (PXR) (Moore et al., 2003). In addition, human CAR has been suggested as being activated by phenobarbital leading to not only activation of the CYP2B enzyme, but also in the induction of non-P450 genes, such as UGT1A1 (Sugatani et al., 2001). The levels of exposure to phenobarbital in humans from therapeutic uses are comparable to serum levels achieved in animals that produce tumorigenic effects. Based on these analyses, the first key event, induction of CYP isoforms (CYP3A as well as CYP2B), occurs in humans at the levels of exposure that humans attain (Pirttiaho et al., 1982).

A more critical step is the effect on hepatocellular proliferation. It was thought that there is an increase in liver size in humans after prolonged treatment with phenobarbital and that the increased liver size in humans produced by phenobarbital is due to hepatocellular hypertrophy (Holsapple et al., 2006). Thus, it was thought that the same proliferation of smooth endoplasmic reticulum occurred in human liver in response to phenobarbital as is seen in the rat and the mouse. However, limited in vitro studies suggest that human hepatocytes are refractory to the increased proliferative and anti-apoptotic effects of phenobarbital that occur in rodents (Parzefall et al., 1991; Hasmall & Roberts, 1999). Furthermore, dose-dependent increase of DNA synthesis by phenobarbital was detected in rat hepatocytes (Hirose et al., 2009), which is consistent with previous findings (Parzefall et al., 1991; Hasmall & Roberts, 1999), while various concentrations of phenobarbital had no effect on DNA synthesis associated with hepatocellular proliferation in human (Hirose et al., 2009). Most importantly, there is also substantial epidemiological evidence for the non-carcinogenicity of phenobarbital in humans (Olsen et al., 1989, 1995; Whysner, Ross & Williams, 1996; IARC, 2001). Again, the exposure levels in humans are similar to those in rodents; also, administration to humans occurs over many years, frequently beginning in childhood and continuing for essentially the individual's lifetime. Such studies have demonstrated that in human receiving phenobarbital for many years at doses producing plasma concentrations similar to those that are carcinogenic in rodents, there is no evidence of increased liver tumour risk. Thus, there is significant evidence that this key event in CYP2B inducer-produced liver tumours in rodents – increased cell proliferation – does not occur in the human liver. Similarity to phenobarbital and metofluthrin in enhancement of hepatocellular proliferation in rat is therefore not expected to produce an increase in human hepatocellular proliferation, and therefore fenpyrazamine would not result in an increase in liver tumours in human. Based on this analysis, the answer to the question regarding plausibility in humans is likely to be 'no' for fenpyrazamine.

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?*

Continuing the analogy with phenobarbital, fenpyrazamine qualitatively would not be expected to induce cancer in humans. Therefore, quantitative analysis is not necessary. Just to be on the safe side, however, quantitative analysis was also conducted in this section. In contrast to phenobarbital exposures in humans, fenpyrazamine is expected to have exposures considerably lower in humans than those that were used for the animal studies. For both phenobarbital and fenpyrazamine in the animal studies, the effects on the liver occur only at higher doses. This is characteristic for most MOA involving non-DNA-reactive agents, indicating the presence of a threshold (Cohen & Ellwein, 1990, 1991). For phenobarbital, however, this high level of exposure occurs not only in the animal, but also in humans. Also fenpyrazamine produced the key hepatocellular changes only at extremely high exposure levels, 1200 ppm (51.9 mg/kg per day) and above in male rat, but in contrast to phenobarbital, these levels are considerably higher than would be expected for human exposure. Thus, not only is there a qualitative difference between the rodent and human in the response of the liver cells to the CYP inducers regarding induction of tumours, there is also a marked quantitative difference in the level of exposure. Thus, based on quantitative considerations, the confidence in a lack of effect in humans at expected exposures is even stronger than that based only on qualitative consideration.

K. Conclusion: statement of confidence, analysis and implications

Based on the above presentation, there is strong evidence to support an MOA of fenpyrazamine-induced hepatocellular carcinomas in male rats involving CYP2B-induction through CAR activation, an increase in hepatocellular proliferation and, ultimately, a slight increase in liver tumours. This MOA is similar to that demonstrated for phenobarbital, a chemical for which there is strong epidemiological data supporting non-carcinogenicity in humans. The data strongly support the conclusion that qualitatively and quantitatively this MOA would not occur in humans following exposure to fenpyrazamine, similar to the conclusion with phenobarbital.

The data available for fenpyrazamine are considerable and, despite some data gaps such as the lack of available data regarding transmission electron microscopy, and discrepancy such as no development of altered hepatic foci, the MOA for possible liver tumour induction by fenpyrazamine would be by CYP2B-induction and increased cell proliferation. The various discrepancies may be attributable to the low potency of fenpyrazamine relative to phenobarbital. Based on the evidence, including a comparison with the results with phenobarbital acting by a similar MOA, it is reasonable to conclude that fenpyrazamine will not have any hepatocarcinogenic hazard in humans.

Analysis for MOA in thyroid

A. Postulated MOA for the induction of thyroid follicular cell carcinoma in male rat

The postulated MOA for possible induction of thyroid follicular cell tumours by fenpyrazamine involves the perturbation of homeostasis of the pituitary–thyroid axis by an extrathyroidal mechanism. Specifically, fenpyrazamine induces hepatic thyroxine-uridine diphospho-glucuronosyltransferase (T_4-UGT) activity, leading to enhanced metabolism of T_4 by conjugation and increased biliary excretion of the conjugated hormone. The result of this enhanced liver metabolism is a decrease in serum T_4 (and sometimes T_3) half-life. The pituitary gland responds to a decrease in circulating serum levels of T_4 by enhancing the output and serum level of thyroid-stimulating hormone (TSH). Prolonged elevation of circulating TSH levels stimulates the thyroid gland to deplete its stores of thyroid hormone and continues to induce hormone production. Thus, the thyroid follicular cells enlarge (hypertrophy) and are induced to proliferate at an increased rate and to increase in number (hyperplasia). With chronic exposure, thyroid hyperplasia eventually progresses to neoplasia.

B. Key events in experimental animals

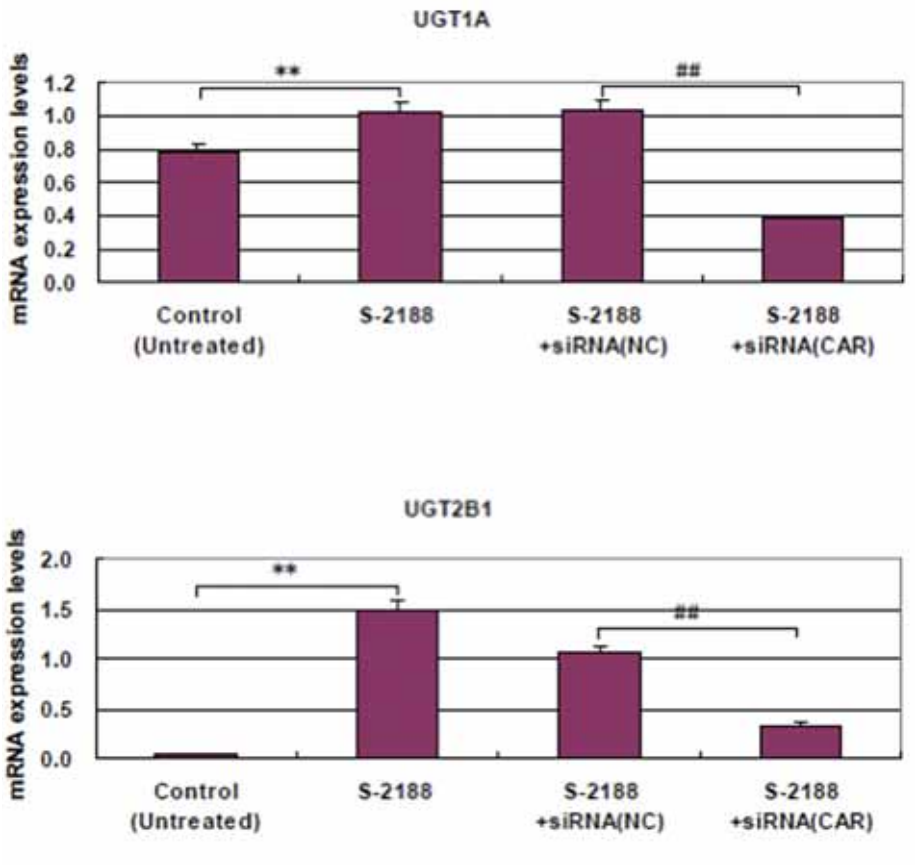
The sequence of key events in the MOA for effects of fenpyrazamine on thyroid includes: induction of hepatic UGT activity, increase in hepatic metabolism and biliary excretion of T_4, decrease in serum T_4 half-life and concentration, increase in circulating TSH concentration, and thyroid follicular cell hypertrophy and hyperplasia.

To determine whether fenpyrazamine works via disruption of thyroid–pituitary status by increasing hepatic clearance of circulating thyroid hormone, several parameters were evaluated in male rats in short-term and subchronic studies, and at interim and terminal kills in a chronic study (Table A3).

i. Activation of the nuclear CAR

Phenobarbital has been shown to produce thyroid tumours in rodents (Hiasa et al., 1982; McClain et al., 1988) by an MOA that involves activation of CAR (Qatanani & Moore, 2005; Qatanani, Zhang & Moore, 2005). Since a CAR knockout rat has, to date, not been developed, the role of CAR in the MOA for fenpyrazamine UGT induction was determined in rat hepatocytes using the RNAi technique. The treatment of rat hepatocytes with CAR-siRNA significantly reduced CAR mRNA in the presence of fenpyrazamine as mentioned in the preceding liver section, resulting in a significant reduction in the magnitude of induction of UGT1A/2B1 mRNA levels by fenpyrazamine (Fig. A4). These findings demonstrate that fenpyrazamine induces UGT1A/2B1 through the CAR, fenpyrazamine thus having activating effect on CAR in rat hepatocytes, similar to phenobarbital.

Figure A4. mRNA levels[a] of UGT1A and UGT2B1 induced by fenpyrazamine[b] in hepatocytes with or without CAR knockdown



CAR: constitutive androstene receptor; Control (Untreated): without fenpyrazamine or siRNA;
mRNA: messenger ribonucleic acid; siRNA(CAR): short-interfering ribonucleic acid for constitutive androstene receptor;
Control (Untreated): without fenpyrazamine or siRNA; SD: standard deviation;
siRNA: short-interfering ribonucleic acid; siRNA(NC): short-interfering RNA for negative control;
UGT: uridine 5'-diphospho-glucuronosyltransferase;
**: $P < 0.01$, statistically different from Control (Untreated) group;
##: $P < 0.01$, statistically different from fenpyrazamine + siRNA(NC) group

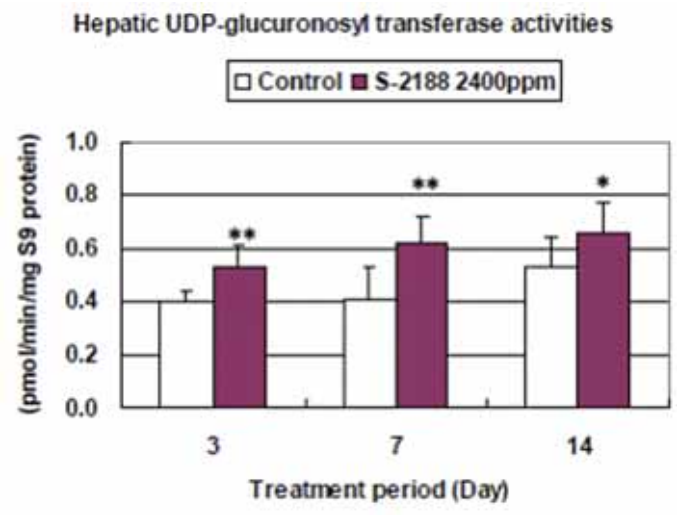
[a] Columns present expression level of mRNA. Mean ± SD, $N = 4$.

[b] Concentration of fenpyrazamine was 50 μmol/L.

ii. Enzyme induction

UGT isoform induction has been shown to be associated with thyroid tumour formation in rodents exposed to phenobarbital and related compounds (Hiasa et al., 1982; McClain et al., 1988; Whysner, Ross & Williams, 1996; Finch et al., 2006). It involves activation of nuclear receptors, particularly the CAR (Qatanani & Moore, 2005; Qatanani, Zhang & Moore, 2005). Hepatic CYP2B and UGT were both induced by phenobarbital via CAR (Yamamoto et al., 2004; Qatanani & Moore, 2005; Qatanani, Zhang & Moore, 2005; Holsapple et al., 2006; Deguchi et al., 2009; Yamada et al., 2009). Significant increases of hepatic T_4-UGT activity was observed in rat treated with 2400 ppm of fenpyrazamine for 3, 7 and 14 days (Fig. A5).

Figure A5. Hepatic uridine 5′-diphospho-glucuronosyltransferase activities toward thyroxine (T_4) [a]



ppm: parts per million; SD: standard deviation; UDP: uridine 5′-diphospho-glucuronosyltransferase;
*: $P < 0.05$, **: $P < 0.01$, statistically significant differences from the control

[a] The activities were determined in the liver from rats treated with fenpyrazamine for 3, 7 or 14 days. Values represent group mean ± SD, $N = 10$.

Additional evidence that microsomal enzyme induction is occurring at biologically relevant levels is supported by the observation of hepatocellular hypertrophy, particularly in the centrilobular region of the liver lobule ((Kondo, 2010; Table A3). This is a characteristic change of microsomal enzyme inducers, such as phenobarbital and related compounds. Thus, there is both direct and indirect evidence for microsomal enzyme induction by fenpyrazamine administration with biologically important consequences.

iii. Circulating levels of thyroid hormone and TSH

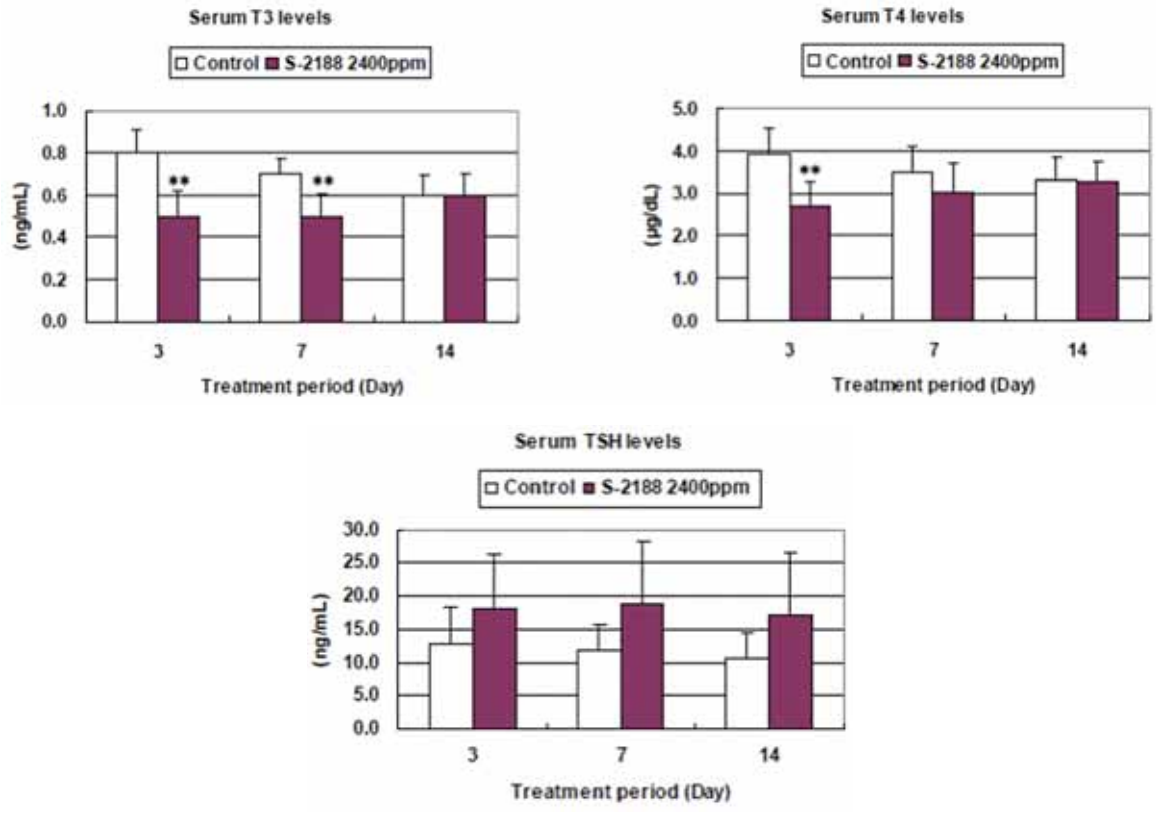
Induction of hepatic T_4-UGT activity results in an increase in hepatic metabolism and biliary excretion of T_4 and consequently a decrease in serum T_4 half-life and concentration. While hepatic metabolism and biliary excretion of T_4 were not determined, reduced serum levels of T_4 and T_3 and a tendency of increase of serum TSH level were observed in fenpyrazamine-treated rats within 3 days (Fig. A6).

iv. Thyroid morphology

Increased thyroid follicular cell hypertrophy was observed at 2400 ppm after 7- and 14-day treatment (Table A3). This was also observed in a 90-day study (3000 ppm) and 2-year study (2400 ppm). In addition, in the 2-year study, increased incidence of thyroid follicular cell hyperplasia was also observed in rats treated with fenpyrazamine (1200 and 2400 ppm). Thus, there is direct and strong evidence in support of the key events of increased thyroid follicular cell hypertrophy and hyperplasia by fenpyrazamine. Especially, time-course alteration of the thyroid morphology is similar to the T_4-UGT inducer, phenobarbital.

It should be noted that for all of these effects, the response to fenpyrazamine was relatively weak in comparison to those usually seen with a strong inducing agent such as phenobarbital, but the tumorigenic effect (if exists) was also quite weak even at doses of the MTD (2400ppm).

Figure A6. Serum hormone levels (T_3, T_4 and TSH)[a]



ppm: parts per million; S-2188: fenpyrazamine; SD: standard deviation; T_3: triiodothyronine; T_4: tetraiodothyronine; TSH: thyroid-stimulating hormone;
**: $P < 0.01$ statistically significantly different from the control

[a] Changes in T_3, T_4 and TSH levels were determined in serum from rats treated with fenpyrazamine for 3, 7 or 14 days. Values represent group mean ± SD, $N = 10$.

Thus, based on the key events listed, biological indicators of MOA by fenpyrazamine should include changes in liver metabolism, alterations in hormone levels, increases in thyroid growth and lesion progression in the thyroid.

C. Dose–response relationship

The parameters described above were not evaluated with respect to dose in the Kondo (2010) MOA study. However, some of the parameters can be evaluated using data from the 90-day study (0, 300, 600, 1000 and 3000 ppm) and the 2-year study (0, 100, 300, 1200 and 2400 ppm) (Table A3).

i. Enzyme induction

In the Kondo (2010) MOA study, increased T_4-UGT activity with hepatocellular hypertrophy was observed in males at the dose level at which thyroid follicular cell tumours were observed in the 2-year study (2400 ppm). Although T_4-UGT activity was not examined, hepatocellular hypertrophy, which indicated occurrence of enzyme induction (Cohen, 2010), was observed at dose levels of 3000 ppm in the 90-day study and at 1200 and 2400 ppm on weeks 52 and 104 in the 2-year study.

ii. Circulating levels of thyroid hormone and TSH

Decreased serum levels of T_4 and T_3 and increased serum TSH levels were observed in males at the dose level (2400 ppm) at which thyroid follicular cell tumour was observed in 2-year study. Although these parameters were not examined with respect to dose response, alterations in thyroid morphology, the next key event of the increased TSH, were evaluated with respect to dose response.

iii. Thyroid morphology

Increased thyroid follicular cell hypertrophy was observed at 2400 ppm; increased incidence of thyroid follicular cell hyperplasia was also observed at 1200 and 2400 ppm in rats treated with fenpyrazamine.

iv. Tumour induction

Thyroid follicular cell carcinoma was observed in male rats given fenpyrazamine at 2400 ppm but not observed in other groups, including control males, or in females. The various parameters described above that relate to the key events in the induction of thyroid tumours were observed at or below the dose levels at which these tumours occurred in males in the 2-year study with fenpyrazamine (Table A3). Generally, effects on liver enzymes/weight and pituitary–thyroid hormone concentrations would be expected to occur at doses at least as low as those that produce thyroid weight changes and increases in thyroid tumour incidence, given that this thyroid disruption MOA is a threshold phenomenon (Dellarco et al., 2006).

Taken together, there are strong parallels in the dose response for the key events and the tumour response. Again, it is noteworthy that the degree of changes observed in each of the parameters was relatively mild, consistent with the slight increase in tumour incidences, particularly when compared not only to the current controls but to the range of historical controls.

Table A3. Dose–response relationship of changes in thyroid and liver of male rats treated with fenpyrazamine

Parameter	Measure per dose level							
	0 ppm	**100 ppm**	**300 ppm**	**600 ppm**	**1000 ppm**	**1200 ppm**	**2400 ppm [a]**	**3000 ppm**
Thyroid tumours in 2-year study [b]								
Thyroid follicular cell carcinoma	0/50	1/50	1/50	ND	ND	0/50	3/49 (6.1%)	ND
Thyroid follicular cell adenoma	2/50	4/50	2/50	ND	ND	350	3/49	ND
Hepatic T_4-UGT induction (pmol/min per mg S9 protein)								
Day 3 in 14-day MOA study	0.4	ND	ND	ND	ND	ND	0.53** (133%)	ND
Day 7 in 14-day MOA study	0.41	ND	ND	ND	ND	ND	0.62** (151%)	ND
Day 14 in 14-day MOA study	0.53	ND	ND	ND	ND	ND	0.66* (125%)	ND
Hepatocellular hypertrophy [b]								
Day 3 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	5/10* (50%)	ND
Day 7 in 14-day MOA study	1/10	ND	ND	ND	ND	ND	6/10* (60%)	ND
Day 14 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	9/10** (90%)	ND
90-Day study	1/12	ND	1/12	0/12	1/12	ND	ND	4/12* (33%)
Week 52 in 2-year study	1/20	3/20	7/20* (35%) [c]	ND	ND	10/20** (50%)	8/20** (40%)	ND
Week 104 in 2-year study	0/50	2/50	2/50	ND	ND	7/50* (14%)	6/50* (12%)	ND

Parameter	Measure per dose level							
	0 ppm	100 ppm	300 ppm	600 ppm	1000 ppm	1200 ppm	2400 ppm [a]	3000 ppm
Relative liver weight (g/100g bw)								
Day 3 in 14-day MOA study	4.60	ND	ND	ND	ND	ND	5.49** (119%)	ND
Day 7 in 14-day MOA study	4.45	ND	ND	ND	ND	ND	5.38** (121%)	ND
Day 14 in 14-day MOA study	4.07	ND	ND	ND	ND	ND	5.20** (128%)	ND
90-Day study	2.58	ND	2.54	2.52	2.63	ND	ND	3.15** (112%)
Week 52 in 2-year study	2.49	2.43	2.38	ND	ND	2.69* (108%)	2.98** (120%)	ND
Week 104 in 2-year study	2.34	2.38	2.38	ND	ND	2.44	2.85** (122%)	ND
Serum T_3 levels (ng/mL)								
Day 3 in 14-day MOA study	0.8	ND	ND	ND	ND	ND	0.5** (63%)	ND
Day 7 in14-day MOA study	0.7	ND	ND	ND	ND	ND	0.5** (71%)	ND
Day 14 in 14-day MOA study	0.6	ND	ND	ND	ND	ND	0.6	ND
Serum T_4 levels (ng/mL)								
Day 3 in 14-day MOA study	3.95	ND	ND	ND	ND	ND	2.72** (69%)	ND
Day 7 in 14-day MOA study	3.49	ND	ND	ND	ND	ND	3.02 (87%)	ND
Day 14 in 14-day MOA study	3.31	ND	ND	ND	ND	ND	3.26 (98%)	ND
Serum TSH levels (ng/mL)								
Day 3 in 14-day MOA study	12.8	ND	ND	ND	ND	ND	17.9 (140%)	ND
Day 7 in 14-day MOA study	11.7	ND	ND	ND	ND	ND	18.7 (160%)	ND
Day 14 in 14-day MOA study	10.7	ND	ND	ND	ND	ND	17.1 (160%)	ND
Relative thyroid weight (mg/100g bw)								
Day 3 in 14-day MOA study	6.2	ND	ND	ND	ND	ND	6.3	ND
Day 7 in 14-day MOA study	6.0	ND	ND	ND	ND	ND	7.8** (130%)	ND
Day 14 in 14-day MOA study	7.2	ND	ND	ND	ND	ND	8.1 (113%)	ND
90-Day study	7	ND	6	6	7	ND	ND	8

Parameter	Measure per dose level							
	0 ppm	100 ppm	300 ppm	600 ppm	1000 ppm	1200 ppm	2400 ppm [a]	3000 ppm
Thyroid follicular cell hypertrophy; diffuse [b]								
Day 3 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	7/10** (70%)	ND
Day 7 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	9/10** (90%)	ND
Day 14 in 14-day MOA study	0/10	ND	ND	ND	ND	ND	9/10** (90%)	ND
90-Day study	0/12	ND	0/12	1/12	1/12	ND	ND	4/12* (33%)
Week 104 in 2-year study	1/50	0/50	1/50	ND	ND	1/50	2/49 (4.1%)	ND
Thyroid folicullar-cell hyperplasia; diffuse [b]								
Week 104 in 2-year study	5/50	5/50	8/50 [c]	ND	ND	7/50 (14%)	11/49 (22.4%)	ND
Thyroid folicullar-cell hyperplasia; focal [b]								
Week 104 in 2-year study	1/50	0/50	0/50	ND	ND	1/50	3/49 (6.1%)	ND

bw: body weight; MOA: mode of action; ND: not determined; ppm: parts per million;
T3: triiodothyronine; T4: tetraiodothyronine; TSH: thyroid-stimulating hormone;
UGT: uridine 5'-diphospho-glucuronosyltransferase; *: $0.01 < P < 0.05$, **: $P < 0.01$, in comparison to control

[a] Highest dose of the 2-year study.

[b] Results presented as number of animals with finding / number of animals examined.

[c] This change is considered not treatment related because of a lack of a dose response.

Sources: Kondo (2010); Sommer (2009b)

D. Temporal association

It is critical in the evaluation of MOA that early key events occur before the appearance of tumours, and this is clearly the case with fenpyrazamine. Multiple exposure time data at 3, 7, and 14 days, 52 and 104 weeks are available in which male Wistar rats were offered diets containing 2400 ppm. Liver effects including weight and hepatocellular hypertrophy were increased at all observation times from the earliest time of assessment on day 3. Hepatic T_4-UGT activity was also increased at all observation times examined (days 3, 7 and 14). Biliary excretion of conjugated T_4 was not measured in this experiment; however, serum T_4 was reduced at early phase of treatment such as days 3 and 7. Increases in circulating TSH were observed at all sampling times, although the increase was not statistical significant. Slight but continuous increase of circulating TSH is considered enough to alter thyroid morphology such as follicular cell hypertrophy and hyperplasia (Hood et al., 1999). Increases in thyroid weight were also observed at 7 days after treatment began. Histopathologically, there was a time-related increase in follicular cell hypertrophy beginning at 3 days. The appearance of follicular cell hyperplasia and carcinoma occurred only after chronic administration of fenpyrazamine. Thus, there is a logical temporal response for the key events of thyroid follicular cell tumour formation in which all key events precede tumour development.

E. Strength, consistency and specificity of association of tumour response with key events

Strength, consistency and specificity of the association can be established from the studies described earlier. The quantifiable precursor events, fundamental to the proposed MOA, are relatively consistent with the emergence of thyroid follicular cell tumours. Observation of liver weight increase and induction of hepatic T_4-UGT in rats receiving fenpyrazamine in the diet would be consistent with perturbation of homeostasis of the pituitary–thyroid axis by an extrathyroidal mechanism. An increase in hepatic T_4-

UGT activity is a step that occurs before the other key biochemical changes and before thyroid follicular cell hypertrophy and hyperplasia. Fenpyrazamine treatment clearly results in a decrease in circulating T_4 and an increase in TSH following enhanced liver metabolism of T_4. Furthermore, the development of hypertrophy/hyperplasia were shown to be increased under the same conditions of dose and time as the appearance of changes in thyroid hormone levels.

F. Biological plausibility and coherence

There are considerable data from studies in laboratory rodents demonstrating the relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by TSH and the progression of thyroid follicular cells to hypertrophy, hyperplasia, and eventually neoplasia (McClain, 1995; Hard, 1998; Hurley, Hill & Whiting, 1998; IARC, 2001). Increased secretion of TSH may result of several mechanisms, including increased hepatic clearance of T_4, as is the case with fenpyrazamine. Circulating levels of T_4 are monitored by the thyrotropic cells of the pituitary gland, which are responsible for the synthesis of TSH. In the pituitary gland, T_4 is metabolized by 5-deiodinase type II to T_3, which then binds to specific receptors in the cell nucleus. A decrease in T_3 receptor occupancy results in stimulation of TSH synthesis and secretion. Studies in vivo have shown that injection of rats with TSH leads to reductions in thyroid follicular cell nuclear statin, a non-proliferation specific nuclear antigen, indicating that these cells were leaving the non-dividing state to resume the cell cycle (Bayer et al., 1992). This study showed that low, repeated doses of TSH (0.25 IU/rat twice daily) produced a cumulative response in nuclear statin levels over 10 days, and the response returned to normal resting levels within 5 days of cessation of TSH injections. Reduction in nuclear statin is also an early event that parallels the earliest known pinocytotic response to TSH. These data are consistent with increased TSH concentrations alone causing thyroid follicular cells of rats to enter a state of pre-proliferation. Therefore, the suggestion that the high dose of fenpyrazamine causes thyroid follicular cell neoplasms in rats by initially inducing hepatic T_4-UGT is in line with the known physiology of the hypothalamus–pituitary–thyroid dynamic control system, at least to the stage of hypertrophy and hyperplasia. The fenpyrazamine-induced key events summarized above are similar to those seen with phenobarbital (Dellarco et al., 2006; Finch et al., 2006). We can therefore conclude that fenpyrazamine has a similar MOA to that of phenobarbital for rat thyroid tumour formation.

G. Other MOAs

As described in the earlier discussion of liver, it can be concluded that fenpyrazamine has no genotoxic potential.

Additional effects on the hypothalamic–pituitary–thyroid axis or disruption of other pathways of thyroid hormone metabolism (e.g. blockade of T_4 synthesis, receptor blockade, etc.) are other possibilities of altering thyroid homeostasis. These variations would not differ in any fundamental way from the one that has been proposed for fenpyrazamine, in that all would lead to prolonged TSH stimulation with continuous exposure.

H. Uncertainties, inconsistencies and data gaps

For the most part, the data are quite consistent with key events at several time points and at several doses, as noted in Table A3. However, there are some apparent inconsistencies. The increased thyroid weight is a key event in the MOA for thyroid tumours induced by enhancement of thyroid hormone metabolizing enzyme (Dellarco et al., 2006). However, the increased thyroid weight was not observed at 3000 ppm in the 90-day rat study with fenpyrazamine. Thyroid weight data are sometimes unreliable because of the small size, and thyroid histopathology tends to be a more reliable indicator than thyroid weight (DeVito et al., 1999; Hood et al., 1999; Yamada et al., 2004). Since the carcinogenic potential of fenpyrazamine is very weak (if indeed real), and the incidence of thyroid folicullar cell carcinoma observed at 2400 ppm of fenpyrazamine was only marginally above comparable historical control data, the increased thyroid weight may not have been detectable. Although increased thyroid weight was not observed after chronic treatment with fenpyrazamine, the Meeting did not believe that this is essential because direct evidence of histopathological alterations of thyroid such as hypertrophy/hyperplasia, suggesting increased thyroid weight, was observed.

It is well accepted that TSH stimulation resulted in increased thyroid weight, thyroid follicular cell hypertrophy and proliferation (Capen, 1997; Dellarco et al. 2006). The higher levels of serum TSH, although not statistically significant, were observed at all time points examined – 3, 7 and 14 days after treatment began. It was reported that small increases in serum TSH can be sufficient to stimulate thyroid follicular cell proliferation (Hood et al., 1999). Therefore the slight and increase of TSH (without statistical significance) by fenpyrazamine is considered to be a biologically meaningful change.

Although data for T_4 biliary elimination was not collected, the Meeting considered that this is not essential because the direct evidence for reduced serum T_4 and of increased hepatic T_4-UGT by fenpyrazamine administration was obtained.

Lastly, the tumour response elicited by fenpyrazamine is typical of a rodent thyroid carcinogen in that thyroid follicular cell tumours are found in male rats but not in female rats or mice. Rats tend to be more sensitive to thyroid carcinogenesis than mice, and male rats are frequently found to be more sensitive than female rats with respect to the proportion of chemicals that induce thyroid tumours (Hurley, Hill & Whiting, 1998). In keeping with this, TSH levels are typically higher in male rats than in females (Hill et al., 1989). In addition, male rats are sometimes more prone to hepatic enzyme induction than females of the same strain, but this depends on the enzyme in question, the dose of the inducing compound, and the age of the animals (Sundseth & Waxman, 1992; Agrawal & Shapiro, 1996; Oropeza-Hernández, López-Romero & Albores, 2003).

I. Assessment of postulated MOA

As described above, the key events in the MOA for fenpyrazamine have been well documented, with a strong dose and temporal consistency. In addition, this is an established MOA, and the various parameters essential for documenting this MOA have been presented for fenpyrazamine. Thus, we consider that the level of confidence in the postulated MOA is high.

J. Human applicability of the proposed MOA

Human applicability of the proposed MOA for thyroid tumour by fenpyrazamine is also evaluated by the IPCS Human Relevance Framework (Meek et al., 2003) modified based on discussions by the IPCS Cancer Working Group (Boobis et al., 2006).

1. *Is the weight of evidence sufficient to establish an MOA in animals?*

As described in detail earlier, there is clear evidence that fenpyrazamine alters thyroid homeostasis by UDP-glucuronosyltransferase induction causing reduced serum T_4 levels and consequently elevating serum TSH.

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?*

The current understanding of the regulation of thyroid hormone homeostasis in humans and of the role of increased TSH levels (as a result of altered thyroid homeostasis) as a risk factor for thyroid cancer was considered in order to assess the human relevance of the key events in animal mode of carcinogenic action by fenpyrazamine. Although there are substantial quantitative dynamic differences (discussed later), the fundamental mechanisms involved in the function and regulation of the hypothalamic–pituitary–thyroid axis in rats are qualitatively similar to those in humans (Bianco et al., 2002). Therefore, an agent that decreases T_4 levels in rats could likewise reduce T_4 in humans; this, in turn, could potentially lead to an increase in TSH levels. There are data showing that rodents and humans respond in a similar fashion to perturbations of pituitary–thyroid function. For example, it is established that iodine deficiency, which leads to decreased thyroid hormone levels, stimulates thyroid cell proliferation in humans, leading to goitre. If left untreated, iodine deficiency may lead to tumour formation, albeit rarely (Thomas & Williams, 1999). Although there is no evidence of increased susceptibility to thyroid cancer, a number of pharmaceuticals (e.g. propylthiouracil, lithium, amiodarone, iopanoic acid) that disrupt thyroid homeostasis by acting directly on the thyroid gland (for example, by inhibiting hormone synthesis or release or by blocking the conversion of T_4 to T_3) are known to lead to hypothyroidism and increases in TSH in humans (Ron et al., 1987).

In contrast to rats, no increases in TSH levels have been found in humans following exposure to agents that induce hepatic microsomal enzymes and reduce circulating T_4 levels (Meek et al., 2003). For example, the pharmaceutical compounds phenytoin, rifampin and carbamazepine induce hepatic microsomal enzymes, including UGT, and reduce circulating T_4 levels, but TSH levels are unchanged (Curran & DeGroot, 1991); agents that produce thyroid tumours in rats by increasing glucuronidation and biliary excretion of T_4 at high experimental doses (e.g. omeprazole, lansoprazole and pantoprazole) produce no changes in thyroid hormones at clinical doses in humans (Masubuchi, Hakusui & Okazaki, 1997). Thus, there appears to be a substantial difference between rats and humans in the dose–response relationship for altered homeostasis of the pituitary–thyroid axis. This observation is based on the quantitative dynamic differences between rats and humans in the basic physiological processes underlying pituitary–thyroid function.

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?*

The primary effect of fenpyrazamine is on hepatic metabolizing enzymes, and the increase in metabolic activity indirectly increases the systemic clearance of T_4, leading to the hypothyroid state and the compensatory increase in TSH found in rats. Although there are no chemical-specific data on the potential for fenpyrazamine to disrupt thyroid hormone homeostasis in humans, a number of other microsomal enzyme inducers have been extensively studied, such as phenobarbital (Meek et al., 2003). As discussed earlier; agents that produce hypothyroidism by altering hepatic clearance of T_4 do not appear to result in elevated TSH levels in humans. Presumably, TSH is not increased because a critical reduction of T_4 is not reached. There are several important physiological and biochemical differences between rats and humans related to thyroid function. Rats have a smaller reserve capacity of thyroid hormones compared with humans. The rat has a much shorter thyroid hormone half-life than humans. The half-life of T_4 is about 12 hours in rats, compared to 5–9 days in humans (Döhler, Wong & von zur Mühlen, 1979). The shorter half-life in rats is likely related to the absence of a high-affinity binding globulin for T_4 that is present in humans (Hill, et al., 1989). In rats, the increased clearance contributes to the need for a higher rate of production of T_4 (per unit of body weight) to maintain normal levels of T_4. In contrast, in humans, the binding of thyroid hormone to this globulin accounts for a slower metabolic degradation and clearance that in turn results in the thyroid gland being less active than in rats. The constitutive TSH levels are approximately 25 times higher in rats than in humans, reflecting the higher activity of the pituitary–thyroid axis in rats (Döhler, Wong & von zur Mühlen, 1979; McClain, 1992). Therefore, humans are quantitatively less sensitive than rats to agents that reduce T_4 and lead to elevated TSH. There is no increased risk of thyroid tumour development if TSH is not elevated.

Another difference between rats and humans is the histological appearance of the thyroid. The rat thyroid is more "functionally active", with the rate of production of T_4 needed to maintain a consistent serum concentration higher in rats than in primates including humans (McClain, 1995). More of the follicular epithelium in the rat is stimulated to synthesize thyroglobulin, and therefore more of the follicular cells are tall cuboidal and appear to be active in synthesis. In contrast, more of the follicular cells in humans tend to be short cuboidal or almost squamous in appearance, suggesting they are quiescent. Because rat follicular cells are already generally active, under stimulation from TSH, they will respond with hyperplasia more readily than human follicular cells. Because of the greater storage capability of the human thyroid and the greater numbers of cells in a quiescent state, human thyroid follicular cells will be roused from their quiescent state to synthesize and secrete additional thyroid hormone without the need for a hyperplastic response to re-establish homeostasis. Therefore, the primary response in the human thyroid gland would be thyroglobulin reabsorption and cellular hypertrophy rather than hyperplasia.

In short, there is much greater buffering capacity in the biochemistry of the human than the rat thyroid. Even though certain agents can cause a reduction in thyroid hormone levels in humans, there is no clear evidence that these agents increase susceptibility to thyroid cancer (Ron et al., 1987). For example, epidemiological studies with phenobarbital do not show any increased risk of thyroid cancer (Olsen et al., 1989, 1993). Studies of individuals with conditions that would lead to elevated TSH (patients with Graves' disease or goitre) indicate the occurrence of thyroid cancer is rare in these circumstances (Mazzaferri, 2000; Gabriele et al., 2003). A study of environmental and heritable causes of cancer among 9.6 million individuals, using the Nationwide Swedish Family Cancer

Database, found that the environment did not appear to play a principal causative role in thyroid cancer (Czene, Lichtenstein & Hemminki, 2002). The only known human thyroid carcinogen is radiation, a mutagenic exposure. As summarized in Table A4, there is sufficient evidence in the general literature on the biochemical and physiological differences in thyroid function to indicate differences between rats and humans in tumour susceptibility. In contrast to humans, rats are very susceptible to thyroid neoplasia secondary to hypothyroidism. In particular, modest changes in thyroid hormone homeostasis will promote tumour formation in rats. Thus, thyroid tumours in rats treated with fenpyrazamine at 2400 ppm that involve increased hepatic clearance of hormone and altered homeostasis of the pituitary–thyroid axis are considered not relevant to humans, based on quantitative dynamic differences.

K. Conclusion: statement of confidence, analysis and implications

The data available for fenpyrazamine are considerable and, despite some data gaps such as the lack of direct data regarding T_4 biliary elimination, and discrepancy such as no apparent alteration of thyroid weight, it is clear that the MOA for fenpyrazamine-induced rat thyroid tumours (if real) is secondary to enhanced metabolism of T_4 leading to hormone imbalance. Although the possibility that fenpyrazamine may potentially result in hypothyroidism in humans cannot be ruled out, there is sufficient quantitative evidence on the basic physiological processes in the general literature to conclude that thyroid tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to tumour development in humans. Based on the above evidence, it is reasonable to conclude that fenpyrazamine will not have any thyroid carcinogenic activity in humans.

Table A4. A comparison of key events by fenpyrazamine in rats and humans

Key events	Evidence in rats	Evidence in humans
Increase hepatic clearance of T_4	In short-term and chronic rat studies, the liver is found to be the most sensitive target, and evidence of increased T_4 hepatic clearance is provided by studies on T_4-hepatic UGT activity, and liver weights and hypertrophy.	No data available for fenpyrazamine, but microsomal enzyme induction may be plausible.
Decreased serum T_4	Direct experimental evidence.	No data available for fenpyrazamine, but not plausible given that other microsomal enzyme inducers have been shown to reduce T_4 in humans.
Increased TSH levels	Direct experimental evidence.	No data available for fenpyrazamine, but other microsomal enzyme inducers have not been shown to increase TSH levels even when T_4 is decreased.
Increased TSH increases thyroid cell proliferation and tumour formation	Direct experimental evidence.	Induction of thyroid follicular cell tumours secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis. Occurrence of thyroid cancer is rare even in severely hypothyroid individuals.

T_4: tetraiodothyronine; TSH: thyroid-stimulating hormone; UGT: uridine 5'-diphospho-glucuronosyltransferase

Conclusions

The postulated MOAs for possible tumour induction in liver and thyroid of rats were tested against the Bradford Hill criteria and were found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency and specificity that fits with an established MOA for hepatocellular carcinoma and thyroid follicular cell carcinoma. Regarding hepatocellular carcinoma, the postulated MOA would not theoretically operate in humans, as demonstrated by CYP2B inducers. Not only is there a qualitative difference between the rodent and human in the response of the liver cells to the CYP2B inducers regarding induction of tumours, but also a marked quantitative difference in the level of exposure. Thus, based on quantitative considerations, the confidence in a lack of effect in humans at expected exposures is even stronger than that based only on qualitative consideration. As for thyroid follicular cell carcinoma, although the postulated MOAs could theoretically operate in humans, there are marked quantitative differences in the inherent susceptibility for neoplasia to thyroid hormone imbalance in rats and humans. Therefore, even though the hepatocellular carcinoma and thyroid follicular cell carcinoma observed in the rat 2-year study is treatment related, the findings from the MOA analysis allow for the conclusion that fenpyrazamine does not pose a carcinogenic hazard to humans.

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FENPYROXIMATE

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Explanation

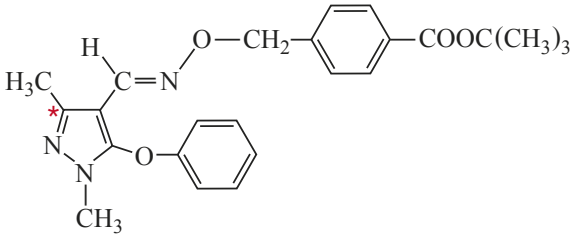
Fenpyroximate is the International Organization for Standardization (ISO)–approved common name for *tert*-butyl (*E*)-alpha-(1,3)-dimethyl-5-phenoxy-1*H*-pyrazol-4-yl methyleneamino-oxy)-*para*-toluate (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) number 134098-61-6 (*E* isomer) and 111812-58-9 (unspecified stereochemistry).

Fenpyroximate (Fig. 1) is a phenoxypyrazole acaricide for application to leaves infested with phytophagous mites. Fenpyroximate inhibits mitochondrial NADH-coenzyme Q reductase of the electron transport chain in mites. It is very active against phytophagous mites, relatively less active against predacious mites and inactive against animal parasitic and soil mites. The only insect against

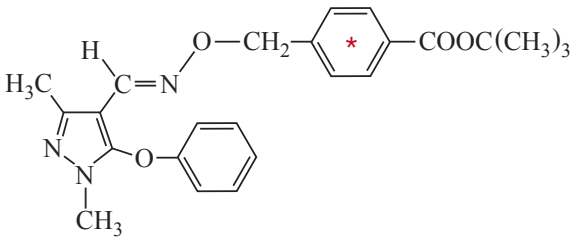
which it is active is *Empoasca onukii* (tea green leafhopper) (Taninaka, 1993). Fenpyroximate inhibits mitochondrial NADH-coenzyme Q reductase on the electron transport chain in *Tetrachynus urticae* (two-spotted spider mite) and in rats (Motoba, Suzuki & Uchida, 1992).

Fenpyroximate was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1995, when an accepTable daily intake (ADI) of 0–0.01 mg/kg bw was established based upon a no observed-adverse-effect level (NOAEL) of 1 mg/kg bw per day in a 104-week study in rats and a safety factor of 100. The critical effect in that study was a reduction in body weight gain and plasma protein concentration. Fenpyroximate was re-evaluated by the 2004 JMPR in order to determine an acute reference dose (ARfD). The ARfD of 0.01 mg/kg bw was established based on the marginal lowest-observed-adverse-effect level (LOAEL) of 2 mg/kg bw per day for the marginal induction of diarrhoea at the beginning of the 13-week toxicity study in dogs using a safety factor of 200 as no NOAEL was identified in the study. The ARfD was re-evaluated by the 2007 JMPR. The 2007 Meeting established an ARfD of 0.02 mg/kg bw based on the NOAEL of 2 mg/kg bw identified from a study of fenpyroximate, where diarrhoea was observed after a single dose in dogs, and using a safety factor of 100. Furthermore, the Meeting also stated that it remained unclear whether the diarrhoea observed in dogs was the result of fenpyroximate being a direct irritant or its pharmacological effects; it was not possible to consider a modification in the safety factor.

Figure 1. Structure of the radiolabelled fenpyroximate



* Indicates position of ^{14}C label in [pyrazole-^{14}C]fenpyroximate



* Indicates position of ^{14}C label in [benzyl-^{14}C]fenpyroximate

Fenpyroximate was reviewed by the present Meeting under the periodic review programme of Codex Committee on Pesticide Residues (CCPR). New studies included a phototoxicity study, acute and short-term neurotoxicity studies in rats, immunotoxicity study, single- and repeated-dose toxicity studies in dogs, gene mutation studies on the parent and metabolites and an in vitro metabolism study.

All studies evaluated in this monograph were performed by laboratories that were certified for good laboratory practice and that complied, where appropriate, with the relevant Organisation for Economic Co-operation and Development (OECD) test guidelines or similar guidelines of the European Union or United States Environmental Protection Agency, unless otherwise indicated. A search of the open literature did not retrieve any relevant publications.

Evaluation for acceptable intake

1 Biochemical aspects

Absorption, distribution, metabolism and excretion of fenpyroximate were studied in rats following a single oral low dose, a single oral high dose and a single oral low dose repeated daily for 14 days and followed by a radioactive dose. A biliary excretion study used [pyrazole-^{14}C]- and [benzyl-^{14}C]-labelled fenpyroximate (Fig. 1).

1.1 Absorption, distribution and excretion

(a) Oral administration

A pharmacokinetic study was conducted in Sprague Dawley (Crl:CD(SD)BR) rats using [pyrazole-^{14}C]-labelled fenpyroximate (purity 99.6%). In this study, rats (5/sex per group) received a single gavage dose of approximately 2 mg/kg bw or 400 mg/kg bw of [pyrazole-^{14}C]fenpyroximate suspended in 1% aqueous Tween 80. Two control groups received the vehicle only. Blood samples were taken from the low-dose animals up to six days after dosing and from the high-dose animals up to nine days after dosing. Control rats were killed at three hours after dosing (Sharp, 1990a). In a separate study, rats (5/sex per dose) were administered a single gavage dose of [benzyl-^{14}C]-labelled fenpyroximate suspended in 1% aqueous Tween 80 at 2 or 400 mg/kg bw. Blood samples were taken from the low-dose animals for six days after dosing and from the high-dose animals for nine days after dosing.

Calculated pharmacokinetic parameters for both single oral doses of the two radiolabels at 2 and 400 mg/kg bw are shown in Table 1. In rats given [pyrazole-^{14}C]-labelled fenpyroximate at 2 mg/kg bw, the maximum concentration of radioactivity in blood was reached (T_{max}) at approximately 11 hours, with mean maximum concentrations (C_{max}) of 0.152 and 0.176 µg equiv./g for males and females, respectively. By 72 hours after dosing, the concentration of radioactivity was at or below the limit of detection (LOD; 0.003 µg equiv./g). In rats given 400 mg/kg bw [pyrazole-^{14}C]-labelled fenpyroximate, the T_{max} was approximately 101 hours for males (4.67 µg equiv./g) and 90 hours for females (4.69 µg equiv./g). By 216 hours after dosing,the concentration of radioactivity was at or below the LOD (0.549 µg equiv./g).

No sex differences were apparent in the pharmacokinetics of [pyrazole-^{14}C]-labelled fenpyroximate. Absorption of the high dose was greatly delayed relative to the low dose, and the half-life was much longer (Sharp, 1990a).

In rats given [benzyl-^{14}C]-labelled fenpyroximate at 2 mg/kg bw, the maximum concentration of radioactivity in blood was reached at 7.8 hours for males (0.097 µg equiv./g) and 7.2 hours for females (0.181 µg equiv./g). By 48 hours after dosing, the concentration of radioactivity was at or below the LOD (0.003 µg equiv./g). In rats given [benzyl-^{14}C]-labelled fenpyroximate at 400 mg/kg bw, the maximum concentration of radioactivity in blood was reached at approximately 28.8 hours for males (5.10 µg equiv./g) and 86.4 hours for females (8.88 µg equiv./g). By 168 hours after dosing, the concentration of radioactivity was at or below the LOD (0.575 µg equiv./g).

No sex differences were apparent in the pharmacokinetics of [benzyl-^{14}C]-labelled fenpyroximate. Absorption of the high dose was greatly delayed relative to the low dose, and the half-life was much longer (Sharp, 1990b).

Table 1. Pharmacokinetic parameters following a single oral dose of [pyrazole-^{14}C]- or [benzyl-^{14}C]-labelled fenpyroximate at 2 or 400 mg/kg bw in rats

Dose	Sex	Pharmacokinetic parameters [a]			
		C_{max} (µg equiv./g)	T_{max} (hours)	$t_{½}$ (hours)	AUC (µg × hour/mL)
[pyrazole-14C] label					
2 mg/kg bw	Male	0.152	11.0	8.9	3.49
	Female	0.176	11.4	8.9	3.82
400 mg/kg bw	Male	4.67	101	48.7	377
	Female	4.69	90.0	45.3	411
[Benzyl-14C] label					
2 mg/kg bw	Male	0.097	7.8	6.1	1.80
	Female	0.181	7.2	7.9	3.01
400 mg/kg bw	Male	5.10	28.8	47.0	425
	Female	8.88	86.4	35.4	728

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; eqiv.: equivalents; $t_{½}$: half-life; T_{max}: time to reach maximum concentration (C_{max})

[a] Mean of five animals/sex per dose.

Source: Sharp (1990a,b)

In a separate study, Sprague Dawley CD rats (5/sex per group) received a single oral dose of [pyrazole-^{14}C]-labelled fenpyroximate at 2 or 400 mg/kg bw. A separate group of rats (5/sex) received 14 consecutive daily doses of fenpyroximate suspended in 1% aqueous Tween 80 followed by a single dose of [pyrazole-^{14}C]-labelled fenpyroximate at 2 mg/kg bw. Urine and faeces were collected at various intervals up to 168 hours post dosing, at which time the rats were killed. Groups of animals were also killed at 24, 96 and 120 hours post dosing.

The overall recoveries (including cage washes/rinses) were 99.3%, 91.2% and 93.4% in males and 93.8%, 98.7% and 93.8% in females at low dose, repeated low dose and high dose of [pyrazole-^{14}C]-labelled fenpyroximate, respectively. The majority of the radioactivity was excreted in faeces: 84.8%, 75.6% and 76.7% of the applied dose for males and 69.7%, 79.9% and 75.1% of the applied dose for females at low dose, repeated low dose and high dose, respectively. Urine was the other important route of excretion, containing 12.2%, 13.4% and 10.8% of the applied dose in males and 17.8%, 17.5% and 11.7% of the applied dose in females at low dose, repeated dose and high dose, respectively (Table 2).

The tissue residues in general were very low at the 168-hour kill time. The tissues containing the highest concentrations of radioactivity 12 hours after dosing with 2 mg/kg bw of [pyrazole-^{14}C]-labelled fenpyroximate were liver (0.620 µg/g in males and 0.951 µg/g in females), kidneys (0.210 µg/g in both sexes), heart (0.132 µg/g in males and 0.135 µg/g in females) and urinary bladder (0.435 µg/g in males and 0.068 µg/g in females). Tissue levels started decreasing after 12 hours post dosing; at 168 hours, most of the tissue had no detecTable radioactivity except fat (0.025 µg/g in males and 0.011 µg/g in females).

In the repeated-dose group given [pyrazole-^{14}C]-labelled fenpyroximate at 2 mg/kg bw, 168 hours after the radioactive dose was administered tissue levels were highest in fat (0.016 µg/g in males and 0.008 µg/g in females), liver (0.005 µg/g in males and 0.003 µg/g in females) and carcass (0.003 µg/g in males and 0.001 µg/g in females). The tissue that contained the highest concentration of radioactivity 12 hours post dosing with [pyrazole-^{14}C]-labelled fenpyroximate at 400 mg/kg bw was liver (16.1 µg/g in males and 18.7 µg/g in females). The concentration of radioactivity in liver at 24 hours was 29.1 and 35.2 µg/g in males and females, respectively; at 96 hours, was 45.7 and 26.0 µg/g, respectively; and at 168 hours, was 5.30 and 8.83 µg/g, respectively. In high-dose animals, the distribution radioactivity was delayed (96 h) compared to low-dose animals (12 h).

Table 2. Excretion of radioactivity by rats dosed orally with [pyrazole-^{14}C]-labelled fenpyroximate

Collection time (h)	% radioactive dose applied [a]											
	Single dose 2 mg/kg bw				Repeated dose 2 mg/kg bw				Single dose 400 mg/kg bw			
	Male		Female		Male		Female		Male		Female	
	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
0–6	0.03	0.91	< 0.01	1.56	< 0.01	0.91	< 0.01	0.87	0.01	0.09	< 0.01	0.13
6–12	0.83	1.86	< 0.01	2.16	0.06	1.46	5.97	2.07	0.09	0.19	1.38	0.22
12–24	69.8	5.68	53.1	7.79	51.8	5.67	52.2	8.04	8.35	0.38	9.79	0.35
24–48	12.1	2.68	14.3	4.72	20.9	3.57	17.8	4.58	8.32	1.26	5.56	0.84
48–72	1.33	0.55	1.74	1.12	1.95	1.00	2.26	1.04	15.9	1.55	8.43	1.20
72–96	0.38	0.23	0.36	0.26	0.51	0.34	0.36	0.52	20.1	1.55	8.13	1.42
96–120	0.17	0.15	0.13	0.15	0.17	0.21	0.17	0.19	10.2	1.29	9.25	1.39
120–144	0.10	0.10	0.05	0.06	0.12	0.14	0.07	0.13	9.41	2.14	16.6	2.24
144–168	0.04	0.04	0.04	0.03	0.08	0.07	0.11	0.06	4.38	2.32	15.9	3.93
Total	84.8	12.2	69.7	17.8	75.6	13.4	79.0	17.5	76.7	10.8	75.1	11.7

bw: body weight

[a] Mean of 5/sex per group.

Source: Sharp (1991a)

In all groups for which the concentration of radioactivity was above the LODs, the concentration in plasma was about double that of blood, indicating that nearly all of the radioactivity in the blood was associated with plasma. Also, in nearly all groups, some portions of the gastrointestinal tract contained high concentration of radioactivity. High-dose animals showed delayed progression of [pyrazole-^{14}C]-labelled fenpyroximate along the gastrointestinal tract relative to low-dose animals. The majority of the radioactivity in the high-dose group was still in the stomach at 24 hours, whereas in the low-dose group nearly all of the radioactivity had been excreted at 24 hours after dosing.

In summary, following a single oral dose of [pyrazole-^{14}C]-labelled fenpyroximate to male and female rats, the majority (> 69%) of the dose was excreted in faeces independent of sex or dose level. Urine contained 11–18% of the dose. Overall recoveries of radioactivity were 91–99% of the dose. Tissue residues were generally low by 168 hours after dosing (Sharp, 1991a).

In a separate study, Sprague Dawley CD rats (5/sex per group) received a single oral dose of [benzyl-^{14}C]-labelled fenpyroximate (purity 99%) at 2 or 400 mg/kg bw. A separate group of rats (5/sex) received 14 consecutive daily doses of fenpyroximate (suspended in 1% aqueous Tween 80) at 2 mg/kg bw followed by a single dose of [benzyl-^{14}C]-labelled fenpyroximate at 2 mg/kg bw. Urine and faeces were collected at various intervals up to 168 hours post dosing. The animals were killed at 24, 96 and 120 hours post dosing.

The overall recoveries (including cage washes/rinses) were 108%, 93.7% and 96.2% in males and 97.7%, 101% and 94.8% in females at low dose, repeated low dose and high dose of [benzyl-^{14}C]-labelled fenpyroximate, respectively. The majority of the radioactivity was excreted in faeces: 91.6%, 77.9% and 80.0% of the applied dose in males and 82.7%, 90.2% and 80.1% of the applied dose in females at low dose, repeated dose and high dose of [benzyl-^{14}C]-labelled fenpyroximate, respectively. Urine was the other important route of excretion, containing 13.2%, 13.8% and 11.3% of the applied dose in males and 12.5%, 9.21% and 9.47% of the applied dose in females at low dose, repeated dose and high dose of [benzyl-^{14}C]-labelled fenpyroximate, respectively (Table 3).

Table 3. Excretion of radioactivity by rats dosed orally with [benzyl-^{14}C]-labelled NNI-850

Collection time (h)	% radioactive dose applied [a]											
	2 mg/kg bw				Repeated dose 2 mg/kg bw				400 mg/kg bw			
	Male		Female		Male		Female		Male		Female	
	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
0–6	< 0.01	0.99	< 0.01	0.35	< 0.01	1.09	< 0.01	0.96	< 0.01	0.09	< 0.01	0.06
6–12	0.08	2.22	4.16	2.94	1.71	2.08	5.07	1.57	1.04	0.13	2.50	0.11
12–24	63.6	5.18	54.8	4.89	57.9	5.30	65.4	3.11	9.95	0.36	10.8	0.27
24–48	23.3	3.22	20.6	3.17	14.9	3.58	17.4	2.53	4.45	1.43	4.78	0.86
48–72	3.00	0.86	2.39	0.78	2.41	0.97	1.68	0.53	16.1	2.19	9.19	1.14
72–96	0.76	0.37	0.48	0.16	0.71	0.28	0.37	0.23	25.1	2.62	11.6	1.25
96–120	0.39	0.22	0.12	0.11	0.17	0.19	0.15	0.11	16.2	2.07	24.4	1.18
120–144	0.17	0.09	0.06	0.06	0.09	0.16	0.12	0.12	4.24	1.16	10.7	1.73
144–168	0.27	0.05	0.05	0.05	0.06	0.11	0.05	0.06	2.92	1.23	6.06	2.87
Total	91.6	13.2	82.7	12.5	77.9	13.8	90.2	9.21	80.0	11.3	80.1	9.47

bw: body weight; NNI-850: fenpyroximate

[a] Mean of 6 rats/sex.

Source: Sharp (1991b)

The tissue residues in general were very low at the 168-hour kill time. The tissue containing the highest concentration of radioactivity 12 hours after dosing with [benzyl-^{14}C]-labelled fenpyroximate at 2 mg/kg bw was liver (3.51 µg/g in males and 4.35 µg/g in females). Tissue levels started decreasing after 12 hours post dosing; at 168 hours, most of the tissue had no detecTable radioactivity except fat (0.114 µg/g in males and 0.064 µg/g in females) and adrenals (0.018 µg/g in males and 0.025 µg/g in females).

In the repeated-dose group given [benzyl-^{14}C]-labelled fenpyroximate at 2 mg/kg bw, 168 hours after the radioactive dose was administered, tissue levels were highest in fat (0.049 µg/g in males and 0.046 µg/g in females), adrenals in males (0.013 µg/g) and ovaries in females (0.015 µg/g). The tissue that contained the highest concentration of radioactivity 12 hours after dosing with [benzyl-^{14}C]-labelled fenpyroximate at 400 mg/kg bw was liver (13.9 µg/g in males and 21.0 µg/g in females). The concentration of radioactivity in liver at 24 hours was 23.0 and 31.3 µg/g in males and females, respectively; at 96 hours, was 32.2 and 30.9 µg/g, respectively; and at 168 hours, was 2.32 and 2.31 µg/g, respectively. In high-dose animals, the distribution radioactivity was delayed (96 h) compared to low-dose animals (12 h).

In all groups with concentration of radioactivity above the LODs, the concentration in plasma was about double that in blood, indicating that nearly all of the radioactivity in the blood was associated with plasma. Also, in nearly all groups, some portions of the gastrointestinal tract contained high concentration of radioactivity. High-dose animals showed a delayed progression of [benzyl-^{14}C]-labelled fenpyroximate along the gastrointestinal tract relative to low-dose animals. The majority of the radioactivity in the high-dose group was still in the stomach at 24 hours, whereas in the low-dose group nearly all the radioactivity had been excreted at 24 hours after dosing.

In summary, following a single oral dose of [benzyl-^{14}C]-labelled fenpyroximate to male and female rats, the majority, accounting for 78–92% of the dose, was excreted in faeces independent of sex or dose level. Urine contained 9–14% of the dose. Tissue residues were generally low by 168 hours after dosing (Sharp, 1991b).

The biliary excretion of [pyrazole-^{14}C]- and [benzyl-^{14}C]-labelled fenpyroximate was determined after a single oral administration to bile duct–cannulated Sprague Dawley rats (6 rats/sex) at a dose level of 2 mg/kg bw. The bile was sampled at 12, 24, 36 and 48 hours post dosing. Urine and faeces were also collected at various intervals.

After oral administration of [pyrazole-^{14}C]-labelled fenpyroximate, about 55% and 47% of the radioactivity was excreted in bile at 48 hours post dosing by males and females; about 5% and 10% in urine; and about 28% and 17% in faeces, respectively (Table 4). The amount of radioactivity excreted via bile and urine was about 60.5% for male rats and about 56% for female rats. Based on this biliary excretion study, oral absorption of [pyrazole-^{14}C]-labelled fenpyroximate was approximately 27.7% and 17% in males and females, respectively, since radioactivity found in faeces, which just passes through the gastrointestinal tract, can be regarded as not systemically available (Lin, 1992a).

Table 4. Excretion of radioactivity from bile duct–cannulated rats after a single oral dose of [pyrazole-^{14}C]- or [benzyl-^{14}C]-labelled fenpyroximate

Fenpyroximate label	% ± SD of radioactive dose [a]							
	Male				Female			
	Bile	Urine	Faeces	Total	Bile	Urine	Faeces	Total
[pyrazole-^{14}C]	55.165 ± 11.117	5.335 ± 2.118	27.749 ± 8.633	88.25 ± 10.83	46.554 ± 6.828	9.725 ± 2.952	17.134 ± 12.333	73.41 ± 16.94
[Benzyl-^{14}C]	50.977 ± 7.071	6.173 ± 1.895	40.018 ± 6.268	97.17 ± 6.88	46.562 ± 9.745	7.978 ± 2.684	28.455 ± 6.895	82.99 ± 14.05

bw: body weight; SD: standard deviation

[a] Mean value for six rats/sex at 48 hours after single oral dose of 2 mg/kg bw of radiolabelled fenpyroximate.

Source: Lin (1992a).

After oral administration of [benzyl-^{14}C]-labelled fenpyroximate, radioactive materials excreted in bile 48 hours post dosing were about 51% and 46.5% for males and females; in urine, about 6% and 10%; and in faeces, about 40% and 28.5%, respectively (Table 4). The amount of radioactivity excreted via bile and urine was about 57.1% for male rats and about 54.5% for female rats. Based on the biliary excretion study, oral absorption of [benzyl-^{14}C]-labelled fenpyroximate was approximately 40% in males and 28.5% in females based on radioactivity found in faeces that just passed through the gastrointestinal tract and can therefore be regarded as not systemically available. The T_{max}, C_{max} and half-lives of the radiolabel in the blood of cannulated rats were similar to those of rats with no cannulae (Lin, 1992b).

1.2 Biotransformation

(a) Oral administration

Urine and faeces from the Sharp (1991a,b) study were pooled for metabolite identification (6 rats/sex per dose). For the low-dose group (2 mg/kg bw) and repeated-dose group (2 mg/kg bw), the 0–24 and 24–48 hour time-point samples underwent metabolite analysis. For the high-dose group (400 mg/kg bw), the 0–24, 24–48, 48–120 and 120–168 hour time points were analysed. Urine was lyophilized and reconstituted. Unhydrolysed urine or β-glucuronidase- or sulfatase-hydrolysed urine was concentrated, reconstituted and mixed with standard compounds and spotted on thin layer chromatography (TLC) plates.

The major urinary metabolite in the 2 mg/kg bw single-dose pyrazole-labelled fenpyroximate group was M-8 (1,3-dimethyl-5-phenoxypyrazole-4-carboxylic acid), representing 6.50–14.2% of the urinary radioactivity. Hydrolysis of urine with glucuronidase increased M-8 to 18.5–28.3% of the urinary radioactivity; hydrolysis with sulfatase increased M-8 slightly. Glucuronidase hydrolysis also formed:

M-2 (*tert*-butyl (*E*)-4-{[1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]-methyleneaminooxymethyl}benzoate),

M-5 ((*E*)-4-{[(1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]methyleneaminooxymethyl benzoic acid),

M-10 (1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbonitrile) and

M-3 ((*E*)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl] benzoic acid) in small amounts.

M-21 (4-cyano-1-methyl-5-phenoxypyrazole-3-carboxylic acid) was also observed (Table 5).

(For IUPAC names, CAS numbers and structures of the metabolites, see Appendix 1.)

Faeces from the 2 mg/kg bw single-dose pyrazole-labelled fenpyroximate group contained the parent compound (fenpyroximate, or NNI-850) and M-1 (*tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate). NNI-850 represented 1.64–14.8% of the unhydrolysed faecal radioactivity. The major metabolites observed were:

M-3 (10.1–24.5% of the faecal radioactivity),

M-11 (1,3-dimethyl-5-phenoxypyrazole-4-carbonitrile)combined with U-1 (2.45–15.7% of the faecal radioactivity) and

M-22 ((*E*)-2-[4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoyloxy]-2-methylpropanoic acid; 2.25–15.5% of the faecal radioactivity).

Minor amounts of:

M-2, M-4 ((*Z*)-4-[(1,3-dimethyl-5-phenocypyrazol-4-yl) methyleneaminooxymethyl]benzoic acid),

M-5, M-6 (1,3-dimethyl-5-phenoxypyrzole-4-carbaldehyde),

M-7 (1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde), M-8,

M-9 (3-methyl-5-phenoxypyrzole-4-carbaldehyde), M-10 and

M-14 (3-methyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde) were also observed.

M-7 and M-9 were mostly observed after enzymatic hydrolysis.

The major urinary metabolite in the 2 mg/kg bw repeated-dose pyrazole-labelled fenpyroximate group was M-8, representing 9.12–15.8% of the unhydrolysed urinary radioactivity. Hydrolysis of the urine with either glucuronidase or sulfatase increased M-8 to 10.3–24.4% of the urinary radioactivity. M-21 represented 2.60–7.97% of the urinary radioactivity. Minor metabolites included M-7, M-10 and M-13 ((*E*)-1,3-dimethyl-5-phenoxypyrazole-4-carbaldehyde oxime). M-7, M-10 and M-13 all increased slightly with glucuronidase or sulfatase hydrolysis. M-3, M-5 and M-9 were observed only after enzymatic hydrolysis.

The major faecal metabolite in the 2 mg/kg bw repeated-dose pyrazole-labelled fenpyroximate group was the parent (fenpyroximate), which constituted 3.91–31.1% of the unhydrolysed faecal radioactivity. M-3 (3.15–15.8% of the faecal radioactivity) and M-22 (0.24–16.7% of the faecal radioactivity) were also observed. Minor metabolites included M-1, M-2, M-4, M-6, M-7, M-8, M-9, M-10, M-11, M-13 and M-14. The minor metabolites generally increased upon enzymatic hydrolysis.

The major urinary metabolite in 400 mg/kg bw pyrazole-labelled fenpyroximate group was also M-8, representing 3.51–16.7% of the unhydrolysed urinary radioactivity. Hydrolysis of urine with glucuronidase increased M-8 to 9.89–29.3% of the urinary radioactivity. Glucuronidase hydrolysis also formed small amounts of M-3, M-7 and M-10, all observed to a greater extent in female samples.

Faeces from the 400 mg/kg bw pyrazole-labelled fenpyroximate group also contained the parent fenpyroximate (47.4–82.0% of the unhydrolysed faecal radioactivity). Amounts of M-1, M-3, M-6, M-7, M-8, M-9, M-10, M-11, M-14, M-21 and M-22 were also found in some samples.

The metabolic pathway of fenpyroximate is shown in Fig 2. The major pathways of metabolism of the pyrazole moiety of fenpyroximate included cleavage of the benzyl moiety from the pyrazole ring, forming M-11 (perhaps with M-13 as an intermediate), with further oxidation to M-21 and M-10; oxime hydrolysis to M-6. With subsequent transformation to M-7, M-8 and M-9; and ester hydrolysis to M-3. Fenpyroximate was also isomerized to M-1, followed by ester hydrolysis to M-4. Another pathway of metabolism is 4-hydroxylation of the 5-phenoxy ring to form M-2, accompanied by ester cleavage to form M-5. Oxime hydrolysis to M-7 with subsequent transformation to M-14 then occurs. Although M-12 was not unequivocally found in urine and faeces it is very possible that M-12 was an intermediate to M-9 and M-14 which were found as faecal metabolites. Another pathway was oxidation of the *t*-butyl group to form M-22. In general, there was no change in the metabolism of pyrazole-labelled fenpyroximate upon repeated-dose or low-dose administration relative to the high dose. The increases in the parent compound in the faeces are due to lower percentages of absorption at the high dose (Sharp, 1991a).

Table 5. Distribution of metabolites in rat urine and faeces after oral doses of [pyrazole-^{14}C]-labelled fenpyroximate

Metabolite	% of administered dose[a]											
	2 mg/kg bw; 0–48 h				2 mg/kg bw after 14 daily doses; 0–48 h				400 mg/kg bw; 0–168 h			
	Male		Female		Male		Female		Male		Female	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Fenpyroximate	–	6.95	–	6.32	–	10.8	–	20.2	–	51.9	–	52.4
M-1	–	0.39	–	0.67	–	0.75	–	0.93	–	1.77	–	1.69
M-2	(0.05)	0.39	–	0.54	–	0.13	–	–	–	–	–	
M-3	(0.14)	8.15	(0.15)	7.61	–	4.69 (0.48)	(0.02)	7.93	–	0.27	0.04	1.28 (0.35)
M-4	–	1.58	–	0.27 (1.33)	–	1.68	–	0.58 (0.48)	–	–	–	–
M-5	(0.08)	2.58	(0.16)	–	–	4.27	–	0.58 (0.80)	–	–	–	–
M-6	–	1.20	–	1.81 (0.64)	–	1.08	–	2.14	–	0.54	–	0.61 (0.06)
M-7	–	(0.36)	–	(0.20)	0.07	0.44	(0.08)	0.16 (0.12)	(0.03)	–	(0.01)	0.06
M-8	1.27 (0.72)	0.20 (0.21)	1.29 (2.59)	0.62 (0.55)	1.42 (0.57)	0.41 (0.04)	1.96 (1.54)	0.38	0.70 (0.78)	–	1.24 (1.38)	0.34
M-9	–	0.10 (0.35)		0.30 (0.45)	(0.08)	0.28 (0.30)	(0.11)	0.57	–	(0.05)	–	–
M-10	(0.11)	0.13 (0.01)	(0.19)	0.56 (0.13)	0.14 (0.05)	0.72	0.09 (0.39)	1.05	(0.13)	0.07 (0.21)	0.11 (0.27)	0.17 (0.12)
M-11	–	1.69 (0.04)	–	5.24	–	1.55	–	2.67	–	0.83 (0.26)	–	1.04 (0.53)
M-13	–	0.06	–	–	0.03 (0.02)	(0.07)	(0.07)	(0.06)	–	–	(0.02)	–
M-14	–	0.41	–	–	–	(0.63)	–	–	–	–	–	–
M-21	1.80	0.09	2.38	–	0.52 (0.31)	–	1.00	–	1.35	–	1.30	–
M-22	–	2.81 (1.89)	–	2.05 (0.16)	–	2.61 (1.07)	–	2.74	(0.01)	0.11 (0.06)	–	0.55 (0.18)
Origin	8.05	22.0	13.6	15.3	6.98	14.6	10.5	15.2	8.92	3.79	7.90	5.33

(*Continued on next page*)

Metabolite	% of administered dose[a]											
	2 mg/kg bw; 0–48 h				2 mg/kg bw after 14 daily doses; 0–48 h				400 mg/kg bw; 0–168 h			
	Male		Female		Male		Female		Male		Female	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
PUU1	0.74	–	0.16 (0.14)	–	0.09 (0.04)	–	0.10 (0.03)	–	0.35 (0.07)	–	0.26 (0.13)	–
PUU2	(0.05)	–	(0.04)	–	0.30	–	0.50	–	0.49	–	0.59	–
PUU3	(0.21)	–	0.34	–	0.20	–	(0.45)	–	–	–	0.19	–
PFU1	–	0.21	–	1.51	–	0.61	–	0.69	–	0.82	–	2.23
PFU2	–	3.28	–	0.30 (1.88)	–	8.89	–	6.74	–	1.85	–	0.67 (0.21)
PFU3	–	7.84	–	3.13 (0.69)	–	7.19	–	5.73	–	2.72	–	(0.07)
Total not identified	9.05	33.3	14.28	21.8	7.34	31.3	11.58	31.4	9.83	9.19	9.07	8.51
Total activity (%)	12.2	84.4	17.8	69.7	13.4	75.5	17.5	79.0	10.7	76.7	11.7	75.1

[a] Values in parenthesis are the % increases after hydrolysis with β-glucuronidase.
bw: body weight;
M-1: *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate;
M-2: *tert*-butyl (*E*)-4-{[1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]-methyleneaminooxymethyl}benzoate;
M-3: (*E*)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid;
M-4: (*Z*)-4-[(1,3-dimethyl-5-phenocypyrazol-4-yl) methyleneaminooxymethyl]benzoic acid;
M-5: (*E)*-4-{[(1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]methyleneaminooxymethyl}benzoic acid);
M-6: 1,3-dimethyl-5-phenoxypyrzole-4-carbaldehyde;
M-7: 1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde;
M-8: (1,3-dimethyl-5-phenoxypyrazole-4-carboxylic acid);
M-9: 3-methyl-5-phenoxypyrzole-4-carbaldehyde;
M-10: 1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbonitrile;
M-11: 1,3-dimethyl-5-phenoxypyrazole-4-carbonitrile;
M-13: (*E*)-1,3-dimethyl-5-phenoxypyrazole-4-carbaldehyde oxime;
M-14: 3-methyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde;
M-21: 4-cyano-1-methyl-5-phenoxypyrazole-3-carboxylic acid;
M-22: (*E*)-2-[4- [(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoyloxy]-2-methylpropanoic acid;
NNI-850: fenpyroximate; PUU1, PUU2, PUU3 [pyrazole-^{14}C]-labelled NNI-850 Urine Unknown metabolites (1–3);
PFU: [pyrazole-^{14}C]-labelled NNI-850 Faeces Unknown metabolite (1–3)
Source: Sharp (1991a)

The major urinary metabolite in the 2 mg/kg bw single-dose benzyl-labelled fenpyroximate group was M-18 (terephthalic acid), representing 45.2–73.4% of the urinary radioactivity. Other metabolites identified were M-16 (4-hydroxymethylbenzoic acid), M-5, M-3 and M-4. The amounts of M-3 and M-4 were increased slightly by hydrolysis with β-glucuronidase or sulfatase. M-2 and 4-formylbenzoic acid (M-17) were also present in small amounts (Table 6).

Faeces from the 2 mg/kg bw single-dose benzyl-labelled fenpyroximate group contained the parent compound fenpyroximate (3.76–14.7% of the faecal radioactivity) and M-1 (<0.01–1.42% of the faecal radioactivity). M-3 (5.23–15.6% of the faecal radioactivity), M-4 (0.50–8.93% of the faecal radioactivity) were observed as well as M-5 and M-16. M-22 (1.90–17.7% of the faecal radioactivity) was also detected. Small amounts of M-2, M-15 (*tert*-butyl 4-hydroxymethylbenzoate), M-17 and M-18 were also observed in some samples.

The major urinary metabolite in the 2 mg/kg bw repeated-dose benzyl-labelled fenpyroximate group was M-18, representing 3.62–39.4% of the urinary radioactivity. Other metabolites were M-5, M-16, M-3 and M-4. M-2 and M-17 were also observed in some samples. Hydrolysis of the urine with either glucuronidase or sulfatase increased M-3 and M-4 slightly, but otherwise there was no change in the profile after enzymatic hydrolysis.

The major faecal metabolite in the 2 mg/kg bw repeated-dose pyrazole-labelled fenpyroximate group was the parent fenpyroximate, which constituted 2.13–20.3% of the faecal radioactivity. M-1 (1.12–2.55% of the faecal radioactivity), M-3 (0.96–13.8% of the faecal radioactivity) and M-4 (0.72–13.3%; combined with M-3 at the 24- to 48-hour time point) were also observed. M-18 (5.24%) was also detected.

The major urinary metabolite in the 400 mg/kg bw benzyl-labelled fenpyroximate group was also M-18, representing 8.90–70.5% of the urinary radioactivity.

Faeces from the 400 mg/kg bw benzyl-labelled fenpyroximate group contained the parent fenpyroximate (47.8–91.9% of the faecal radioactivity). Small amounts of M-1, M-2, M-3, M-4, M-5, M-12, M-15, M-16, M-17 and M-22 were also found in some samples (Sharp, 1991b).

The major pathways of metabolism of the benzyl moiety of fenpyroximate included cleavage of the benzyl moiety from the pyrazole ring either before (forming M-15) or after (forming M-16) the hydrolysis of the *tert*-butyl ester. The product of cleavage of the *tert*-butyl ester of fenpyroximate and M-1, which was formed through isomerization of NNI-850, were found as both the *E*- and *Z*-isomers (M-3 and M-4, respectively). M-16 and M-15 were converted into M-17 and M-18 by ester cleavage of M-15 to M-16, followed by oxidation. Other metabolic pathways were 4-hydroxylation of the 5-phenoxy ring to form M-2, accompanied by ester cleavage to form M-5, as well as oxidation of the *tert*-butyl group from M-22. This type of biotransformation is extremely unusual. With the exception of a slight increase of M-3, M-4 and M-5 in some samples, treatment with glucuronidase or sulfatase did not affect the metabolite profile. The same metabolite pathways found in urine also appeared in faeces. However, M-18 was not excreted in large amounts via the faeces. The increases in the parent compound in the faeces are due to a lowered percentage of absorption at the high dose (Sharp, 1991b).

Table 6. Distribution of metabolites in rat urine and faeces after oral doses of [benzyl-^{14}C]-labelled fenpyroximate

Metabolite	% of administered dose [a]											
	2 mg/kg bw; 0–48 h				2 mg/kg bw after 14 daily doses; 0–48 h				400 mg/kg bw; 0–168 h			
	Male		Female		Male		Female		Male		Female	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Fenpyroximate	–	6.51	–	8.68	–	8.43	–	12.9	–	51.7	–	50.2
M-1	–	< 0.01		0.76		0.60		1.40		2.16		1.96
M-2	–					0.69		0.93				
M-3	–	6.64	0.31	6.49	0.06	4.65 (0.31)	0.05	6.90		0.67	0.04	0.75
M-4	–	3.89		3.94		2.60		6.80 [b]		0.09		0.14
M-5 + M-16	2.57	8.28 (1.30)	1.44 (0.37)	3.21 (0.84)	0.37	7.15	0.64	3.49	0.36	1.73	0.35	1.42
M-12 [c]	–	–	–	–	–	(0.12)	–	(0.07)	–	–	–	–
M-15	–	–	–	0.22	–	0.38	–	0.23 (0.10)	–	0.68 (0.26)	–	0.94 (0.20)
M-17	–	–	–	–	–	(0.05)	–	–	–	–	–	–
M-18	6.04 (0.32)	0.41	6.37	1.18	4.24	(5.18)	1.21 (0.84)	–	4.56 (2.81)	–	6.31	–
M-22	–	4.24	–	2.27 (1.04)	–	2.96	–	2.13 [b] (0.44)	–	0.55	–	0.32
Origin	2.25	19.8	3.46	18.3	4.87	8.34	4.02	14.6	3.65	4.52	1.91	6.49

Metabolite	% of administered dose[a]											
	2 mg/kg bw; 0–48 h				2 mg/kg bw after 14 daily doses; 0–48 h				400 mg/kg bw; 0–168 h			
	Male		Female		Male		Female		Male		Female	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
BUU1	0.12	–	0.11	–	0.08[b]	–	0.04[b]		0.13 (0.14)[b]	–	0.06	–
BUU2	–	–	0.52	–	0.08[b]	–	0.04[b]		0.08 (0.08)[b]	–	0.03	–
BUU3	(0.28)	–	–	–	0.50 (0.69)	–	0.69		0.34 (0.22)	–	0.11 (0.25)	–
BFU3	–	17.3	–	9.00 (3.70)	–	10.7 (7.10)	–	10.1 (2.40)	–	3.53	–	2.60 (0.79)
Total unidentified	2.65	37.1	4.09	31.0	6.22	26.1	4.79	27.1	4.64	8.05	2.36	9.88
Total activity (%)	13.2	91.6	12.5	82.7	13.8	77.9	9.21	90.2	11.3	80.0	9.47	80.1

bw: body weight; *Source*: Sharp (1991b)

BFU3: Benzyl-14C NNI-850 Faeces Unknown metabolite 3 (BFU3 is considered to be the same as BUU3);

BUU1, BUU2, BUU3: [Benzyl-14C]NNI-850 Urine Unknown metabolites 1–3;

M-1: tert-butyl (Z)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-p-toluate;

M-2: tert-butyl (E)-4-{[1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]-methyleneaminooxymethyl}benzoate;

M-3: (E)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid;

M-4: (Z)-4-[(1,3-dimethyl-5-phenocypyrazol-4-yl) methyleneaminooxymethyl]benzoic acid;

M-5: (E)-4-{[(1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]methyleneaminooxymethyl}benzoic acid);

M-12: tert-butyl (E)--4-[(3-methyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoate;

M-15: tert-butyl 4-hydroxymethylbenzoate;

M-16: 4-hydroxymethylbenzoic acid; M-17: 4-formylbenzoic acid;

M-18: terephthalic acid;

M-22: (E)-2-[4- [(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoyloxy]-2-methylpropanoic acid;

NNI-850: fenpyroximate

[a] Values in parenthesis are % increases after hydrolysis with β-glucuronidase.

[b] The metabolites could not be separated in at least one time point.

[c] Evidence of M-12 was firm only in the repeated-dose group B. In the single low and single high dose groups, all metabolites co-eluting with both standards M-12 and M-15 were reported as M-15.

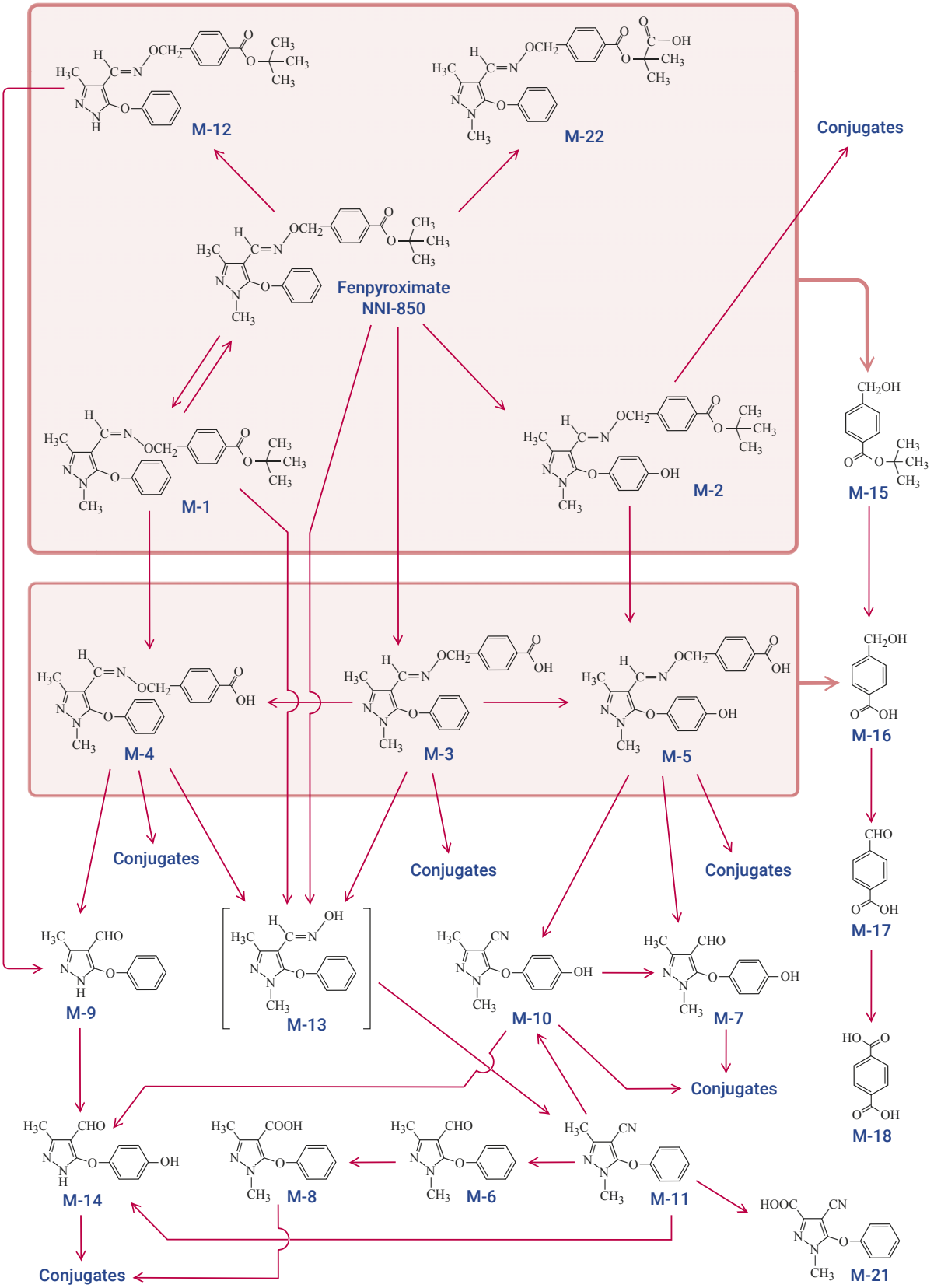
Bile and faecal samples from the Lin (1992a,b) study (section 1.1a) by were pooled for metabolite identification (6 rats/sex).

In bile of rats treated with pyrazole-labelled fenpyroximate, the major biliary metabolite was M-22. In addition, M-3/M-4, M-5, M-6, M-8, M-13 and M-14 were also detected as minor metabolites. Furthermore, M-2, M-3/M-4, M-5, M-6, M-7, M-8, M-9, M-10, M-13 and M-14 increased by hydrolysis using sulfatase and β-glucuronidase. Therefore, these metabolites were in bile as sulfate- or β-glucuronide. On the other hand, there was no unchanged fenpyroximate in bile. In faeces, unchanged fenpyroximate was the major component (Lin 1992a).

In bile of rats treated with benzyl-labelled fenpyroximate, the major biliary metabolite was M-22. In addition, M-3/M-4, M-5 and M-18 were also detected as minor metabolites, some present as conjugates. There was no unchanged fenpyroximate in bile. In faeces, unchanged fenpyroximate was the major component (Lin, 1992b).

These results indicate that group cleavage of ester bond, hydroxylation at the phenoxypyrazole group and oxidation at the *tert*-butyl group occurred in rats. The possibility for the sulfate and glucuronide conjugation is favourable for M-3/M-4, M-5 and M-8.

Figure 2. Proposed metabolic pathways of fenpyroximate in rats



(*For abbreviations footnote see next page*)

Footnote to Fig.2

NNI-850: fenpyroximate; bw: body weight;
BFU3: [Benzyl-^{14}C]NNI-850 Faeces Unknown metabolite 3 (BFU3 is considered to be the same as BUU3);
BUU1, BUU2, BUU3: [Benzyl-^{14}C] NNI-850 Urine Unknown metabolite 1–3;
M-1: *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate;
M-2: *tert*-butyl (*E*)-4-{[1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]-methyleneaminooxymethyl}benzoate;
M-3: (*E*)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid;
M-4: (*Z*)-4-[(1,3-dimethyl-5-phenocypyrazol-4-yl) methyleneaminooxymethyl]benzoic acid;
M-5: (*E)*-4-{[(1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]methyleneaminooxymethyl}benzoic acid);
M-6: 1,3-dimethyl-5-phenoxypyrzole-4-carbaldehyde; M-8: 1,3-dimethyl-5-phenoxypyrazole-4-carboxylic acid;
M-9: 3-methyl-5-phenoxypyrzole-4-carbaldehyde; M-11: 1,3-dimethyl-5-phenoxypyrazole-4-carbonitrile;
M-12: *tert*-butyl (*E)*--4-[(3-methyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoate;
M-14: 3-methyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde;
M-15: *tert*-butyl 4-hydroxymethylbenzoate; M-16: 4-hydroxymethylbenzoic acid;
M-17: 4-formylbenzoic acid; M-18: terephthalic acid;
M-21: 4-cyano-1-methyl-5-phenoxypyrazole-3-carboxylic acid;
M-22: (*E*)-2-[4- [(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoyloxy]-2-methylpropanoic acid;

In a metabolism study, [pyrazole-^{14}C]-labelled fenpyroximate was administered to male Sprague Dawley rats (n = 5) via gavage at 1.5 mg/kg bw in olive oil. The rats were killed at one (n = 2), 3 (n = 2) and six hours (n = 1) after dosing, respectively, and the liver and the stomach removed. Rat liver microsomes were prepared. Stomach tissue including gastric content was minced and extracted three times with methanol/acetone (1:1). Liver microsome metabolism was evaluated at 15, 30, 60 and 120 minutes of exposure. The metabolic identification was conducted using TLC autoradiography method.

M-2, M-3 M-12 and M-13 were identified as the metabolites in in vitro metabolism study of pyrazole-labelled fenpyroximate using rat liver microsomes. This indicates that the primary metabolic pathway was hydrolysis of the *tert*-butyl ester, elimination of *N*-methyl moiety, 4-hydroxylation of the 5-phenoxy ring and cleavage of the benzyl moiety from the pyrazole ring. In addition, it was revealed that pyrazole-labelled fenpyroximate was isomerized to M-1 under acidic condition, and M-1 was detected in the stomach of rats orally dosed with NNI-850. NNI-850, M-1, M-3, M-5, M-8 and M-12 were identified as the metabolites in the liver, confirming the metabolic pathway described above. In a rat metabolism study with [phenyl-^{14}C], M-12 and M-13 were not detected in the urine and faeces. However, the present study indicated that these metabolites were present as intermediates through the metabolism process in rats (Suzuki, 1990).

An in vitro study was conducted using pyrazole-labelled fenpyroximate to clarify hydrolytic fate and transformation of fenpyroximate and its *Z*-isomer, M-1 under acidic condition, simulating gastrointestinal tract conditions of animals (pH 1.2 at 37°C).

The result of this study shows that both of fenpyroximate and M-1 were isomerized under gastrointestinal tract conditions. The isomerization rate of M-1 to fenpyroximate is much greater than the rate from fenpyroximate to M-1. As a consequence, fenpyroximate predominates over M-1 at equilibrium state with 10-fold isomer ratio regardless of starting isomers. The evidence strongly suggests that similar isomer ratio of fenpyroximate is likely to be present in gastrointestinal tract after oral application regardless of dosing isomer (Tadokoro, 2015).

An in vitro comparative metabolism study was conducted using Sprague Dawley rat and human (from both sexes) liver microsomes in the presence and absence of NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form). In this study, both the pyrazole- and benzyl-labelled fenpyroximate was used as the test substance. After the reaction, the reaction mixture was extracted with acetone/methanol (1:1, volume per volume [v/v]), and then analysed by TLC and high-pressure liquid chromatography (HPLC).

Regardless of species and sex, the major metabolites were M-3 and M-12. In addition, M-13, M-15 and several unknown metabolites were also detected. Of these unknown metabolites, "unknown-2" and "unknown-4" were largest components, common to both human and rat microsomes. The results suggest that the metabolic pathway of fenpyroximate in human and rat are qualitatively similar (Yonemura, 2016).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with fenpyroximate (including skin and eye irritation and dermal sensitization studies) are summarized in Table 7.

Table 7. Results of acute toxicity studies with fenpyroximate

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Mouse	CD-1	M + F	Oral	98.0	520 (male) 440 (female)	Blaszcak (1989a)
Rat	Sprague Dawley	M + F	Oral	98.0	480 (male) 245 (female)	Blaszcak (1989b)
Rat	Sprague Dawley	M + F	Dermal	98.0	>2000	Blaszcak (1989c)
Rat	Sprague Dawley	M + F	Inhalation (whole body)	89.4	0.37 (male) 0.4 (female)	Hoffman (1989)
Rat	Sprague Dawley	M + F	Inhalation (nose-only)	88.7	0.21 (male) 0.33 (female)	Hoffman (1991a,b)
Rabbit	NZW	M	Skin irritation	98.4	Non-irritating	Kosaka (1988a)
Rabbit	NZW	F	Eye irritation	98.4	Mildly irritating	Kosaka (1988b)
Guinea pig	Crl: Hartley	F	Sensitization (Magnusson-Kligman test)	98.4	Sensitizer	Kosaka (1988c)
Guinea pig	Dunkin Hartley	F	Sensitization (Buehler test)	98.6	Non-sensitizing	Teale (1990a,b)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NZW: New Zealand White

(a) Oral administration

Mouse

Fasted CD-1 (ICR) BR mice (5/sex per dose) were dosed by gavage with NNI-850 technical (fenpyroximate; purity 98%) as a slurry in 45% Tween 80 (containing 1% methocel) at a single dose of 0, 200, 280, 400, 600 or 800 mg/kg bw. Animals were observed for clinical signs and mortality for up to 14 days post dosing.

Clinical signs seen on the day of dosing in most groups included ataxia, hypopnoea, hypoactivity and prostration. Most surviving animals had decreased feed consumption on the day after dosing; this continued in some animals through day 5. Additional signs seen in two or more groups included urinary staining and abdominal griping. Hyperpnoea, dyspnoea, hypothermia or hyperactivity was seen in single animals at 800 mg/kg bw. Coarse tremors were seen in animals at 1200 and 1700 mg/kg bw. The body weight gains of surviving animals were comparable to controls. Macroscopic examination of animals found dead showed a variety of changes, primarily in the lungs and gastrointestinal tract.

Based on these results, the acute oral median lethal dose (LD_{50}) of fenpyroximate was 520 mg/kg bw in males, 440 mg/kg bw in females and 500 mg/kg bw combined (Blaszcak, 1989a).

Rat

Fasted Sprague Dawley CD rats (5/sex per dose) were dosed by gavage with NNI-850 technical (fenpyroximate; purity 98%) at a single dose of 0, 200, 280, 400, 600 or 800 mg/kg bw, as a slurry in 45% Tween 80 (containing 1% methocel). Animals were observed for clinical signs and mortality for up to 14 days post dosing.

All deaths occurred within five days of dose administration. Clinical effects included urinary and faecal staining, soft stool, partially closed eyes, hypoactivity, hypopnoea, prostration, dry rales,

dry oral discharge, an unthrifty coat, alopecia, decrease in feed consumption, hypothermia, abdominal griping, emaciation, dyspnoea and ataxia. Effects generally subsided by day 7. The body weights of 7/21 surviving animals (all male) decreased between days 0 and 7 post administration; all surviving animals gained weight between days 7 and 14. Gross necropsy of decedent animals revealed red walls of intestines, discoloration of the lungs, testes in body cavity, red foci on lungs, red and/or black walls of stomach, red and/or black fluid in intestines, and black fluid in stomach. Gross necropsy of animals killed after 14 days revealed no treatment-related abnormalities.

The acute oral LD_{50} of fenpyroximate was 480 mg/kg bw in males, 245 mg/kg bw in female and 350 mg/kg bw combined (Blaszcak, 1989b).

(b) Dermal application

In an acute dermal toxicity study, young adult Sprague Dawley-derived CD rats (5/sex) were dermally exposed to NNI-850 technical (purity 98.0%; fenpyroximate) at 2000 mg/kg (limit dose) for 24 hours. The test substance was applied onto a piece of 8-ply gauze and moistened with approximately 0.5 mL of 0.9% saline. The gauze was applied to approximately 10% of the total body surface area. After 24 hours, the wrapping and gauze were removed and the test site wiped free of excess test material. Animals were observed for clinical signs of toxicity and mortality for up to 14 days after dosing.

All animals survived the 14-day study. Clinical effects included decreased feed consumption in up to 10/10 animals between days 3 and 8, and dry red nasal discharge in one female on day 3. All animals appeared normal by day 9. No severe dermal effects were observed. No treatment-related effect on body weight was observed in males. In contrast, 2/5 females lost weight either during the first or second week of the study, and only slight increases in body weights were observed overall (ranging from 1.3% to 7.7%). Necropsy after 14 days revealed a swollen uterus in one female.

Based on these results, the acute dermal LD_{50} of fenpyroximate was greater than 2000 mg/kg bw in male and female rats (Blaszcak, 1989c).

(c) Exposure by inhalation

In an acute inhalation toxicity study, young adult Sprague Dawley-derived CD albino rats (5/sex per group) were exposed by whole-body inhalation to NNI-850 (fenpyroximate; purity 89.4%) at analytically determined concentrations of 0.067, 0.14, 0.36, 0.74 or 0.78 mg/L for four hours. Animals were observed for clinical signs of toxicity and mortality for up to 14 days post exposure.

The mass median aerodynamic diameters (MMADs) were 3.5, 4.2, 4.2, 3.4 and 6.8 µm and the mean geometric standard deviations (GSDs) were 2.1, 2.5, 2.3, 1.9 and 2.5 µm for analytically determined concentrations of 0.067, 0.14, 0.36, 0.74 or 0.78 mg/L, respectively. At an average of 6.8 µm, the MMAD for the 0.78 mg/L exposure exceeds the ideal respirable range of 1–4 µm. Mortality occurred in 1/20 animals tested at 0.14 mg/L or less, 2/10 animals tested at 0.36 mg/L and 19/20 animals tested at 0.74 mg/L and above. Although the highest number of spontaneous deaths occurred on the day of exposure, a delayed pattern of mortality was evident at 0.78 mg/L, with 5/10 deaths occurring between days 8 and 11. Signs of respiratory irritation (laboured breathing, gasping) were commonly noted during all exposures, with most deaths occurring during the exposures. Clinical signs of toxicity generally abated in surviving animals after the first week of the treatment. No significant effect on body weight was observed in surviving males at 0.067 and 0.14 mg/L. All males at 0.36 mg/L lost weight during the first week after exposure, but recovered during the second week. The single surviving male from the 0.74 mg/L group also lost weight during the first week after exposure and recovered slightly during the second week. In surviving females, 6/14 animals lost weight during the first week after exposure, and all gained weight during the second week. Findings at necropsy were generally unremarkable in surviving animals.

Based on these results, the acute inhalation median lethal concentration (LC_{50}) for fenpyroximate was 0.37 mg/L in males (95% confidence limit [CL]: 0.18–0.75 mg/L), 0.40 mg/L in females (95% CL: 0.27–0.60 mg/L) and 0.40 mg/L combined (95% CL: 0.21 to 0.80 mg/L) (Hoffman, 1989).

In an acute inhalation toxicity, young adult Sprague Dawley-derived CD albino rats (5/sex per group) were exposed by nose-only inhalation to NNI-850 (fenpyroximate; purity 88.7%) at analytically determined concentrations of 0.11, 0.22 or 0.58 mg/L for 4 hours. Animals were observed for clinical signs of toxicity and mortality for up to 14 days post exposure.

Particle size distribution measurements showed the MMAD to range from 2.5 to 3.0 µm, with a GSD ranging from 2.2 to 2.3. Most of the measurements indicated that about 95% of particles were 10 µm or less. Mortality occurred within one day of exposure in 10/19 animals at 0.22 mg/L and higher concentrations. The surviving animals from all three exposures showed various responses including laboured breathing, rales, gasping, nasal discharge and anogenital staining. No significant effect on body weight was observed in surviving males and females at 0.11 and 0.22 mg/L. Both surviving males and the single surviving female from the 0.58 mg/L group lost weight during the first week after exposure and gained weight during the second week.

Notable findings upon necropsy of decedent animals were red, oedemic and/or firm lungs; white frothy fluid in the trachea; dilated pelvis of the kidneys; and red fluid-filled bronchi. Similar effects were observed upon necropsy of animals after 14 days and included red, edemic and/or red foci of the lungs; frothy fluid (often white) in the trachea; and/or red fluid and red gelatinous material in the thoracic cavity.

Based on these results, the acute inhalation LC_{50} for fenpyroximate was 0.21 mg/L in males (95% CL: 0.035–1.2 mg/L), 0.33 mg/L in females (95% CL: 0.20–0.55 mg/L) and 0.31 mg/L combined (95% CL: 0.18 to 0.52 mg/L) (Hoffman, 1991a).

(d) Dermal irritation

In a study of primary dermal irritation, six male New Zealand White rabbits were dermally exposed to 0.5 g of NNI-850 technical (fenpyroximate; purity 98.4%) slightly moistened with distilled water and placed on one side on the back of each rabbit. The other side was left untreated to serve as a control site. A gauze patch was placed on each site of the skin and held in place with a polyethylene sheet and non-irritating adhesive tape. The test material was in contact with the skin for four hours. After removal of the patch, the treated application site was rinsed with water to remove the test compound, and the dermal irritation was scored at 24, 48 and 72 hours after patch removal using the Draize method.

There was neither erythema nor oedema at any time point. No animals showed decreases in body weights.

Based on the results of this study, fenpyroximate was not irritating to the skin of rabbits (Kosaka, 1988a).

(e) Ocular irritation

In a primary eye irritation study, a sample of 100 mg of NNI-850 technical (fenpyroximate; purity 98.4%) was instilled into the left eye of six male New Zealand White rabbits. The eyes were not washed in rabbits in Group I while the eyes of three rabbits in Group II were washed for 30–60 seconds with lukewarm water 2–3 minutes after the treatment. Ocular reactions were graded at 1, 2, 3, 24, 48, 72 and 96 hours up to seven days after treatment.

No changes in the cornea and iris were seen at any time point in unwashed or washed eyes. All animals in groups I and II showed redness at grade 1 in conjunctivae (some blood vessels definitely hyperaemic) from one to three hours after the treatment. At the same time points, all animals in group I and one animal in group II showed chemosis of grade 1 (any swelling above normal) or grade 2 (obvious swelling with partial eversion of lids). Animals showing chemosis also had discharge of grade 1 (any amount different from normal) or grade 2 (discharge with moistening of the lids and hairs just adjacent to lids). All of these changes due to irritation in the conjunctivae disappeared by one day after the treatment in both groups. None of the animals in either group had decreases in body weights.

Based on the results of this study, fenpyroximate was mildly irritating to the eyes of rabbits (Kosaka, 1988b).

(f) Dermal sensitization

The skin sensitization potential of NNI-850 technical (fenpyroximate; purity 98.4%) was investigated in 50 female Crl: guinea pigs using the maximization test. Animals in groups A and C received two 0.05 mL volumes of three injections (water in oil emulsion of Freund's complete adjuvant in salt solution; suspension of the test compound [or 2,4-dinitrochlorbenzene] in paraffin oil; or water in oil emulsion of the test compound [or 2,4-dinitrochlorbenzene] suspended in Freund's complete adjuvant in salt solution). Animals in groups B and D received injections without the test compound. Six days after intradermal injections, the shaved shoulder region of each animal was treated by open topical application with 10% sodium laurylsulfate. The next day, animals in groups A and C were exposed to the test compound and 2,4-dinitrochlorbenzene, respectively, mixed with white petrolatum by closed patches on the shoulder region for 48 hours. Animals in groups B and D were treated with white petroleum only. For the challenge phase, groups A and B were treated with the mixture of the test compound and white petrolatum on the left flank by closed patches for 24 hours following 13 days after topical application. Animals in groups C and D were treated by the same method with the mixture of 2,4-dinitrochlorbenzene and white petrolatum. The right flanks of all animals were treated with white petrolatum alone. Skin reactions were scored 24, 48 and 72 hours after challenge application. Body weights were measured periodically.

None of the animals in any of the groups had decreases in body weight during the test period. In this study, the sensitization rate was 36% in the test compound-treated group. The rate is classified as Grade III (moderate) when the criteria of the guinea pig maximization test are applied.

Fenpyroximate had moderate dermal sensitizing potential in guinea pigs as determined by the Magnusson–Kligman test (Kosaka, 1988c).

In a dermal sensitization study conducted with NNI-850 technical (fenpyroximate; purity 98.6%), 20 young adult female Dunkin Hartley albino guinea pigs were tested using methods based on those derived by Buehler. An additional 20 females served as challenge treatment-only controls. A concurrent positive control study was conducted in the same manner using 2,4-dinitrochlorobenzene and 10 test and 10 control animals.

No dermal irritation was observed 24 through 72 hours after challenge exposures with 50% and 25% NNI-850 technical in either previously induced or control animals. Acceptable positive control data were provided to validate the test methodology. No significant differences were apparent between the recorded body weights of the test and control groups during the study period.

Under the conditions of the Buehler test, there was no indication of delayed contact hypersensitivity induced by fenpyroximate in guinea pigs (Teale, 1990a,b).

(g) Phototoxicity

Fenpyroximate (purity 99.2%) was assayed for phototoxicity to Balb/c 3T3 fibroblast cells using the neutral red uptake assay in the absence and presence of ultraviolet light.

Under conditions of this in vitro test system, fenpyroximate was not phototoxic when tested up to 100 µg/mL, the maximum non-precipitating concentration (Valin, 2016).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

No data submitted.

Rat

In a 90-day toxicity study, NNI-850 (fenpyroximate; purity 99%) was administered to CD rats (10/sex per dose) in the diet at dose levels of 20, 100 or 500 ppm (equal to 1.3, 6.57 and 35.22 mg/kg bw per day for males and 1.65, 8.29, 38.60 mg/kg bw per day for females, respectively) for 13 weeks.

One female at 20 ppm and two males at 500 ppm died during routine blood sampling after week 12. At 500 ppm, 3/10 males and 7/10 females had facial staining, 7/10 males had encrustations of the muzzle, 7/10 females developed large areas of dorsal/ventral hair loss and 6/10 females had skin encrustations and/or exfoliation. No treatment-related effects were observed in the 20 ppm treatment groups. Both sexes at 100 and 500 ppm had impaired growth performance, reduced feed intake and decreased body weights and body weight gains. Body weights for the males at 100 ppm were comparable to the controls through week 10, and then were reduced to 90% of the control weight during weeks 11–13 ($P < 0.05$). body weights for females at 100 ppm were 90–93% of the control weight throughout the study ($P < 0.05$ or $P < 0.001$) except during weeks 0–2 and 10, when they were comparable to the control weight. Body weight gains for both sexes at 100 ppm were 15% lower than the respective control gains. For males and females at 500 ppm, body weights were 49–73% and 62–84%, respectively, of the corresponding control weights during the study ($P < 0.001$), and body weight gains were 32% and 37%, respectively, of the corresponding control gains. Feed consumption for both sexes at 100 ppm was 4–5% lower than the respective controls. Mean feed consumption for the males and females at 500 ppm was 59% and 65%, respectively.

No significant abnormality was found on ophthalmoscopy.

At 100 ppm, there was a decrease in total leukocyte count in males. In males at 500 ppm, haematocrit, haemoglobin and red blood cell (RBC) counts were higher and white blood cell counts were 40% lower than the control values. In females at 500 ppm, haemoglobin, haematocrit, RBC counts and platelet counts were higher than the control values. Total plasma proteins were 10% lower in males at 500 ppm and 6% and 16% lower in females at 100 and 500 ppm. Females at 500 ppm had alkaline phosphatase activity 123% higher and plasma butyrylcholinesterase and plasma acetylcholinesterase activities 72–73% lower than the control values. Sodium levels were statistically significantly increased in females at all doses; however, there was no clear dose response and the magnitude of change was very small (143, 146, 145 and 146 mequiv./L at 0, 20, 100 and 500 ppm, respectively). At 500 ppm, males had lower urine volume and pH values.

Differences attributed to the effect of fenpyroximate on growth rather than a direct treatment-related effect were lower relative adrenal gland weights in females at 20 ppm; higher relative heart weights in males and females at 100 ppm; and, in males and females at 500 ppm, depressed absolute organ weights for all weighed organs (not statistically significant for testes) and increased relative brain, heart, kidneys (statistically significant for females only), liver, lungs and spleen weights, and in the males only, adrenal glands, pituitary (not statistically significant) and testes weights ($P < 0.05$ or $P < 0.01$). Treatment-related effects noted in the gross pathology of the 500 ppm groups were facial staining in both sexes; encrustations of the muzzle and persistent hyaloid arteries in males; and, dorsal/ventral hair loss, skin encrustations, skin masses, perineal staining and skin exfoliation in females. Minimal hepatocytic hypertrophy was observed in both sexes at 100 and 500 ppm.

The NOAEL was 20 ppm (equal to 1.3 mg/kg bw per day) based on decreased body weight and body weight gains, reduced feed consumption and minimal hepatocytic hypertrophy in both sexes at the LOAEL of 100 ppm (equal to 6.57 mg/kg bw per day) (Aughton, 1987).

Dog

In a single-dose study, fenpyroximate (purity 99.8%) suspended in 0.5% w/v methylcellulose in purified water was administered as a single gavage dose to beagle dogs (4/sex per dose) at 0, 2 or 5 mg/kg bw. Prior to test article administration, each dog was sham-dosed with the vehicle (0.5% w/v methylcellulose in purified water) for seven days. Blood analysis (haematology, chemistry), urine analysis and neurological examination were performed. Dogs were killed one day post dosing and histopathologically examined.

All animals survived to the scheduled necropsy. There were no treatment-related effects on body weights and feed consumption or on haematology, blood biochemistry or urine analysis parameters. There were no consistent treatment-related effects on any neurological parameters in the assessment battery at 2 or 5 mg/kg bw (day 1). Organ weight analysis and macroscopic and microscopic analysis showed no treatment-related effects. Soft faeces were observed in 9/24 animals (day −1 and day −2) during the seven-day pretreatment period with vehicle. During the post-dosing period, a single female treated at 5 mg/kg bw vomited two hours after dosing. At 5 mg/kg bw, liquid faeces were seen in 2/4 males and 2/4 females, with onset as early as 2 hours post dosing and persisting in some cases up to 24 hours after dosing. At 2 mg/kg bw, liquid faces were seen in 2/4 male dogs with onset at 21 hours after dosing.

No NOAEL could be identified, as effects were observed at all doses. The LOAEL was 2 mg/kg bw, the lowest dose tested, based on liquid faeces (diarrhoea) (Harvey, 2006a).

In a dose escalation study, fenpyroximate (purity 99.8%) suspended in 0.5% w/v methylcellulose in purified water was administered orally via bolus gavage to beagle dogs (2/sex). During the maximum tolerated dose (MTD) phase, the animals were dosed with a single dose of 2 mg/kg bw on day 1. The same animals were then dosed with 5 mg/kg bw on day 8 and with 20 mg/kg bw on day 15. On day 23 the same four beagles were dosed with 5 mg/kg bw per day (fixed-dose phase) for five consecutive days (days 23–27) and killed. Each dog was dosed with vehicle for seven days prior to the initiation of the treatment to establish the incidence of diarrhoea and the reaction to the vehicle.

There were no treatment-related effects on body weights, feed consumptions, neurological assessment battery, haematology or clinical chemistry parameters during the MTD and fixed-dose phases. No treatment-related effects were observed on organ weights at scheduled kill. Diarrhoea was caused by a single dose of 20 mg/kg bw in the MTD phase and also after three hours by a single dose of 5 mg/kg bw in 1/2 male and female dogs on the first day and all dogs on five days in the fixed-dose phase of the study. According to the study author, it was unclear if this resulted from residual effect due to prior treatment with 20 mg/kg bw in the MTD phase. However, diarrhoea was not observed from day 16 to 22 after the application of the 20 mg/kg bw dose. Several dogs had macroscopic findings in the gastrointestinal tract including slightly pale stomach, slightly dark caecum, severe red caecum with slightly red ileum and yellow colon with red focus. According to the study author, it was unclear whether these findings resulted from a residual effect due to prior treatment with 20 mg/kg bw in the escalating dose. There were no concurrent controls. Histopathological examinations were not conducted. No diarrhoea was observed after the single application of 2 mg/kg bw.

The NOAEL was 2 mg/kg bw based on diarrhoea observed at the LOAEL of 5 mg/kg bw (Harvey, 2006b).

In a 90-day oral toxicity study, NNI-850 (fenpyroximate; purity 98.4–98.9%) was administered via capsule to beagle dogs (4/sex per dose) at dose levels of 2, 10 or 50 mg/kg bw per day for 13 weeks.

During weeks 4 or 5, two females at 50 mg/kg bw per day were euthanized in extremis after a period of treatment-related inappetence and body weight loss. Diarrhoea occurred most frequently in females at 10 mg/kg bw per day and in both sexes at 50 mg/kg bw per day (Table 8). One female at 2 mg/kg bw per day was emaciated during the latter part of the study. At 50 mg/kg bw per day, 3/4 males were emaciated during the latter half of the study and 4/4 females were emaciated beginning at week 2. Emesis was observed in 2/4 males and 4/4 females at 10 mg/kg bw per day and in 4/4 males and 4/4 females at 50 mg/kg bw per day. Emesis was generally observed between 30 minutes and five hours after dosing, with the greatest incidence occurring during week 1. Torpor was observed in 1/4 females at

2 mg/kg bw per day, 2/4 females at 10 mg/kg bw per day, and 2/4 males and 4/4 females at 50 mg/kg bw per day. Slight bradycardia was noted in several dogs from all treatment groups 2 and 24 hours after dosing, and was primarily observed in the 10 and 50 mg/kg bw per day treatment groups.

Table 8. Overview of 13-week oral toxicity study in dogs treated via capsule with fenpyroximate

	Group incidence of diarrhoea per sex and dose (%)							
	Males				Females			
Week number	0 mg/kg bw per day	2 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day	0 mg/kg bw per day	2 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day
−2	0	0	0	0	0	0	5	0
−1	0	0	5	0	10	5	0	5
1	10	25	40	80	10	45	45	45
2	5	25	45	75	0	55	50	35
3	5	30	20	65	5	35	40	45
4	5	25	15	70	0	15	65	40
5	15	30	15	80	5	40	30	40
6	5	10	20	70	5	35	40	50
7	10	20	15	70	10	40	70	65
8	5	20	30	65	0	20	50	30
9	0	10	10	70	0	20	30	35
10	10	30	10	70	0	35	65	70
11	20	20	25	70	0	30	55	70
12	5	30	25	55	0	30	60	55
13	15	20	5	75	0	0	50	45

bw: body weight

Source: Broadmeadow (1988)

Body weights and body weight gains for the males at 2 and 10 mg/kg bw per day were comparable to the controls. body weights for the males at 50 mg/kg bw per day were nonstatistically significantly lower than the controls. body weights for all female treatment groups were lower than the controls, reaching statistical significance in the 50 mg/kg bw per day group ($P < 0.05$). Total body weight gain for the females at 2 mg/kg bw per day was 6% lower than the control weight gain, and was attributed to weight loss in one female that had short periods of inappetence. Feed consumption was decreased by 5–7% for the females at 2 and 10 mg/kg bw per day and by 34% for the females at 50 mg/kg bw per day. Feed consumption in males was unaffected by the treatment. Water consumption was not affected by the treatment. No treatment-related ophthalmological abnormalities were observed in any of the treatment groups. The females at 50 mg/kg bw per day had 34.2–34.7% lower total white blood cell counts and 19–34% longer partial thromboplastin time with kaolin (PTTK) at weeks 6 and 12 ($P < 0.05$, 0.01 or 0.001) and 20% higher platelet counts at week 12 ($P < 0.05$) compared to the controls. No treatment-related effects on clinical chemistry parameters were observed in males at 2 mg/kg bw per day. Males at 10 and 50 mg/kg bw per day exhibited 7–11% lower glucose levels at weeks 6 and/or week 12 compared to the controls ($P < 0.05$). No treatment-related effects were observed in females at 2 or 10 mg/kg bw per day. The females at 50 mg/kg bw per day had 32–46% higher blood urea concentrations ($P < 0.05$) at weeks 6 and 12 compared to the controls. The two decedents (both females at 50 mg/kg bw per day) had high blood urea concentrations and low plasma butyrylcholinesterase activities, and one had low concentration of blood glucose. No intergroup differences were seen in urine analysis results. Absolute and relative adrenal gland weights were 25–36.8% higher and relative liver weights were 13% higher in males at 50 mg/kg bw per day. Relative adrenal gland and liver weights were 23% and 32.7% higher, respectively, in females at 50 mg/kg bw per day. Females at 50 mg/kg bw per day had depleted hepatocytic glycogen and fine vacuolation of the cell cytoplasm in the renal medullary rays. One or both of the females at 50 mg/kg bw per day that were euthanized in extremis exhibited first

and second degree heart block, increased urea concentration, low glucose concentration, disturbances in plasma electrolyte levels, depleted hepatocytic glycogen and fine vacuolation of the cell cytoplasm in the renal medullary rays.

The LOAEL was 2 mg/kg bw per day, the lowest dose tested, based on slight bradycardia and an increased incidence of diarrhoea in both sexes and reduced feed consumption, body weight, body weight gain, emaciation and torpor in females only. No NOAEL could be identified (Broadmeadow, 1988).

In a one-year toxicity study, NNI-850 (fenpyroximate; purity 98.0%) was administered to beagle dogs (4/sex per dose) via capsule at dose levels of 0, 0.5, 1.5, 5.0 or 15.0 mg/kg bw per day for 52 weeks.

There were no mortalities during the study. Diarrhoea was observed in animals from all groups, including controls, but was more frequent in males at 5.0 mg/kg bw per day and in males and females at 15 mg/kg bw per day. Salivation in a few dogs from all treated groups and emesis in isolated animals at 1.5 mg/kg bw per day or above were also noted. Vomiting after dosing (≤5 hours) was observed with one female at 1.5 mg/kg bw per day (day 1); one female at 5.0 mg/kg bw per day (days 1 and 3); and one male and two females at 15.0 mg/kg bw per day (all at day 1, one female at days 2 and 3). Three of the four male dogs at 15.0 mg/kg bw per day exhibited a slower weight gain during the first three months of the study than dogs in the control group. The difference in average weight gain (33% lower in the treated animals) was statistically significant ($P < 0.05$). The body weight gains of male dogs at 0.5, 1.5 and 5.0 mg/kg bw per day and female dogs at 0.5, 1.5, 5.0 and 15 mg/kg bw per day were not significantly different from the control. Periods of inappetence, occasionally marked, were recorded for three males and one female at 15.0 mg/kg bw per day. Neither the feed consumption of animals at 0.5, 1.5 and 5.0 mg/kg bw per day nor the water consumption of all animals was affected by treatment. Veterinary examination confirmed increased salivation and thin appearance in some treated dogs. There were no treatment-related ocular lesions. Compared to the control dogs, all four male dogs treated with NNI-850 at 15 mg/kg bw per day had significantly ($P < 0.05$ or 0.01) slower heart rates 2 and 24 hours after dosing. The heart rates of females at the highest dose were also slower than the control; however, the difference was not statistically significant.

Table 9. Group incidence of diarrhoea in one-year study in dogs treated with fenpyroximate

Week	Group incidence of diarrhoea per sex and dose (%)									
	Males					Females				
	0 mg/kg bw per day	0.5 mg/kg bw per day	1.5 mg/kg bw per day	5 mg/kg bw per day	15.0 mg/kg bw per day	0 mg/kg bw per day	0.5 mg/kg bw per day	1.5 mg/kg bw per day	5 mg/kg bw per day	15.0 mg/kg bw per day
−2 to −1	0	0	0	0	0	0	0	0	0	0
1–4	15	20	25	45	65	15	25	0	20	65
5–8	10	5	20	30	65	15	20	0	15	55
9–12	15	20	25	30	45	15	20	5	20	60
13–16	10	15	20	45	60	10	20	10	15	60
17–20	0	5	20	40	50	5	15	0	5	50
21–24	5	10	5	15	35	0	5	0	5	40
25–28	0	10	5	25	40	0	15	0	0	60
29–32	5	10	20	30	55	15	20	0	10	55
33–36	5	10	5	20	45	5	5	0	0	50
37–40	0	0	5	15	40	0	5	0	5	50
41–44	0	0	10	20	30	5	5	0	10	45
45–48	0	0	0	15	30	0	5	0	5	30
49–52	5	0	5	15	20	5	0	0	0	35

bw: body weight

Source: Broadmeadow (1989)

Most haematological differences between the control and treated dogs occurred randomly and could be attributed to normal variation. Both sexes in all treatment groups had blood cholesterol concentrations 26–45% lower than those in the control groups after 50 weeks of dosing; no accompanying changes in liver function or pathology were noted. Urinary analysis indicated no treatment-related changes. Males in all treated groups had prostate glands an average of 60% heavier than those in the control group, but with no dose response. There were no macroscopic or histopathological treatment-attribuTable findings.

The NOAEL was 1.5 mg/kg bw per day based on increased incidences of diarrhoea at the LOAEL of 5.0 mg/kg bw per day (Broadmeadow, 1989).

(b) Dermal application

In a dermal toxicity study, fenpyroximate (purity 99%) was administered for 6 hours/day for 21 days to Sprague Dawley rats (5/sex per group) at dose levels of 0, 100, 300 or 1000 mg/kg bw per day. The test substance was placed on a gauze dressing dampened with deionized water. Additional deionized water was added to form a paste; the paste was evenly applied to the skin and covered with gauze, which was secured with an elastic bandage.

No premature deaths occurred during the treatment interval. At 1000 mg/kg per day, red nose/mouth/nasal discharge was noted in 2/5 females on days 17 through 21 versus 0/5 controls. These clinical findings are not considered treatment related due to the low frequency. Decreases ($P \leq 0.05$) in body weights were observed in the males throughout the study (↓10–20%, days 8–22) and in the females on days 15 and 22 (↓12–15%). Decreases in feed consumption were noted in the males and females on day 2 (↓54% and 33%, $P \leq 0.05$); day 16 (males, ↓25%, $P \leq 0.05$); and day 21 (↓26% and 23%; $P \leq 0.05$ males only). Compared to concurrent controls, no treatment-related findings were noted in haematological parameters at any dose level tested. No treatment-related findings were observed in clinical chemistry parameters compared to control values. Increases were noted in absolute liver weights of females (20%, $P \leq 0.01$) at 1000 mg/kg bw per day. The relative liver weights were 127.1% and 142.2% of the controls in males and females, respectively, at 1000 mg/kg bw per day. In addition, focal/multifocal acute hepatocellular necrosis was observed in 3/5 females versus 1/5 controls. This necrosis may have been treatment related because there was a corresponding increase in liver weight at this dose. No other treatment-related changes were noted in histopathological examination. No treatment-related changes were noted in gross pathology at any dose level tested. No treatment-related changes were observed in animals at 100 and 300 mg/kg per day.

The NOAEL was 300 mg/kg bw per day based on clinical signs in the females; decreased body weights, body weight gains and feed consumption in both sexes; and increased absolute liver weights and a possible increase in hepatocellular necrosis in the females at the LOAEL of 1000 mg/kg bw per day (Wilkinson, Ryan & Peters, 1992).

(c) Exposure by inhalation

In a four-week nose-only inhalation toxicity study, fenpyroximate (NNI-850; purity 88.7%) when administered via the inhalation route as a dust to Sprague Dawley CD rats (5/sex in Groups II and III, 10/sex in Groups I and IV) for 6 hours/day, 5 days/week, for four weeks at target concentrations of 0 (Group I), 2 (Group II), 10 (Group III) and 50 (Group IV) mg/m^3. The cumulative mean analytical exposure concentrations, as determined by HPLC, were 0.0030, 1.8, 10 and 51 mg/m^3 for Groups I through IV, respectively. Concentration levels were monitored gravimetrically and analysed by HPLC. Particle size distribution was measured once a week using an aerodynamic particle sizer. Following four weeks of exposure, 5 animals/sex per group were killed. Following a subsequent two-week recovery period, the remaining 5 animals/sex from Groups I and IV were killed. Selected organs were weighed and complete gross examinations were conducted on all animals. Microscopic examination of selected tissues was performed.

Particle size distribution measurements showed the MMAD to range from 2.8 to 3.1 µm with a GSD ranging from 2.1 to 2.2. About 95% of particles were 10 µm or less, indicating that the test substance was respirable to the rats.

All animals survived the study. Group IV (50 mg/m^3) animals showed treatment-related effects with clinical observations consisting of laboured breathing during the last 3 weeks of exposure. Animals in all three treatment groups showed a slightly increased incidence of rales (both moist and dry). Statistically significant ($P < 0.05$) decreases in body weights were observed for Group IV males and females; however, the differences abated during the recovery period for both sexes. Although feed consumption decreased in both sexes in Group IV during the first week of exposure, changes in feed consumption in the different treatment groups showed no clear treatment-related pattern. After four weeks of exposure, statistically significant increases in erythrocyte counts were observed in males in Groups III ($P < 0.05$; ↑106%) and IV ($P < 0.001$; ↑108%) and leukocyte counts in Group IV females ($P < 0.01$; ↑166%). Statistically significant decrease in mean corpuscular volume and mean corpuscular haemoglobin ($P < 0.05$) were seen in Group III males. However, these decreases were not considered adverse since the values in the high-dose group was comparable to control values. Statistically significant increased values of triglycerides in Group IV females ($P < 0.05$; ↑152%) and phosphorus in Group III and IV ($P < 0.001$; ↑139% and ↑135%) females were noted. After the recovery period, Group IV haematological and clinical chemistry differences had all abated. After four weeks of exposure, significant increases of lung weights and/or ratios were seen in both sexes of Group III and IV. There were also significant increases in liver weights and/or ratios (Group IV, both sexes), brain weights (Group IV males) and heart and brain weight ratios (Group IV females). After the recovery period, all differences in organ weights had abated. Few sporadic gross lesions were observed during the gross examinations. High-dose animals killed immediately after the four-week exposure had atrophy of the respiratory and olfactory mucosa in the nasal passages. Squamous metaplasia of the respiratory mucosa was higher in number in mid- and high-dose males and high-dose females killed immediately after the four-week exposure than in controls. The incidences of these effects were either lower or not observed in animals killed after the 14-day recovery period. Microscopic changes were also seen in the tissues examined. The incidences of these findings were either similar to those in the control group or they occurred sporadically.

The NOAEL was 2 mg/m^3 based on increased lung weights and squamous metaplasia of the nasal passage mucosa at the LOAEL of 10 mg/m^3 (Hoffman, 1991c).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a mouse carcinogenicity study, NNI-850 (fenpyroximate; purity 97.9–98.4%) was administered to CD-1 mice (50/sex per group) for up to 78 weeks at 0, 25, 100, 400 or 800 ppm (equal to 0, 2.43, 9.47, 38.02 or 69.63 mg/kg bw per day for males and 0, 2.46, 10.22, 41.46 or 73.10 mg/kg bw per day for females).

An increased incidence of anaemia was observed in high-dose animals compared to the concurrent controls; the incidences were significant in the females (5/49 compared to 0/50 in the controls; $P = 0.05$). Increased hair loss was detected in males and females at 400 ppm (10/50 each vs 3–6/50 in the controls) and females at 100 ppm (11/50). A higher incidence of nodules/masses in the integumentary system was observed in females at 25 ppm (6/50 vs 0/50 in the controls; $P < 0.05$). Percentage survival at 78 weeks in all dose groups of mice was 60–92%. Females in all treatment groups showed no differences in mortality throughout the treatment period. Males at 25, 400 and 800 ppm showed significantly lower mortality at 43 weeks of treatment. Mortality was further decreased in males at 400 ppm at 77 and 78 weeks of treatment.

Table 10. Summary of body weight data for the 18-month carcinogenicity study in mice

	Mean group body weights (g)									
	Males					Females				
Week	Control	25 ppm	100 ppm	400 ppm	800 ppm	Control	25 ppm	100 ppm	400 ppm	800 ppm
0	29.9	29.9	29.9	29.9	29.9	23.0	23.0	23.0	23.0	23.0
1	32.7	32.5	32.3	31.4**	28.2**	24.9	25.1	24.8	23.8**	21.8**
2	34.7	34.7	34.0	32.9**	30.5**	25.9	26.6	26.0	25.2	23.3**
3	36.4	36.3	35.8	35.1	32.4**	28.1	28.1	27.2	26.8	24.6**
4	37.6	37.4	36.2	34.7**	32.2**	28.9	28.7	27.7	27.4*	25.7**
5	39.4	38.8	37.9	36.5**	34.4**	30.7	30.7	28.6**	27.8**	26.3**
6	40.9	40.6	38.9	37.5**	35.3**	30.6	30.3	28.6**	27.5**	26.3**
7	41.8	42.0	39.9	37.8**	35.5**	32.2	30.8	29.4**	28.1**	26.9**
8	41.5	41.6	39.6	37.4**	36.0**	32.1	31.9	29.4*	28.6**	26.9**
9	42.6	43.0	40.0	38.0**	36.6**	33.5	32.7	30.4*	29.2**	27.6**
10	43.5	43.4	41.2	38.5**	36.5**	34.0	33.6	30.8**	29.6**	28.5**
11	44.8	44.6	41.8	39.1**	37.1**	34.8	33.7	31.1**	29.3**	28.5**
12	44.8	45.1	42.3	39.4**	38.2**	36.5	35.7	32.1**	29.4**	29.1**
13	45.4	46.0	42.8	39.7**	38.2**	37.9	36.9	33.6**	31.3**	30.0**
16	48.0	48.1	45.3	41.6**	40.1**	39.9	38.1	34.9**	31.9**	31.2**
20	49.3	49.1	46.8	42.1**	40.5**	42.2	41.5	37.3*	33.0**	31.4**
24	50.5	50.6	48.2	42.6**	40.6**	43.6	42.7	39.0	33.3**	31.6**
28	51.0	50.3	48.2	43.0**	41.0**	45.6	44.7	40.8	34.5**	32.2**
32	51.6	51.9	49.3	43.2**	40.8**	46.5	45.7	41.1*	34.9**	32.4**
36	52.5	52.5	50.1	43.8**	41.3**	47.0	45.9	42.1	35.3**	32.8**
40	52.9	52.8	50.8	44.2**	41.9**	48.5	47.6	43.3	36.0**	33.1**
44	53.1	53.2	50.9	44.6**	42.1**	49.2	49.1	43.8	36.2**	33.5**
48	53.5	54.1	52.1	45.0**	42.2**	49.9	50.1	44.8	37.1**	33.8**
52	53.9	54.2	52.1	45.0**	41.6**	50.5	50.3	45.0	37.0**	33.9**
56	54.1	54.1	51.6	44.7**	40.5**	50.6	50.6	45.5	37.2**	33.6**
60	54.1	54.4	51.3	44.8**	40.8**	50.8	50.8	45.5	37.3**	33.1**
64	54.0	54.4	51.4	44.8**	40.6**	50.9	50.8	46.2	37.8**	33.3**
68	53.6	55.0	51.6	44.8**	41.3**	51.2	50.7	46.3	38.1**	34.0**
72	54.6	53.8	52.5	44.9**	41.8**	50.9	49.7	45.8	38.0**	34.4**
76	54.7	53.4	52.3	44.8**	40.9**	51.0	50.3	46.0	38.6**	34.3**
78	53.9	52.2	51.7	45.0**	41.4**	51.0	50.0	46.7	38.8**	34.3**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ *Source*: Shirasu (1989)

The mean body weights of animals at 800 and 400 ppm were significantly lower than the concurrent controls throughout the dosing period. At termination, the mean body weights of males and females at 800 ppm were 23% and 33% less than the controls, respectively ($P < 0.01$) and of males and females at 400 ppm were 16.5% and 24% less than the controls, respectively ($P < 0.01$). Mean body weights of animals at 100 ppm were also consistently lower than the controls; statistically significant differences were noted from weeks 6–11 in males and 5–32 in females. At the terminal kill, the mean body weights of males and females at 100 ppm were 4% and 8% less than the controls, respectively ($P < 0.01$). There were no treatment-related decreases in mean body weights compared to the concurrent controls in animals treated at 25 ppm.

Mean feed consumption in animals at 400 and 800 ppm was lower than the concurrent controls throughout the dosing period ($P < 0.05$ and < 0.01). For both treatment groups, the decreased feed consumption was most evident after the first week of dosing. Feed consumption in the high-dose males and females was 64% and 54% less than the controls, respectively, at the end of the first week of dosing ($P < 0.01$); in males and females at 400 ppm, mean feed consumption was 42% and 35% less than controls, respectively ($P < 0.01$). At 13 weeks, feed consumption averages in males at 800 and 400 ppm were decreased by 22% and 24%, respectively, relative to the controls ($P < 0.01$), and were decreased by 27% ($P < 0.01$) and 14%, respectively, in females at the same doses. The overall average feed consumption for animals at 100 ppm was 4–5% less than the concurrent controls, and for animals of both sexes receiving 25 ppm, it was similar to the controls. Water consumption was not measured in this study.

Differential leukocyte counts in males at 800 ppm at the 52- and 78-week intervals were similar to controls; blood from male mice dosed at 400, 100 or 25 ppm was not analysed. There was a small decrease in the segmented form of neutrophils in females at 800 ppm at the 52- and 78-week intervals; this was statistically significant ($P < 0.05$) at the 52-week interval. Differential leukocyte counts from females at 400 ppm analysed at the 52-week interval were comparable to the controls. There were no differences in plasma or RBC cholinesterase activities in any treated group compared to concurrent controls. Mean absolute adrenal weights (from 10 animals) in males and females at 800 ppm were 27% and 25%, respectively, lower than the controls ($P < 0.05$); however, adrenal-to- body weight ratios were nearly identical in dosed groups of males or females and in controls. This is due to the significantly decreased mean body weights in the high-dose animals. Relative weights of brain (both sexes), kidneys and liver (females) and testes at 400 and 800 ppm were increased compared to controls. None of these findings were corroborated by macroscopic and histopathological changes. There were no significant differences in mean absolute and relative organ weights in the animals treated at 100 or 25 ppm and concurrent controls. Gross findings at the terminal kill and in those animals euthanized in extremis or found dead during the treatment period included emaciation in 10/50 males at 800 ppm compared to 1/50 ($P < 0.01$) controls. Pulmonary oedema was also observed in 4/50 males at 800 ppm compared to 0/50 controls.

There were no significant dose-dependent increases in the incidence of any neoplasm. The number of tumour-bearing animals in each treated group was also comparable to the controls. Females at 400 and 800 ppm had a significant increase in the overall incidence of ovarian atrophy (incidence: 25, 32, 24, 35 and 35 at 0, 25, 100, 400 and 800 ppm). In males, the incidence of mucosal hyperplasia in the glandular stomach increased significantly in animals euthanized in extremis or found dead during the treatment period. However, the overall incidence of all animals was not increased. Other statistically significant changes in nonneoplastic lesions showed no increased incidences related to dose levels.

The NOAEL was 25 ppm (equal to 2.43 mg/kg bw per day) based on decreased body weights and feed consumption at the LOAEL of 100 ppm (equal to 9.47 mg/kg bw per day). Under the conditions of this study, there was no evidence of carcinogenicity (Shirasu, 1989).

Rat

In a chronic toxicity/carcinogenicity study, NNI-850 (fenpyroximate; purity 97.2%) was administered in the diet to CD rats (50/sex per dose in the carcinogenicity phase and 30/sex per dose in the toxicity phase) for 104 weeks at dose levels of 0, 10, 25, 75 or 150 ppm (equal to, respectively, 0, 0.4, 0.97, 3.00 and 6.20 mg/kg bw per day for males and 0, 0.49, 1.21, 3.18 and 8.01 mg/kg bw per day for females in the carcinogenicity phase, and 0, 0.4, 0.97, 3.08 and 6.18 mg/kg bw per day for males and 0, 0.48, 1.16, 3.79 and 7.57 mg/kg bw per day for females in the toxicity phase). Each group in the toxicity phase was composed of 10 animals/sex per group for the interim kill after 52 weeks of treatment and 20 animals/sex per group for the terminal kill after 104 weeks of treatment.

Treatment-related signs of toxicity were not observed. Statistical analysis revealed significantly lower mortality ($P < 0.05$) in females at 75 ppm and a significant trend for enhanced survival in treated females. Intergroup differences in males were not statistically significant.

Table 11. body weight and feed consumption data in rats in a two-year carcinogenicity study with fenpyroximate

Parameter	Measure per sex and dose level									
	Males					Females				
	0 ppm	10 ppm	25 ppm	75 ppm	150 ppm	0 ppm	10 ppm	25 ppm	75 ppm	150 ppm
Body weight data										
Toxicity phase										
Week 26	540	533	506	481*	432***	228	243	231	201**	188***
Week 52	708	703	670	633**	581***	338	355	330	292*	271***
Week 78	826	714*	726	626***	625***	414	413	455	354	353
Week 104	838	690	677	694	558**	492	478	510	425	362***
Carcinogenicity phase										
Week 26	533	526	533	495***	445***	240	230	237	209***	186***
Week 52	700	681	684	646*	583***	353	336	342	293***	260***
Week 78	729	752	745	697	638**	453	453	432	384***	333***
Week 104	714	748	717	708	617	460	491	503	423	361**
Feed consumption *(% of control)*										
Week 1–104	–	100	95	96	91	–	111	100	95	92
Feed conversion ratio *(feed consumed/unit gain of body weight)*										
Week 1–14	6.1	6.4	6.2	6.5	6.9	10.3	10.3	10.2	11.1	12.0

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$

Males and females at 75 and 150 ppm had decreased mean body weights within the first few weeks of treatment and this effect persisted throughout the study. The statistical significance was not always attained at 75 ppm (Table 11). The total feed intake of animals at 150 ppm was lower than that of controls in animals assigned to both phases of the study. Feed intake was also marginally lower in females at 75 ppm in both phases than in control groups. The efficiency of feed conversion during the first 14 weeks of treatment was lower than that of controls in both carcinogenicity and toxicity phases in males and females at 150 ppm and females at 75 ppm. Water consumption was not measured. No treatment-related ophthalmoscopic abnormalities were noted. There were no important toxicological effects on haematological parameters (24, 49, 76 and 102 weeks of treatment); nearly all values were within the normal range, and no dose–response relationship or compound-related trends were observed. No treatment-related effects on clinical chemistry were observed. Serum glucose levels were about 11–12% lower than controls in males at 150 ppm at 24, 47 and 76 weeks and in females at 150 ppm at 24 weeks. Significant differences at other dose levels were sporadic, and there was no pattern of change with dose. Other blood chemistry parameters that occasionally showed significant differences from controls included creatinine, bilirubin, cell acetylcholinesterase, plasma α_1-globulin, sodium, calcium, inorganic phosphorus and chloride, but none showed a consistent treatment-related pattern. No treatment-related urinary effects were observed although there was a tendency towards low urine volume with associated low pH and high specific gravity in males at 150 ppm, with the effect was most marked after 49 weeks of treatment. Similar effects on females were not observed. There were no significant differences in urinary parameters between control and treated animals at 23 weeks of feeding, and the differences observed at subsequent periods were generally sporadic and lacked a dose response. For the carcinogenicity phase animals at 104 weeks, the liver weights were 6%, 20% and 29% lower than controls in females receiving 25, 75 and 150 ppm ($P < 0.01$ at 75 and 150 ppm), but the relative liver weights were only 12% lower than controls (nonsignificant) in each of the treatment groups (25, 75, 150 ppm). The increased liver weights were not considered adverse since there were no collaborating changes in blood biochemistry and morphological correlates. Other changes in organ weights that achieved a level of significance at 150 ppm were not accompanied by changes in organ:body weight ratios, indicating that the changes may be associated with decreases in terminal body weights. There were no treatment-related increases

in tumour incidence compared to controls at the doses tested. Nonsignificant nonneoplastic microscopic changes in pituitary of carcinogenicity phase males and some female group, and in the stomach, uterus, ovaries and mesenteric lymph nodes occurred with greater frequency than in controls.

The NOAEL for systemic toxicity was 25 ppm (equal to 0.97 mg/kg bw per day) based on decreased body weights, feed intake and feed conversion and decreased plasma total protein at the LOAEL of 75 ppm (equal to 3 mg/kg bw per day). There was no evidence for carcinogenicity of fenpyroximate (Aughton, 1989).

2.4 Genotoxicity

The results of studies of genotoxicity of fenpyroximate are summarized in Table 12.

Table 12. Overview of genotoxicity tests with fenpyroximate

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 *Escherichia coli* WP2uvrA	50, 158, 500, 1 580, 5000 µg/plate in DMSO (± S9)	97.3	Negative	May (1988)
Mammalian cytogenetic test	Chinese hamster V79 lung cells	3, 10, 30, 100, 330 µg/mL in acetone (± S9)	97.3	Negative	Hodson-Walker (1988a)
Mammalian cell gene mutation	Human lymphocytes	1.25, 5, 20 µg/mL in acetone (± S9)	97.3	Negative	Hodson-Walker (1988b)
Mammalian cytogenetic assay	Mouse lymphoma L5178Y $tk^{+/-}$	0.059–30 µg/mL in CMC sodium (10% v/v) (± S9)[a]	99.2	Negative	Kajiwara (2016a)
DNA repair test	*Bacillus subtilis* (H17 (rec+), M45 (rec-))	10, 20, 50, 100, 200, 500 µg/disk in DMSO (± S9)	97.3	Negative	Watanabe (1990a)
Unscheduled DNA synthesis	Rat hepatocytes	0.025, 0.051, 0.102, 0.255, 0.509, 1.02 µg/mL in DMSO	97.3	Negative	Cifone (1989)
In vivo					
Micronucleus test in mice	CD-1 mice	80, 400 or 2000 mg/kg bw in 0.5% methylcellulose	97.3	Negative	Hodson-Walker (1988c)

bw: body weight; CMC: carboxymethylcellulose; DMSO: dimethyl sulfoxide; v/v: volume per volume

S9: 9000 × *g* supernatant fraction from induced rats (metabolic activation);

tk: thymidine kinase locus;

[a] At 3 hours, −S9: 0, 0.059, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15, 30 µg/mL;
at 3 hours, +S9: 0, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15, 30 µg/mL;
at 24 hours –S9, 0, 0.029, 0.059, 0.12, 0.23, 0.47, 0.94, 188, 3.75, 7.5, 15 µg/mL.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, groups of male and female CD rats were given diets containing fenpyroximate (purity 97.3%) at concentrations of 0, 10, 30 or 100 ppm (equal to 0, 0.67, 1.99 and 6.59 mg/kg bw per day for the F_0 males; 0, 0.83, 2.44 and 8.60 mg/kg bw per day for the F_0 females; 0, 0.78, 2.33 and 8.45 mg/kg bw per day for the F_1 males; and 0, 0.96, 2.82 and 9.92 mg/kg bw per day for the F_1 females). F_0 rats (24/sex per group) received the test diets for 14 weeks before being paired to produce the F_1 litters; treatment of the F_0 parents was continued throughout mating and until weaning of the F_1 offspring, at which time the F_0 males were killed, while the females were killed about 10 days later. At each dietary concentration, and where possible, the size of these litters was reduced to eight (4/sex where the numbers of each sex in the litter made this possible; where the litter size was < 8, culling was not carried out). Twenty-four males and 24 females were selected as the F_1 generation (if possible, one male and one female being selected from each litter) and given diets containing fenpyroximate, as described above, for 14 weeks, after which they were paired to produce the F_2 litters. Treatment of the F_1 parents via the diet continued throughout mating and until weaning of the F_2 offspring, at which time the F_1 males and the F_2 offspring were killed, while the females were killed about 10 days later.

One F_0 female in the 30 ppm treatment group was euthanized for humane reasons on day 23 post coitum after showing prostration, pallor and reduced respiratory rate. One F_1 female receiving fenpyroximate at 100 ppm was killed in extremis on gestation day 24 and was found to have a ruptured uterus. There were no apparent treatment-related clinical signs at any dose level of the F_0 and F_1 generations. At 100 ppm, mean body weights for the F_0 males and females were slightly decreased throughout most of the premating period (5–6%), and body weight gains at mating were 8% and 11% ($P < 0.05$) lower than controls for males and females. In the F_0 females at 30 ppm, there was slightly reduced body weight gain during gestation. In F_1 females at 100 ppm, mean body weight at gestation day 20 was 7% lower than in controls ($P < 0.05$); body weight gain was decreased at gestation days 6 and 13, but was similar to control gain at gestation day 20. At 100 ppm, there was a reduction in body weight gain in F_1 males. In F_0 males at 100 ppm, feed consumption was slightly reduced compared with that of the controls, but feed consumption was not reduced in males receiving fenpyroximate at lower dietary concentration nor in any group of females. No treatment-related effects on estrous cycle length or periodicity, male fertility, sexual maturation, reproductive performance, gestation length or gestation index were reported in this study. At 100 ppm, the conception rate and fertility index were slightly reduced in F_0 animals (Table 13). Litter size at birth and viability up until the time of weaning were not affected by treatment. In F_0, absolute and relative organ weights of the reproductive organs were similar in all groups. In F_1, absolute organ weights of testes were increased at all treatment levels, however there was no clear dose dependency. When testes weights were expressed relative to body weight, only an increase at 100 ppm was observed. As this occurred in the presence of a decrease in body weight and had no effect on mating performance, the decreases were not considered to be treatment related. The weight of the other reproductive organs did not consistently differ from the control group. No treatment-related macroscopic or microscopic observations were reported.

There were no treatment-related viability effects or clinical signs of toxicity reported in either F_1 or F_2 offspring. body weight means of both F_1 and F_2 pups on day 1 postnatal was similar in all groups. body weight gains during weaning were significantly lower ($P < 0.01$) at 100 ppm. No treatment-attributable intergroup differences in sex ratio were observed. Necropsy of adult F_1 rats, of F_2 offspring that died before weaning and of F_1 offspring that were culled on postnatal day 4 or that were killed at termination after weaning did not show any treatment-related macroscopic abnormalities. No macroscopic or microscopic abnormalities were reported for pups in this study.

The NOAEL for parental toxicity was 30 ppm (equal to 1.99 mg/kg bw per day) based on reduced body weights of both sexes during the premating period, decreased feed consumption and increased testicular (absolute) and epididymal weights (absolute and relative to body weight) in males at the LOAEL of 100 ppm (equal to 6.59 mg/kg bw per day). The NOAEL for reproductive toxicity was 100 ppm (equal to 6.59 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 30 ppm (equal to 1.99 mg/kg bw per day) based on reduced pup body weights at the LOAEL of 100 ppm (equal to 6.59 mg/kg bw per day) (Higgins, 1989a).

Table 13. Overview of mating performance and fertility in parental generations of rats treated via the diet with fenpyroximate

	Measure per sex and dose level							
	Males				Females			
Mating performance and fertility	**0 ppm**	**10 ppm**	**30 ppm**	**100 ppm**	**0 ppm**	**10 ppm**	**30 ppm**	**100 ppm**
F_0 generation								
No. paired/no. mated	24/23	24/24	24/23	24/22	24/24	24/24	24/24	24/24
No. of pregnancies	22	22	22	19	23	22	23	21
% mating index	96	100	96	92	100	100	100	100
% conception rate	96	92	96	86	96	92	96	88
% fertility index	92	92	92	79	96	92	96	88
F_1 generation								
No. paired/no. mated	24/20	24/24	24/22	24/24	24/22	24/24	24/22	24/24
No. of pregnancies	18	21	18	24	20	21	18	24
% mating index	83	100	92	100	92	100	92	100
% conception rate	90	88	82	100	91	88	82	100
% fertility index	75	88	75	100	83	88	75	100
Historical control data from 33 studies								
% mating	Range: 83–100%; Mean: 96%				Range: 90–100%; Mean: 98%			
% conception rate	Range: 71–100%; Mean: 88%				Mean: 72–100%; Mean: 88%			
% fertility index	Range: 67–100%; Mean: 85%				Range: 67–100%; Mean: 85%			

F_0: parental generation; F_1: first filial generation; no.: number; ppm: parts per million *Source*: Higgins (1989a)

(b) Developmental toxicity

Rat

In a developmental toxicity study, NNI-850 (fenpyroximate; purity 97.6%) was administered to CD Sprague Dawley female rats (22/dose) by gavage at dose levels of 0, 1.0, 5.0 or 25 mg/kg bw per day from gestation day 6 through 15. The test material was administered as a suspension in aqueous 1% (w/v) carboxymethylcellulose (CMC) and 0.1% (w/v) Tween 80. The surviving rats were killed on gestation day 20 for macroscopic examination and examination of their uterine contents. The numbers of corpora lutea per ovary, implantation sites, resorption sites and live and dead fetuses were recorded. The fetuses were weighed, sexed and examined for external abnormalities. Approximately two-thirds of the animals in each litter were processed and stained (with alizarin red) for skeletal examination and the remaining one-third were fixed in Bouin's fixative and serially sectioned.

There were no treatment-related effects noted in mortality or clinical signs. Three dams at 5 mg/kg bw per day, but none in the other groups, were found dead after dosing on gestation day 12. Although the cause of death was not determined, a pale precipitate was found on the exterior surface of the lungs in all three animals upon necropsy. The effect was not considered to be treatment related because there were no deaths in the group at the highest dose. Decreases (↓3–4%) in mean body weights of the high-dose dams were noted on the first day after treatment and persisted until the end of the study. The decrease in body weights did not reach statistical significance. No intergroup differences in body weight or body weight gain were seen in the other groups. Feed consumption at 25 mg/kg bw per day was initially reduced and water consumption increased, but intergroup differences in feed and water consumption were not seen in the other groups. Maternal body weight and feed consumption data were marginally depressed at 25 mg/kg bw per day, and it is apparent that animals could have tolerated higher dose levels of the test material. Slight decrease in body weights (3–4%) were not considered adverse. No treatment-related gross pathological findings were noted in any of the dams.

Caesarean section observations are presented in Table 14. The numbers of corpora lutea, implantations and viable fetuses and the extent of pre- and postimplantation losses were similar in control and treated groups. Fetal and placental weights were also unaffected by treatment. All treated groups showed increases in the number of thoracic ribs, which was greatest at 25 mg/kg bw per day. At the lower doses, there was no clear dose–response relationship (Table 15).

Table 14. Caesarean section observations in rat developmental toxicity study

Observation	Observations per dose level			
	0 mg/kg bw per day	1.0 mg/kg bw per day	5.0 mg/kg bw per day	25 mg/kg bw per day
No. of mated animals	22	22	22	22
No. of pregnant animals (pregnancy rate, %)	22 (100)	22 (100)	21 (95)	22 (100)
No. of nonpregnant animals	0	0	1	0
Maternal wastage				
No. died	0	0	3	0
No. died pregnant	0	0	3	0
No. died nonpregnant	0	0	0	0
No. aborted	0	0	0	0
No. of premature deliveries	0	0	0	0
Total no. of corpora lutea	358	351	290	354
No. of corpora lutea/dam [a]	16.3 ± 2.7	16.0 ± 2.5	16.1 ± 1.6	16.1 ± 1.9
Total no. of implantations	332	332	274	332
No. of implantation/dam [a]	15.1 ± 1.8	15.1 ± 1.9	15.2 ± 1.5	15.1 ± 1.6
Total no. of litters	22	22	18	22
Total no. of live fetuses	318	313	265	311
No. of live fetuses/dam [a]	14.5 ± 1.7	14.2 ± 2.3	14.7 ± 1.6	14.1 ± 1.9
Total no. of dead fetuses No. of dead fetuses/dam	NR	NR	NR	NR
Total no. of resorptions	14	19	9	19
Early	14	19	9	19
Late	0	0	0	0
No. of resorptions/dam [a]	0.6 ± 0.8	0.9 ± 0.9	0.5 ± 0.7	1.0 ± 1.0
Early [a]	0.6 ± 0.8	0.9 ± 0.9	0.5 ± 0.7	1.0 ± 1.0
Late [a]	0	0	0	0
No. of litters with total resorptions	0	0	0	0
Mean fetal weight (g) [a]	3.76 ± 0.07	3.76 ± 0.07	3.83 ± 0.06	3.80 ± 0.06
Mean placental weight (g) [a]	0.52 ± 0.02	0.55 ± 0.02	0.51 ± 0.02	0.55 ± 0.02
Sex ratio (% male)	47	45	55	51
Preimplantation loss (%)	7.5	5.4	5.8	6.7
Postimplantation loss (%)	4.2	5.7	3.3	6.3

[a] Mean ± SD; bw: body weight; NR: not reported *Source*: King (1989)

Table 15. Selected observations at fetal skeletal examination after exposure to fenpyroximate

Observations	% fetuses (no. of litters) with the finding per dose level				Historical control range (mean) [a]
	0 mg/kg bw per day	1 mg/kg bw per day	5 mg/kg bw per day	25 mg/kg bw per day	
Unilateral (ribs 13/14)	2.4% (4)	5.2% (4)	5.0% (5)	8.2% (9)	0.0–4.2% (1.22%)
Bilateral (ribs 14/14)	1.4% (1)	4.8% (5)	1.7% (2)	7.7% (7)	0.0–3.5% (0.48%)

[a] Historical control: 21268 fetuses from 140 studies; bw: body weight; no.: number *Source*: King (1989)

The NOAEL for maternal toxicity was 25 mg/kg bw per day, the highest dose tested. The LOAEL for maternal toxicity was not identified. The study author identified a maternal toxicity NOAEL of 5 mg/kg bw per day based on reduced body weight gain and feed consumption. The NOAEL for developmental toxicity was 5 mg/kg bw per day, based on increases in the number of thoracic ribs at the LOAEL of 25 mg/kg bw per day (Higgins, 1989b).

Rabbit

In a preliminary study of developmental toxicity, pregnant New Zealand White rabbits (4/group) were given fenpyroximate (purity 98.4%) at a dose of 2.5 or 5.0 mg/kg bw per day by gavage from gestation day 6 to 19. An additional group of four pregnant rabbits received the vehicle only (1% w/v aqueous CMC mucilage containing 0.1% w/v Tween 80), and a group of three pregnant rabbits received fenpyroximate at a dose of 1.0 mg/kg bw per day by gavage. On day 29, the rabbits were killed and examined macroscopically; abnormal tissues were retained and fixed. The reproductive tract was dissected out, and the number of corpora lutea in each ovary and the number of implantation sites and resorption sites were determined as was the number and distribution of live and dead fetuses.

There were no deaths. A reduction in faecal output was observed in one female at 5 mg/kg bw per day. A depression in body weight gain was recorded in the high-dose group; however, no statistical significance was achieved. One high-dose female had a reduction in faecal output, progressive depression of body weight gain and a marked reduction in feed and water consumption; the remaining high-dose females had slightly reduced feed and water consumption only during the first half of the treatment period. No treatment-related macroscopic findings were observed. Two of the four females at 5 mg/kg bw per day had increased postimplantation loss. Reduced fetal weight and multiple anomalies were observed in all fetuses of one female at 5 mg/kg bw per day.

The NOAEL for maternal toxicity was 2.5 mg/kg bw per day based on slightly reduced feed and water consumption, reduced faecal output (in one female) and increased postimplantation loss at the LOAEL of 5 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 2.5 mg/kg bw per day based on reduced fetal weight and observed anomalies at the LOAEL of 5 mg/kg bw per day (Bailey, 1989).

In a developmental toxicity study, NNI-850 (fenpyroximate; purity 97.6%) was administered to New Zealand White female rabbits (15/dose) by gavage at dose levels of 0, 1.0, 2.5 or 5.0 mg/kg bw per day from gestation days 6 through 19. An additional group of 15 pregnant rabbits received the vehicle only (1% w/v aqueous CMC mucilage containing 0.1% w/v Tween 80). On gestation day 29, the animals were killed and examined macroscopically. The reproductive tract, including the ovaries, were examined and the numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses were determined. The fetuses and placentas were weighed and examined for external abnormalities. Approximately two-thirds of the animals in each litter were processed and stained (with alizarin red) for skeletal examination and the remaining one-third were fixed in Bouin's fixative and serially sectioned.

Slightly reduced faecal output was noted in the low- and mid-dose females whereas this was marked in high-dose females. These findings were considered equivocal. No other significant clinical signs were noted. No treatment-related effects were noted on mortality. Two mid-dose females were euthanized in extremis because of respiratory distress due to infection or gastrointestinal disturbance. Statistically significant decreases in the body weights of dams from the high-dose group on days 8, 10 and 12 ($P < 0.01$ or < 0.001) were noted. A statistically significant decrease in the mean body weights of the mid-dose dams was noted on day 8 ($P < 0.05$). A possible reduction in feed consumption was noted in the mid- and high-dose groups during treatment, whereas a significant reduction in feed intake (24–35%) was observed in high-dose females. In addition, high-dose females had reduced water intake during the first half of the dosing phase. The numbers of corpora lutea, implantations and viable fetuses and the extent of pre- and postimplantation losses were similar in control and treated groups. Fetal and placental weights were also unaffected by treatment. One mid-dose and one high-dose dam had total litter resorption and one high-dose dam aborted (Table 16).

Table 16. Caesarean section observations in developmental toxicity study in rabbits

Observation	Observations per dose level			
	0 mg/kg bw per day	1.0 mg/kg bw per day	2.5 mg/kg bw per day	5.0 mg/kg bw per day
No. of mated animals	15	15	15	15
No. of pregnant animals (pregnancy rate, %)	15 (100)	14 (93)	15 (100)	15 (100)
No. of nonpregnant animals	0	1	0	0
Maternal wastage				
No. died	0	0	2	0
No. died pregnant	0	0	2	0
No. died nonpregnant	0	0	0	0
No. aborted	0	0	0	1
No. of premature deliveries	0	0	0	0
Total no. of corpora lutea	153	146	136	143
No. of corpora lutea/dam [a]	10.2 ± 1.8	10.4 ± 2.4	10.3 ± 1.9	11.0 ± 2.7
Total no. of implantations	134	118	115	116
No. of implantation/dam [a]	8.9 ± 1.3	8.4 ± 3.7	8.8 ± 1.9	8.8 ± 2.7
Total no. of litters	15	14	12	13
Total no. of live fetuses	120	104	103	104
No. of live fetuses/dam [a]	8.0 ± 1.6	7.4 ± 3.3	8.6 ± 1.9	8.0 ± 2.7
Total no. of dead fetuses	NR	NR	NR	NR
No. of dead fetuses/dam	NR	NR	NR	NR
Total no. of resorptions	14	14	12	12
Early	5	7	3	8
Late	9	7	9	4
No. of resorptions/dam [a]	0.9 ± 1.0	1.0 ± 1.0	0.3 ± 0.5	0.8 ± 0.9
Early [a]	0.3 ± 0.6	0.5 ± 0.7	0.3 ± 0.5	0.5 ± 0.7
Late [a]	0.6 ± 0.8	0.5 ± 0.7	0.0 ± 0.0	0.3 ± 0.6
No. of litters with total resorptions	0	0	1	1
Mean fetal weight (g) [a]	42.8 ± 1.6	41.0 ± 1.9	41.6 ± 1.6	42.7 ± 2.0
Mean placental weight (g) [a]	5.8 ± 0.3	5.6 ± 0.4	5.5 ± 0.2	5.6 ± 0.3
Sex ratio (% male)	57	47	43	50
Preimplantation loss (%)	12.4	19.2	14.5	19.6
Postimplantation loss (%)	10.4	11.9	2.8	9.6

bw: body weight; no.: number; NR: not reported

[a] Mean ± SD.

Source: King (1989)

The incidence of unilateral slightly folded retina was significantly increased relative to the concurrent controls (25.8% vs 8.1%, $P < 0.05$); however, it was also within the historical controls (0–33.3%). The increase in bilateral slightly folded retina was not statistically significant (16.1% vs 10.8% in concurrent controls and 0–16.7% in historical controls), although the values were significantly increased compared with the control group of the study (Table 17).

Table 17. Ocular effects in fetuses after exposure to fenpyroximate [a]

Observations	No. of fetuses (no. of litters) with the finding per dose level				Historical controls range (mean) [a]
	0 mg/kg bw per day	1.0 mg/kg bw per day	2.5 mg/kg bw per day	5.0 mg/kg bw per day	
No. of fetuses (litters) examined	37 (15)	33 (13)	34 (12)	31 (13)	1 120
Incisors erupted	83.8 (14)	84.8 (13)	94.1 (12)	80.6 (12)	41.9–96.4 (68.1)
Unilateral slightly folded retina	8.1 (3)	6.1 (2)	5.9 (2)	25.8 (6)*	0–33.3 (9.91)
Bilateral slightly folded retina	10.8 (3)	6.1 (2)	14.7 (4)	16.1 (5)	0–16.7 (4.82)

bw: body weight; no.: number; *: $P < 0.05$ *Source*: King (1989)

[a] From free-hand serial sectioning of fetal heads.

[b] Mean historical control incidence in parentheses.

The NOAEL for maternal toxicity was 2.5 mg/kg bw per day based on reduced body weight gain, feed and water consumption and reduced faecal output at 5 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 2.5 mg/kg bw per day, based on increased incidence of slightly folded retinas at 5 mg/kg bw per day (King, 1989).

2.6 Special studies

(a) Neurotoxicity

Rat

In an acute neurotoxicity study, groups of Crl:CD(SD) rats (5/sex per dose) were administered a single gavage dose of fenpyroximate (purity 99.8%) in a 1% (w/v) CMC and 0.1% (w/v) Tween 80 aqueous solution at dose levels of 0, 37.5, 150 or 300 mg/kg bw and observed for 14 days. Additional rats (10/sex per dose) were treated similarly and given a neurobehavioural assessment (functional observational battery and motor activity testing) on the day of dosing at peak effect time (8 hours ± 10 minutes) and at 24 hours ± 10 minutes and on days 7 and 15 post dosing. At study termination (day 16), 10 rats/sex per group were killed and perfused in situ for neuropathological examination. Histopathological evaluations of brain and peripheral nervous system tissues of control and high-dose rats were also conducted.

All rats survived to study end. Clinical signs included mild dehydration in 3/10 males at 150 mg/kg bw and in 7/10 males and 4/10 females at 300 mg/kg bw; and coldness to the touch in two males at 300 mg/kg bw on day 2 of the study. There was a dose-related decrease in body weight in both sexes during the first week after dosing (300 mg/kg bw males ↓16% by day 4 post dose and females ↓9% by day 3 post dose) with the males showing the greater effect. Negative body weight gains were observed in all male treatment groups (dose-related) and female treatment groups (no dose response) on days 1–2 after dosing. The reductions in body weight and body weight gain were consistent with the reductions in feed consumption observed in both sexes at 150 and 300 mg/kg.

Treatment-related findings in the functional observational battery and motor activity assessments included (1) a decrease in motor activity (total activity and total time spent in movement) in both sexes at 150 mg/kg and 300 mg/kg dose levels at the 24-hour time interval; and (2) a reduction in auditory startle response in females at these two dose levels at 24 hours post dosing. Consistent with a decrease in activity is the lack of an increase in the number of rears observed in the open field in the females at 150 mg/kg and 300 mg/kg compared to the increase in rears observed in the control and low-dose females at the 8- and 24-hour time intervals. There was no biologically significant decrease in body weight in either sex at the 24-hour time interval. There were no effects on brain weight, and no neuropathological findings were observed in either sex.

The NOAEL for general toxicity was 37.5 mg/kg bw based on decreased motor activity (total activity counts and total time spent in movement) in both sexes and a reduction in auditory startle response in females at 24 hours post dosing, and mild dehydration in males, reduced feed consumptions (about 60%) and statistically significant decreased in body weights and body weight gains in first 3 days in males and females, at 150 mg/kg bw (Barnett, 2011a).

The marginal decreases in motor activity and reduction in auditory response were considered to be effects secondary to the excessive general toxicity rather than the neurotoxic effects. The Meeting noted that the 150 mg/kg bw effect level in this study is approximately 65% of the LD_{50} value for fenpyroximate in females.

In a 90-day neurotoxicity study, fenpyroximate (purity 99.8%) was administered to Crl:CD(SD) rats (10/sex per dose) in the diet at dose levels of 0, 30, 100 or 300 ppm (0, 1.8, 6.1 and 16.4 mg/kg bw per day for males and 0, 2.0, 6.6 and 18.4 mg/kg bw per day for females, respectively) for 90 consecutive days. Neurobehavioural assessments (functional observational battery and motor activity evaluation) were performed in10 rats/sex per group once pretest and at weeks 2, 4, 8 and 13. At study termination, all the rats were killed and perfused in situ for neuropathological evaluation. Of the perfused rats, brain and peripheral nervous system tissues of 5 rats/sex per group underwent histopathological evaluation.

All the treated rats survived to schedule kill. At 300 ppm, there was an increase in the incidence of clinical signs, including (1) dehydration in both sexes, with the males displaying the greater effect (both in incidence and severity); (2) chromorhrinorrhoea (discharge of a pigmented secretion from the nose), which was more prevalent in both sexes compared to the controls; and (3) ungroomed coats in males. Males at 100 ppm had a slight increase in chromorhinorrohea. body weights (males ↓30%, females ↓16%; $P \leq 0.01$) and body weight gains (overall: males ↓54%, females ↓49%) were reduced at 300 ppm for both sexes. Decreased feed consumption/feed efficiency was observed in both sexes at 300 ppm, which is consistent with the observed body weight effects. Reduction in body weights at 100 ppm were not statistically significantly different from the control values. Ophthamological examinations at study end did not show any treatment-related effects.

At 300 ppm, there was a statistically significant dose-related reduction in body temperature in males at week 13, although the magnitude of the change was less than 1°C. Females had an apparent dose-related decrease in the mean number of rears in the open field during the 4- and 8-week time intervals, although statistical significance was not attained and the dose–response relationship was not maintained at the 13-week time interval. There was a significant decrease ($P \leq 0.05$) in the mean hind limb maximum and average grip test score for low-dose males during week 13. There was also a significant increase ($P \leq 0.05$) in the mean hind limb maximum grip test score of male rats at 300 ppm during the week 2 evaluations. This difference was considered incidental and not treatment-related because the average hind limb grip test value was comparable with that of the control group and the values for the other time points were all comparable with the control values. Motor activity measurements for both the male and female rats had no treatment-related statistically significant or biologically relevant differences in the exposure groups for either the total number of movements or total time spent in movement when evaluated during weeks 2, 4, 8 and 13 of exposure. Absolute brain weights were comparable in both sexes in all treatment groups. No treatment-related anomalies were noted in the central or peripheral nervous system tissue, eye (including retina and optic nerve) or skeletal muscle.

The NOAEL for systemic toxicity was 30 ppm (equal to 1.8 mg/kg bw per day) based on increased incidence of chromorhinorrhoea and dehydration in both sexes at the LOAEL of 100 ppm (equal to 6.1 mg/kg bw per day).

The NOAEL for neurotoxicity was 300 ppm (equal to 16.4 mg/kg bw per day), the highest dose tested (Barnett, 2011b).

Hen

In an acute delayed neurotoxicity study, 12 fasted Sterling Ranger hybrid hens, at least 12 months old, received a single gavage dose of fenpyroximate (purity 97.0%) in 0.5% aqueous methylcellulose at 5000 mg/kg (limit test) at days 1 and 22. The hens were observed until day 43–46. Six vehicle controls and six positive controls given triorthocresyl phosphate (TOCP) at 600 mg/kg were also dosed on days 1 and 22 and observed.

There were no treatment-related deaths, clinical signs, gross necropsy observations, histopathological observations or differences in body weight gains. The positive control animals demonstrated neurotoxicity (disturbed balance, abnormal gait and ataxia), decreased body weights and neuropathological changes in the spinal cord and upper cervical bulbs.

Under the conditions of this study, fenpyroximate did not cause acute delayed neurotoxicity in the hen. The LOAEL for delayed neurotoxicity was not observed. The NOAEL was 5000 mg/kg or greater, the limit dose (Cummins, 1989).

(b) Immunotoxicity

In an immunotoxicity study, fenpyroximate (purity 99.8%) was administered to Crl:CD (SD) rats (10/sex per group) at concentrations of 0, 30, 100 and 300 ppm (equal to 0, 2.2, 7.1 and 18.4 mg/kg bw per day for males and 0, 2.6, 7.9 and 21.4 mg/kg bw per day for females) in the diet for 28 days. A concurrent positive control group (8 animals/sex) received 50 mg/kg bw cyclophosphamide by intraperitoneal injection on day 27. All animals received a single intravenous injection of sheep red blood cells (sRBC) (2×10^8 cells/mL in 0.9% saline) on day 25. All rats were killed on day 29. T-cell dependent antibody response (TDAR) was evaluated using an antibody plaque-forming cell (PFC) assay.

There were no deaths. No treatment-related clinical signs were observed during the four-week treatment period. During the first week of treatment, there was a marked reduction in body weight gain in males and females at 300 ppm. The reduction in overall (day 1–29) body weight gain was 32% and 31% of controls in males and females, respectively. Rats at 100 ppm had a more consistently lower weight gain than the controls, and by the end of the treatment period, their overall weight gain was 79% and 76% of controls for males and females, respectively. Similarly, feed consumption was also reduced in males and females at 100 and 300 ppm. Water consumption was reduced in males and females at 300 ppm and was also slightly lower than controls during weeks 3 and 4 by males receiving 100 ppm.

Absolute and relative to body spleen and thymus weights were unaffected by treatment. The macroscopic examinations did not reveal any treatment-related lesions.

There were no immunotoxicologically relevant effects on the humoral T-lymphocyte dependent response against sRBC antigen in any treatment group. Fenpyroximate treatment did not show statistically significant decreases in the number of spleen cells, the specific activity (PFC/10^6 spleen cells) or total activity (PFC/spleen). Animals in the positive control group showed marked decreases in the anti-sRBC PFC response. The positive control group confirmed the validity of the immunotoxicity assay. Natural killer cell activity was not evaluated in this study.

The NOAEL for systemic effects was 30 ppm (equal to 2.2 mg/kg bw per day) based on decreased water consumption in males and feed consumption in males and females, and decreased body weights in males and females at the LOAEL of 100 ppm (equal to 7.1 mg/kg bw per day).

The NOAEL for immunotoxicity was 300 ppm (equal to 18.4 mg/kg bw per day), the highest dose tested (Chambers, 2011).

(c) Studies on metabolites

Acute toxicity - oral

The results of acute toxicity studies with fenpyroximate metabolites are summarized in Table 18.

Table 18. Results of acute toxicity studies with fenpyroximate metabolites

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Metabolite M-1						
Rat	Sprague Dawley	Male Female	Oral	87.7	500–700 (male) 607 (female)	Takeda (1990a)
Metabolite M-12						
Rat	Sprague Dawley	Male Female	Oral	96.4	> 5000 (male and female)	Takeda (1990b)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose;

M-1: *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate;

M-12: *tert*-butyl (*E*)-4-[(3-methyl-5-phenoxypyrazole-4-yl) methylene-ammooxymethyl] benzoate

Acute toxicity - intravenous route

Acute intravenous toxicity of fenpyroximate and its *Z*-isomer (metabolite M-1) were comparatively investigated in eight-week-old female Crl:CD (SD) rats (5/dose per treatment). The intravenous route of administration was selected to avoid possible isomerization of the test articles, which can occur in acidic conditions. A single intravenous injection of fenpyroximate or *Z*-isomer in 10% (v/v) dimethylacetamide (DAMC) and 10% (v/v) Cremophor EL was administered. The test compounds were stable; there was no generation of their isomer in this vehicle at the concentration used. Based on the results of a range-finding study, fenpyroximate was administered at dose levels of 0.69, 0.98 or 1.40 mg/kg bw and the *Z*-isomer at dose levels of 1.37, 1.96 or 2.80 mg/kg bw. A vehicle control was included in the study. Animals were observed for mortality and clinical signs for seven days. Several parameters were evaluated: body weight and body weight gain, feed intake, haematology and blood biochemistry (on days 1 and 7), urine analysis (on days 6 and 7), necropsy and organ weights (on day 7).

Several animals died shortly after injection (within one hour) on day 0 and one animal given fenpyroximate at 0.69 mg/kg was euthanized on day 1 based on its deteriorated condition (diarrhoea, hypothermia, etc.). The resulting mortality is summarized in Table 19. The high mortality of animals injected with fenpyroximate is notable, and the LD_{50} was assumed to be below 0.69 mg/kg; the LD_{50} of M-1was assumed to be over 1.96 mg/kg. Thus, when their acute intravenous LD_{50} values were compared, M-1 is about three times less toxic than fenpyroximate.

Table 19. Mortalities in female rats treated intravenously with fenpyroximate or metabolite M-1

Mortality per dose level in mg/kg bw [a]																	
Fenpyroximate									M-1								
Dose range finding study					Main study				Dose range finding study					Main study			
0	0.25	0.5	1	2	0	0.69	0.98	1.40	0.25	0.5	1	2	4	0	1.37	1.96	2.80
0/3	0/3	0/3	1/3	2/3	0/5	3 [b]/5	5/5	4/5	0/3	0/3	0/3	0/3	3/3	0/5	0/5	2/5	4/5

bw: body weight

[a] Mortality expressed as no. of animals found dead / no. of animals treated.

[b] Includes one animal killed in extremis.

Source: Amanuma (2016)

Several end-points of systemic toxicity were commonly observed for both compounds. The liver is a target for toxicity common to both these test articles. Similar decreases in body weight and feed consumption, clinical signs of toxicity and changes in blood chemistry in both suggest similar toxicological profiles. Based on the results of this study, the rat intravenous LD_{50} values for fenpyroximate and M-1 were less than 0.69 and greater than 1.96 mg/kg bw, respectively. Therefore, M-1 is considered to be about three-fold less toxic than the parent compound, fenpyroximate (Amanuma, 2016).

Genotoxicity

The results of genotoxicity studies with fenpyroximate metabolites are summarized in Table 20.

Table 20. Overview of genotoxicity tests with fenpyroximate metabolites

End-point	Test object	Concentration	Purity (%)	Results	Reference
M-1					
In vitro					
Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2uvrA	0, 313, 625, 1250, 2500 and 5000 µg/plate in DMSO (± S9)	99.7	Negative	Watanabe (1990b)

End-point	Test object	Concentration	Purity (%)	Results	Reference
Mammalian cytogenetic test	Chinese hamster V79 lung cells	6 h −S9: 0–2.5 µg/mL 6 h+S9: 0–40 µg/mL 24 h −S9: 0–0.7 µg/mL Vehicle: DMSO Precipitation at 62.5 µg/mL	99.5	Negative	Kajiwara (2016b)
Mammalian cytogenetic assay	Mouse lymphoma L5178Y *tk*$^{+/-}$	3 h −S9: 0–30 µg/mL 3 h+S9: 0–70 µg/mL 24 h –S9: 0–1.5 µg/mL in DMSO	99.5	Negative	Kajiwara (2016c)
In vivo					
Mouse micronucleus	CD-1 male mice	0.5, 1.0 and 1.5 mg/kg bw iv in saline containing 2.5% dimethylacetamide and Cremophor EL (v/v)	99.8	Negative	Tsukushi (2016)
M-3					
In vitro					
Bacterial gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2uvrA	0, 156, 313, 625, 1250, 2500, 5000 µg/plate in DMSO (± S9)	97.9	Negative	Enomoto (2016)
Mammalian cytogenetic test	Chinese hamster V79 lung cells	6 h −S9: 0–275 µg/mL 6 h +S9: 0–150 µg/mL 24 h −S9: 0–150 µg/mL Vehicle: DMSO Precipitation at 500 µg/mL	97.9	Negative	Kajiwara (2016d)
Mammalian cytogenetic assay	Mouse lymphoma L5178Y *tk*$^{+/-}$	3 h −S9: 0–1000 µg/mL 3 h +S9: 0–1000 µg/mL 24 h –S9: 0–250 µg/mL Vehicle DMSO	97.9	Negative	Munechika (2016)
M-12					
In vitro					
Bacterial gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2uvrA	0, 313, 625, 1250, 2500, 5000 µg/plate in DMSO (± S9)	98.5	Negative	Watanabe (1989)

bw:body weight; DMSO: dimethyl sulfoxide; *tk*: thymidine kinase locus; v/v: volume per volume

S9: 9000 × *g* supernatant fraction from induced rats (metabolic activation); M-1: *tert*-butyl (*Z*)-4-[(1,3-dimethyl-5-phenoxypyrazole-4-yl) methylene-ammooxymethyl] benzoate;

M-3: *E*-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)-methyleneaminooxymethyl] benzoic acid;

M-12: *tert*-butyl (*E*)-4-[(3-methyl-5-phenoxypyrazole-4-yl) methylene-ammooxymethyl]benzoate;

3. Observations in humans

The results of medical surveillance of four male workers involved in the manufacture of a 5% suspension concentrate (SC) formulation of fenpyroximate from 1988 to 1993 were reported. The concentrations of fenpyroximate in the air at the workplace were analysed in 1991 and 1993. In January 1993, air samples from 10 locations (points) were analysed. The analytical values for all points, except point 10, were below 0.0080 mg/m^3. The concentration at point 10 was considerably higher, greater than 2 mg/m^3, but this had decreased to below 0.04 mg/m^3 in May 1993. The average exposure concentration of fenpyroximate during work hours, as determined in November 1991 and January 1993, ranged

from less than 0.0040 to 0.223 mg/m^3. No apparent changes in general health status, blood pressure, urine analysis, haematology, clinical chemistry or cholinesterase activity was detected in the four workers (Nokata, 1994).

In a separate medical surveillance study in 1994–1999, no clear change induced by fenpyroximate was observed in any of the workers handling fenpyroximate 5% SC in the manufacturing plant. Measurement of air samples found the concentration of fenpyroximate to be lower than the occupational exposure limit (0.070 mg/m^3) established voluntarily by Nihon Nohyaku Co. Ltd (Nishizawa & Nokata,v2000).

Eye and skin irritation were reported by some workers in charge of manufacturing fenpyroximate technical grade or fenpyroximate 5% SC in the manufacturing plant and by some farmers using fenpyroximate 5% SC in citrus fields (Nokata, 1992).

A report conducted in 1993 indicated that skin and eye irritation was not observed in workers wearing personal protective clothing and glasses while working with fenpyroximate or 5% SC formulation. The occurrence of eye irritation in farmers applying fenpyroximate 5% SC in 1993 decreased remarkably in comparison with occurrence in 1991 and 1992, after the manufacturer recommended the use of glasses or goggles during spraying operations and requested that farmers avoid excessive exposure to fenpyroximate by spraying on the windward and keeping to the recommended spraying concentration and volume of fenpyroximate (Sano & Nokata, 1993).

Comments

Biochemical aspects

Based on biliary excretion studies using [pyrazole-^{14}C]fenpyroximate and [benzyl-^{14}C]fenpyroximate (2 mg/kg bw) in rats, oral absorption of fenpyroximate was about 60% of the administered dose considering bile excretion (47–55%) and urinary excretion (5–10%) within 48 hours. Oral administration of single or repeated doses of 2 mg/kg bw and 400 mg/kg bw dose of either [pyrazole-^{14}C]fenpyroximate or [benzyl-^{14}C]fenpyroximate resulted in approximately 70–92% of the applied dose being excreted via faeces and approximately 9.0–18% via urine in male and female rats. In the low-dose group (2 mg/kg bw), radioactivity in blood increased slowly with time to reach T_{max} values of about 11.0 hours after applying the pyrazole-labelled compound and about 7.0 hours after applying the benzyl-labelled compound. Radioactivity declined with half-lives between 6.1 and 8.9 hours for both labelled compounds. The mean concentration of radioactivity had decreased to or below the LOD after 72 hours for pyrazole-labelled fenpyroximate and 48 hours for benzyl-labelled fenpyroximate.

At 400 mg/kg bw, radioactivity increased slowly with a T_{max} value of about 90.0 hours after administration of pyrazole-labelled fenpyroximate or 29.0 hours for males and 86.0 hours for females after administration of benzyl-labelled fenpyroximate. Radioactivity declined with a half-life of between 35.4 and 48.7 hours for both labels. The mean concentration of radioactivity had decreased to or below the LOD after 216 hours and 168 hours for the pyrazole-labelled fenpyroximate and for the benzyl-labelled fenpyroximate, respectively.

The high number of identified fenpyroximate metabolites indicates that fenpyroximate was extensively metabolized by, for example, hydrolytic cleavage of the oxime bond, hydrolysis of the *tert*-butyl ester moiety, oxidation of the *tert*-butyl group, hydroxylation of the phenoxy ring and 3-methyl group, by isomerization, *N*-demethylation and conjugation.

The major urinary metabolites were M-8 and M-21; they were also excreted via faeces but not in large amounts. Fenpyroximate represents the major compound in faeces;
M-3 ((*E*)-4- [(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid) and
M-22 ((*E*)-2-[4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoyloxy]-2-methylpropanoic acid) were also excreted in faeces; metabolites of minor abundance were the *Z*-isomer of fenpyroximate (metabolite M-1; *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate), M-6 and M-11. There were no changes in the metabolic profile of [pyrazole-^{14}C]fenpyroximate or [benzyl-^{14}C]fenpyroximate upon repeated dosing or after applying the low or the high dose.

Toxicological data

In rats, the acute oral LD_{50} was 480 and 245 mg/kg bw for males and females, respectively, the acute dermal LD_{50} was greater than 2000 mg/kg bw (Blaszcak, 1989b,c) and the acute inhalation LC_{50} was 0.21 and 0.33 mg/L for male and female, respectively (Hoffman, 1991a). Fenpyroximate was not irritating to the skin of rabbits and mildly irritating to the eyes of rabbits (Kosaka, 1988a,b). It was sensitizing to the skin of guinea-pigs, as determined by the Magnusson-Kligman test (Kosaka, 1988c), and negative for skin sensitization in the Buehler test (Teale, 1990a,b). Fenpyroximate was not phototoxic (Valin, 2016).

In repeated-dose toxicity studies, diarrhoea and decreased body weights in dogs, and decreased in body weights and liver effects in rats were the key findings.

In a 13-week toxicity study in rats using dietary fenpyroximate concentrations of 0, 20, 100 or 500 ppm (equal to 0, 1.3, 6.57 and 35.2 mg/kg bw per day for males and 0, 1.65, 8.29 and 38.6 mg/kg bw per day for females, respectively), the NOAEL was 20 ppm (equal to 1.3 mg/kg bw per day) based on decreased body weight and body weight gains, reduced feed consumption and minimal hepatocytic hypertrophy in both sexes at 100 ppm (equal to 6.57 mg/kg bw per day (Aughton, 1987).

In a single-dose oral toxicity study in dogs, fenpyroximate was administered as a bolus gavage dose of 0, 2 or 5 mg/kg bw. As diarrhoea was seen at all dose levels, the NOAEL could not be determined (Harvey, 2006a).

In a dose escalation oral toxicity study in the same dogs, fenpyroximate was administered via bolus gavage dose of 2, 5, 20 and 25 mg/kg bw. The NOAEL was 2 mg/kg bw per day based on diarrhoea seen at 5 mg/kg bw per day (Harvey, 2006b).

In a 90-day oral toxicity study in dogs using oral (capsule) fenpyroximate doses of 0, 2, 10 or 50 mg/kg bw per day, the LOAEL was 2 mg/kg bw per day, the lowest dose tested, based on slight bradycardia and an increased incidence of diarrhoea in both sexes (weekly observations) and reduced feed consumption, body weight and body weight gain and increased emaciation and torpor in females only (Broadmeadow, 1988).

In a one-year oral toxicity study in dogs using oral (capsule) fenpyroximate doses of 0, 0.5, 1.5, 5.0 or 15.0 mg/kg bw per day, the NOAEL was 1.5 mg/kg bw per day based on increased incidences of diarrhoea at 5 mg/kg bw per (Broadmeadow, 1989).

In a 78-week carcinogenicity study in mice using dietary fenpyroximate concentrations of 0, 25, 100, 400 or 800 ppm (equal to 0, 2.43, 9.47, 38.0 and 69.6 mg/kg bw per day for males and 0, 2.46, 10.2, 41.5 and 73.1 mg/kg bw per day for females, respectively), the NOAEL was 25 ppm (equal to 2.43 mg/kg bw per day) based on decreased body weights and feed consumption at 100 ppm (equal to 9.47 mg/kg bw per day). There were no treatment-related neoplastic findings (Shirasu, 1989).

In a two-year chronic toxicity and carcinogenicity study in rats using dietary fenpyroximate concentrations of 0, 10, 25, 75 or 150 ppm (equal to 0, 0.4, 0.97, 3.00 and 6.20 mg/kg bw per day for males and 0, 0.49, 1.21, 3.18 and 8.01 mg/kg bw per day for females, respectively), the NOAEL was 25 ppm (equal to 0.97 mg/kg bw per day) based on decreased body weights, feed consumption and feed conversion ($P < 0.01$) and decreased plasma total protein at 75 ppm (equal to 3.0 mg/kg bw per day). There were no neoplastic findings that were related to treatment (Aughton, 1989).

The Meeting concluded that fenpyroximate is not carcinogenic in mice or rats.

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

The Meeting concluded that fenpyroximate 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 fenpyroximate is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats using dietary fenpyroximate

concentrations of 0, 10, 30 or 100 ppm (equal to 0, 0.67, 1.99 and 6.59 mg/kg bw per day for males and 0, 0.83, 2.44 and 8.60 mg/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 100 ppm (equal to 6.59 mg/kg bw per day). The NOAEL for parental toxicity was 30 ppm (equal to 1.99 mg/kg bw per day) based on decreased body weights, decreased feed consumption and increased absolute testicular and epididymal weights (absolute and relative to body weight) in males at 100 ppm (equal to 6.59 mg/kg bw per day). The NOAEL for offspring toxicity was 30 ppm (equal to 1.99 mg/kg bw per day) based on decreased pup body weight at 100 ppm (equal to 6.59 mg/kg bw per day) (Higgins, 1989a).

In a developmental toxicity study in rats using oral gavage fenpyroximate doses of 0, 1.0, 5.0 or 25 mg/kg bw per day, the NOAEL for maternal toxicity was 25 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 5 mg/kg bw per day, based on increases in the number of thoracic ribs at 25 mg/kg bw per day (Higgins, 1989b).

In a developmental toxicity study in rabbits using oral gavage fenpyroximate doses of 0, 1.0, 2.5 or 5 mg/kg bw per day, the NOAEL for maternal toxicity was 2.5 mg/kg bw per day, based on reduced body weights, feed and water consumption and faecal output at 5 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 2.5 mg/kg bw per day, based on elevations in the frequency of unilateral and bilateral slightly folded retinas (variations) at 5 mg/kg bw per day (King, 1989).

The Meeting concluded that fenpyroximate is not teratogenic.

In an acute neurotoxicity study in rats administered a single oral gavage dose of fenpyroximate at 0, 37.5, 150 or 300 mg/kg bw, the NOAEL for general toxicity was 37.5 mg/kg bw based on decreased motor activity (total activity counts and total time spent in movement) in both sexes and a reduction in auditory startle response in females at 24 hours post dose, and mild dehydration in males, reduced feed consumptions (about 60%) and statistically significant decreased in body weights and body weight gains in first three days in males and females, at 150 mg/kg bw. The marginal decreases in motor activity and reduction in auditory response were considered to be effects secondary to the excessive general toxicity rather than neurotoxic effects. The Meeting noted that the 150 mg/kg bw effect level in this study is approximately 65% of the LD_{50} value for fenpyroximate in females (Barnett, 2011a).

In a 90-day study of neurotoxicity in rats given diets containing fenpyroximate at a concentration of 0, 30, 100 or 300 ppm (equal to 0, 1.8, 6.1 and 16.4 mg/kg bw per day for males and 0, 2.0, 6.6 and 18.4 mg/kg bw per day for females, respectively), the NOAEL for general toxicity was 30 ppm (equal to 1.8 mg/kg bw per day) based on clinical signs consisting of an increased incidence of chromorhinorrhea and dehydration in both sexes at 100 ppm (equal to 6.1 mg/kg bw per day). The NOAEL for neurotoxicity was 300 ppm (equal to 16.4 mg/kg bw per day), the highest dose tested (Barnett, 2011b).

No evidence of delayed neurotoxicity was seen in an acute delayed neurotoxicity study in hens at doses up to 5000 mg/kg bw (Cummins, 1989).

The Meeting concluded that fenpyroximate is not neurotoxic.

No evidence of immunotoxicity was observed in an immunotoxicity study in male and female rats administered fenpyroximate in the diet at a dose level of 0, 30, 100 or 300 ppm (equal to 0, 2.2, 7.1 and 18.4 mg/kg bw per day for males and 0, 2.6, 7.9 and 21.4 mg/kg bw per day for females, respectively) for 28 days (Chambers, 2011).

The Meeting concluded that fenpyroximate is not immunotoxic

Toxicological data on metabolites and/or degradates

The acute oral LD_{50} value for M-1 metabolite (animal and plant metabolite; *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate) in rats was 500–700 mg/kg bw for males and 607 mg/kg bw for females (Takeda, 1990a). Metabolite M-1 was negative for mutagenicity in the bacterial gene mutation assay (Watanabe, 1990b), mammalian cytogenetic assay in Chinese

hamster V79 lung cells (Kajiwara, 2016b), mammalian cytogenetic assay using mouse lymphoma cells (Kajiwara, 2016c) and in vivo micronucleus in mice (Tsukushi, 2016).

The acute oral LD_{50} value for animal and plant metabolite M-12 was greater than 5000 mg/kg bw in male rats (Takeda, 1990b). M-12 was negative for mutagenicity in the Ames test (Watanabe, 1989).

The animal and plant metabolite M-3 was negative for mutagenicity in the bacterial gene mutation assay (Enomoto, 2016), mammalian cytogenetic assay in Chinese hamster V79 lung cells (Kajiwara, 2016d) and mammalian cytogenetic assay using mouse lymphoma cells (Munechika, 2016).

The major goat metabolite Fen-OH (2-hydroxymethyl-2-propyl(*E*)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)-methylenaminooxymethyl]benzoate) was not found in rats. However, it is plausible that Fen-OH occurs as an unstable intermediate metabolite in the rat when the parent is oxidized at its *t*-butyl moiety to give M-22. No toxicological data were available on Fen-OH but an additional hydroxy group usually renders a molecule more likely to undergo further phase II metabolism and be more rapidly excreted.

The Meeting concluded the toxicity of plant and livestock metabolites M-1, M-3, M-5, M-21, M-22 and Fen-OH would be covered under the toxicity of the parent compound since these metabolites were also detected in rats at significant levels.

Human data

No adverse health effects were reported in workers in fenpyroximate-manufacturing plants (Nokata, 1994; Nishizawa & Nokata, 2000). Eye and skin irritation was reported in some workers in charge of manufacturing fenpyroximate or its 5% soluble concentrate formulation and also in some farm workers handling 5% soluble concentrate formulation (Sano & Nokata, 1992).

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

Toxicological evaluation

The Meeting withdrew the existing ARfD and established an ARfD of 0.01 mg/kg bw on the basis of the LOAEL of 2 mg/kg bw for the induction of diarrhoea seen in a newly submitted single bolus gavage study and a 13-week study of toxicity in dogs. A safety factor of 200 was used since no NOAEL was identified. It was unclear whether the diarrhoea was the result of a direct irritant or pharmacological effect of fenpyroximate; however, histopathological examination of gastrointestinal tract did not reveal any evidence of irritation in the available database.

This ADI and ARfD can be applied to M-1, M-3, M-5, M-21, M-22 and Fen-OH.

Levels relevant to risk assessment of fenpyroximate

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a]	Toxicity	25 ppm, equal to 2.43 mg/kg bw per day	100 ppm, equal to 9.47 mg/kg bw per day
		Carcinogenicity	800 ppm, equal to 69.63 mg/kg bw per day [b]	–
Rat	Acute neurotoxicity study [c]	Neurotoxicity	300 mg/kg bw per day [b]	–
	Ninety-day neurotoxicity study [a]	Neurotoxicity	300 ppm, equal to 16.4 mg/kg bw per day [b]	–
	Two-year studies of toxicity and carcinogenicity [a,d]	Toxicity	25 ppm, equal to 0.97 mg/kg bw per day	75 ppm, equal to 3.0 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 6.20 mg/kg bw per day [b]	–
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	100 ppm, equal to 6.59 mg/kg bw per day [b]	–
		Parental toxicity	30 ppm, equal to 1.99 mg/kg bw per day	100 ppm, equal to 6.59 mg/kg bw per day
		Offspring toxicity	30 ppm, equal to 1.99 mg/kg bw per day	100 ppm, equal to 6.59 mg/kg bw per day
	Developmental toxicity study [c]	Maternal toxicity	25 mg/kg bw per day [b]	–
		Embryo/fetal toxicity	5 mg/kg bw per day	25 mg/kg bw per day
Rabbit	Developmental toxicity study [c]		2.5 mg/kg bw per day	5 mg/kg bw per day
			2.5 mg/kg bw per day	5 mg/kg bw per day
Dog	Single dose, escalating dose and 13-week study [c,d,e]	Toxicity	–	2 mg/kg bw per day

[a] Dietary administration.

[b] Highest dose tested.

[c] Gavage administration.

[d] Two or more studies combined.

[e] Capsule administration.

Estimate of acceptable daily intake (ADI; applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22 and Fen-OH, expressed as fenpyroximate)

0–0.01 mg/kg bw

Estimate of acute reference dose (ARfD; applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22 and FEN-OH, expressed as fenpyroximate)

0.01 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 fenpyroximate

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Approximately 60%, based on excretion via bile (47–55%) and urine (5–10%) within 48 hours; T_{max} about 11 and 90 h at 2 and 400 mg/kg bw, respectively
Dermal absorption	No data
Distribution	Widely distributed, the highest amounts of radioactivity were found in the gastrointestinal tract, liver, fat and kidney; 168 h after administration tissue residues below LOD
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid and complete within 48 h at 2 mg/kg bw, mainly in bile
Metabolism in animals	Extensive; cleavage of the benzyl moiety from the pyrazole ring with further oxidation, oxime hydrolysis, ester hydrolysis; other pathways of metabolism were 4-hydroxylation of the 5-phenoxy ring and oxidation of the *t*-butyl moiety
Toxicologically significant compounds in animals and plants	Parent and M-1
Acute toxicity	
Rat, LD_{50}, oral	480 and 245 mg/kg bw for male and female, respectively
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	0.21 and 0.33 mg/L male and female, respectively
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Mildly irritating
Guinea pigs, dermal sensitization	Sensitizing (Magnusson-Kligman) and non-sensitizing (Buehler)
Short-term studies of toxicity	
Target/critical effect	Reduced feed intake, decreased body weight gain (rat); diarrhoea, salivation, emesis, bradycardia (dogs)
Lowest relevant oral NOAEL	1.3 mg/kg bw per day (rat); 1.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	300 mg/kg bw per day (rat) [b]
Lowest relevant inhalation NOAEC	2.0 mg/m^3 (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Lower body weight gain, feed intake and feed conversion efficiency; decreased total protein
Lowest relevant NOAEL	0.97 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice or rats [a]
Genotoxicity	No evidence of genotoxicity [a]
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	1.99 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	1.99 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	6.59 mg/kg bw per day (rat) [b]

(*Continued on next page***)**

Developmental toxicity	
Target/critical effect	Increased incidence of slightly folded retinas in rabbits
Lowest relevant maternal NOAEL	2.5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	5.0 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	300 mg/kg bw (rat)[b]
Subchronic neurotoxicity NOAEL	16.4 mg/kg bw per day (rat)[b]
Delayed neurotoxicity NOAEL	5000 mg/kg bw (hen)[b]
Other toxicological studies	
Immunotoxicity	18.4 mg/kg bw per day (rat)
Studies on toxicologically relevant metabolites	
M-1	Oral LD_{50}: 500 and 607 mg/kg bw for males and females, respectively (rats)
	Ames test, mammalian cytogenetic assay in Chinese Hamster lung cells, mouse lymphoma assay and micronucleus test: negative
M-3	Bacterial gene mutation assay, mammalian cytogenetic assay in Chinese hamster lung cells and mouse lymphoma
M-12	Oral LD_{50}: > 5000 mg/kg bw for male rats
	Negative for mutagenicity in the bacterial gene mutation assay
Human data	Eye and skin irritation reported in workers at manufacturing plants and agricultural workers

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

[b] Highest dose tested.

Summary

	Value	Study	Safety factor
ADI[a]	0–0.01 mg/kg bw	104-study of toxicity (rats)	100
ARfD[a]	0.01 mg/kg bw	Single dose toxicity and 13-week study of toxicity (dog)	200

[a] Applies to fenpyroximate and to M-1, M-3, M-5, M-21, M-22 and Fen-OH, expressed as fenpyroximate

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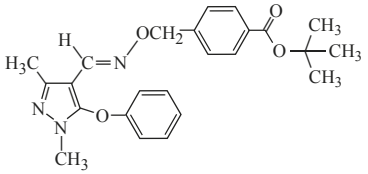
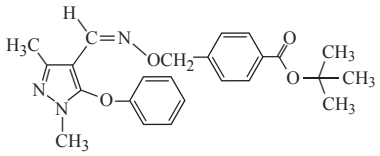
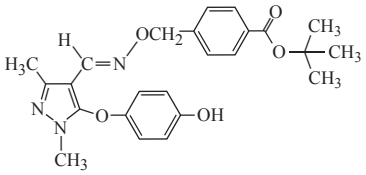
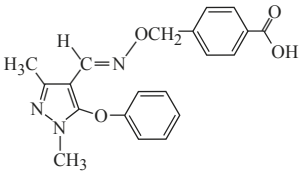
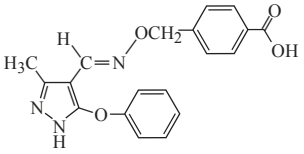
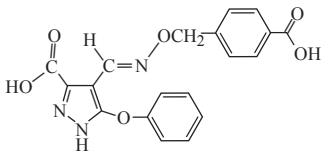
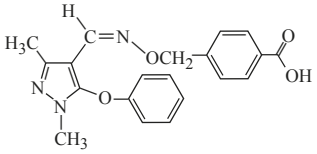
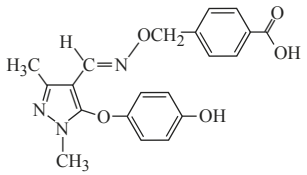
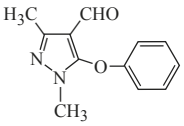
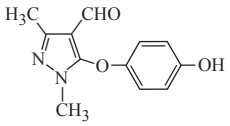
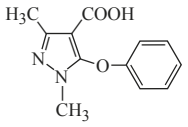
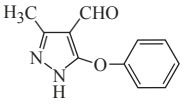
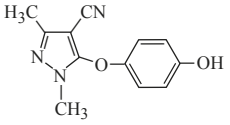
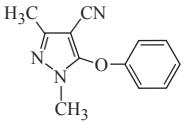
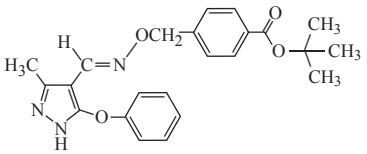
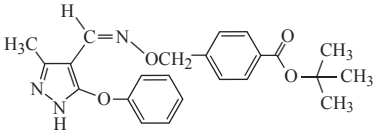
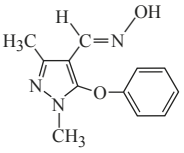
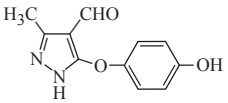
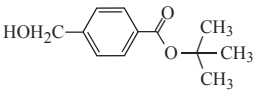
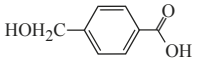
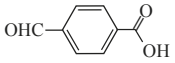
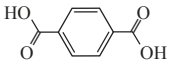
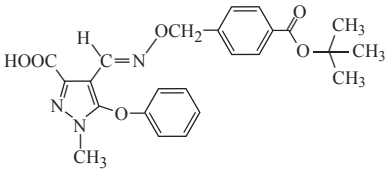
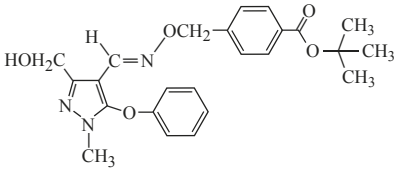
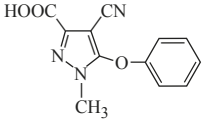
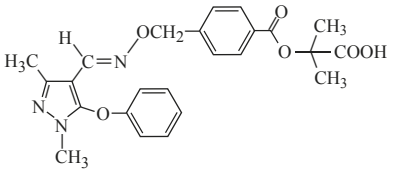
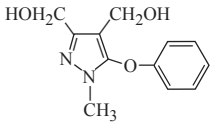
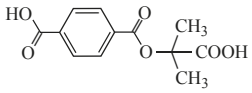
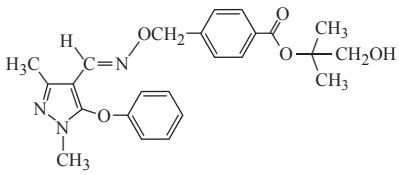
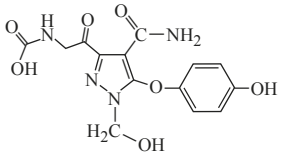
Appendix 1: Substance and metabolites; structures codes, synonyms

Name/Code no. (Synonyms)	Description	Compound found in:	Structure
Fenpyroximate (*E* isomer)	IUPAC: *tert*-butyl (*E*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate CAS: 1,1-dimethylethyl (*E*)-4-[[[[(1,3-dimethyl-5-phenoxy-1*H*-pyrazol-4-yl)methylene]amino]oxy]methyl]benzoate	NA	
M-1 (*Z*-isomer)	IUPAC: *tert*-butyl (*Z*)-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneamino-oxy)-*p*-toluate CAS: 1,1-dimethylethyl (*Z*)-4-[[[[(1,3-dimethyl-5-phenoxy-1*H*-pyrazol-4-yl)methylene]amino]oxy]methyl]benzoate	Soil photolysis, aqueous photolysis, surface water, citrus leaves, citrus rind, apple leaves, apple fruit, grape leaves, grape fruit, snap beans, Swiss chard leaves, goat, rotated crops, rat	
M-2	*tert*-butyl (*E*)-4-{[1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]-methyleneaminooxymethyl}benzoate	Citrus leaves, rat	
M-3	(*E*)-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid	Aerobic soil, anaerobic soil, groundwater, surface water, sediment, apple leaves, goat, rotated crops, rat	
N-desmethyl M-3	(*E*)-4-[(3-methyl-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid	Goat	
N-desmethyl M-3 acid	(*E*)-4-[(3-carboxy-5-phenoxypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid	Goat	
M-4	(*Z*)-4-[(1,3-dimethyl-5-phenocypyrazol-4-yl)methyleneaminooxymethyl]benzoic acid		
M-5	(*E*)-4-{[(1,3-dimethyl-5-(4-hydroxyphenoxy)pyrazol-4-yl]methyleneaminooxymethyl}benzoic acid	Goat (also present as phenoxy glucuronide), rotated crops, rat	

Name/Code no. (Synonyms)	Description	Compound found in:	Structure
M-6	1,3-dimethyl-5-phenoxypyrzole-4-carbaldehyde	Apple leaves, grape leaves, rat	
M-7	1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde		
M-8	1,3-dimethyl-5-phenoxypyrazole-4-carboxylic acid	Aerobic soil, anaerobic soil, surface water, sediment, apple leaves, grape leaves, rotated crops, rat	
M-9	3-methyl-5-phenoxypyrzole-4-carbaldehyde	Citrus leaves, apple leaves, grape leaves, rat	
M-10	1,3-dimethyl-5-(4-hydroxyphenoxy)pyrzole-4-carbonitrile	Rat	
M-11	1,3-dimethyl-5-phenoxypyrazole-4-carbonitrile	Aerobic soil, anaerobic soil, aqueous photolysis, surface water, sediment, apple leaves, grape leaves, rat	
M-12	*tert*-butyl (*E*)-4-[(3-methyl-5-phenoxypyrazol-4-yl) methyleneaminooxymethyl] benzoate	Soil photolysis, citrus leaves, citrus rind, apple leaves, grape leaves, rotated crops, rat	
M-12 isomer	*tert*-butyl (*Z*)-4-[(3-methyl-5-phenoxypyrazol-4-yl) methyleneaminooxymethyl] benzoate	Soil photolysis	
M-13	(*E*)-1,3-dimethyl-5-phenoxypyrazole-4-carbaldehyde oxime	Rat, citrus leaves	
M-14	3-methyl-5-(4-hydroxyphenoxy)pyrzole-4-carbaldehyde	Rat	
M-15	*tert*-butyl 4-hydroxymethylbenzoate	Citrus leaves, rat	
M-16	4-hydroxymethylbenzoic acid	Rat	

Name/Code no. (Synonyms)	Description	Compound found in:	Structure
M-17	4-formylbenzoic acid	Citrus leaves, citrus rind, rat	
M-18	Terephthalic acid	Rat	
M-19	(*E)*-4-[(4-*tert*-butoxycarbonylphenyl) methyloxyiminomethyl]-1-methyl-5-phenoxypyrazole-3-carboxylic acid	Citrus leaves, apple leaves, grape leaves	
M-20	*tert*-butyl (*E)*-4-[(3-hydroxymethyl-1-methyl-5-phenoxypyrazol-4-yl) methyleneaminooxymethyl] benzoate	Citrus leaves, apple leaves, grape leaves	
M-21	4-cyano-1-methyl-5-phenoxypyrazole-3-carboxylic acid	Goat, rat	
M-22	(*E*)-2-[4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl) methyleneaminooxymethyl] benzoyloxy]-2-methylpropanoic acid	Goat, rat	
M-24	3, 4-di(hydroxymethyl)-1-methyl-5-phenoxy-1*H*-pyrazole	Apple leaves	
MTBT	Mono-(*tert*-butyl) terephthalate	Soil photolysis, surface water, sediment	
Fen-OH	2-hydroxymethyl-2-propyl (*E)*-4-[(1,3-dimethyl-5-phenoxypyrazol-4-yl)-methylenaminooxymethyl] benzoate	Goat	
Metabolite 2	2-(5-(4-hydroxyphenoxy)-1-(hydroxymethyl)-1*H*-pyrazole-4-carbamoyl-3-carbonylamino)acetic acid	Goat	

FOSETYL-ALUMINIUM

First draft prepared by
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Explanation

Fosetyl-aluminium (fosetyl-Al) is the International Organization for Standardization–approved common name for aluminium ethyl hydrogen phosphonate, with the Chemical Abstracts Service number 39148-24-8. Fosetyl-Al belongs to a group of the phosphonate class of compounds used as fungicides and bactericides. Phosphonates disrupt phosphorus metabolism and interrupt the synthesis of complex molecules and specific enzymes by the targeted pathogen. Fosetyl-Al is used as a protectant on a variety of crops including vines, fruits (citrus, pineapples, avocados, berries, stone and pome fruit), vegetables and salads and hops.

Fosetyl-Al has not been previously 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) or Organisation for Economic Co-operation and Development (OECD) test guidelines or other validated guidelines because the data were generated before the implementation of these guidelines. The studies were considered adequate for the evaluation.

Evaluation for accepTable daily intake

1. Biochemical aspects

Structures of the parent compound, metabolites and degradates of fosetyl-Al are shown in Table 1, and a schematic of the metabolism of fosetyl-Al in animals is shown in Fig. 1. The absorption, distribution, metabolism and excretion (ADME) of fosetyl-Al by Sprague Dawley rats has been investigated using [^{14}C]ethyl and [^{32}P]phosphonate radiolabels (Fig. 2). The ADME properties of the metabolite phosphonic acid, which is formed in plants and the environment (water and soil), have also been investigated.

In rats, orally administered [^{14}C]fosetyl-Al is essentially completely absorbed, widely distributed, extensively metabolized and then rapidly excreted. The metabolic steps involve dissociation of fosetylAl and hydrolysis to phosphonate and ethanol. The ethanol is oxidized via acetaldehyde and acetate to CO_2 and then excreted with expired air. The phosphonate is partly excreted (along with some unchanged fosetyl) with the urine. The major portion of phosphonate is excreted with the faeces. Orally administered phosphonate is partly (approximately 10–30%) oxidized to phosphate before it is excreted in faeces (Unsworth, 1978). A small proportion of the administered radioactivity associated with the two-carbon metabolites (ethanol, acetate) would have been available for introduction into normal anabolic processes and thus account for the appearance of radioactivity in endogenous molecules.

Only one of the ADME studies of fosetyl-Al was performed according to GLP (Cameron, 2001).

Figure 1. Metabolic pathway of fosetyl-Al in animals

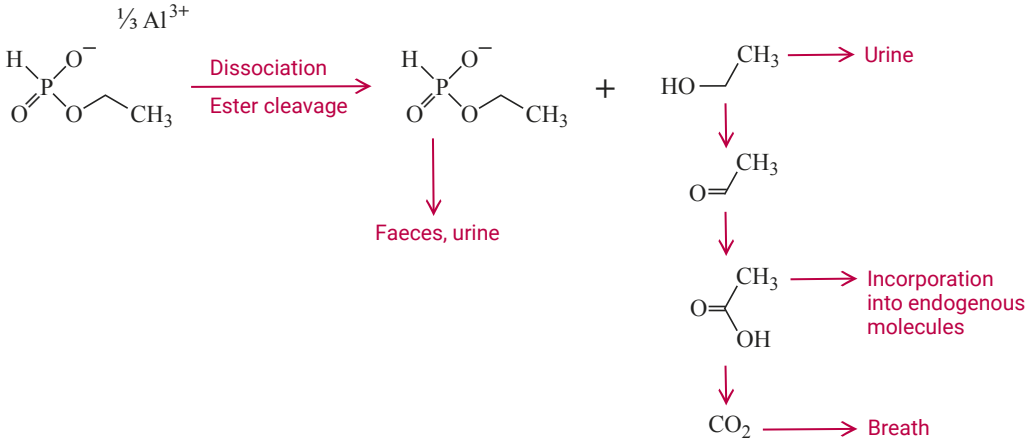
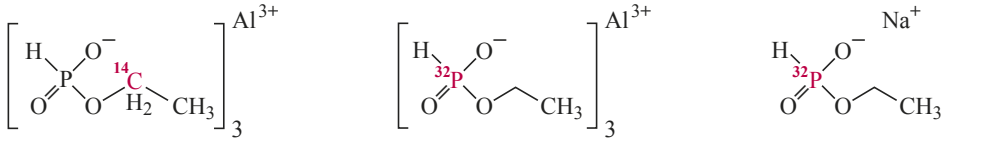
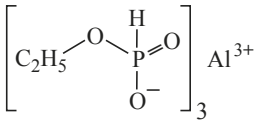
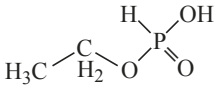
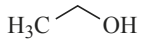
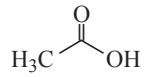
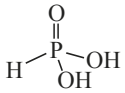


Figure 2. Positions of radiolabelling in ADME studies



^{14}C-Fosetyl-Al ^{32}P-Fosetyl-Al Sodium ^{32}P-phosphonate

Table 1. Structures of the parent compound and metabolites and degradates of fosetyl-Al in various crops and animals

Common name	Chemical name	Structure
Fosetyl-aluminium	Ethyl hydrogen phosphonate, aluminium salt	
Fosetyl (free acid after dissociation of the test substance)	*O*-ethyl phosphonic acid	
Ethanol (metabolite in rats, large animals and plants)	Ethyl alcohol, ethanol	
Acetic acid (metabolite in rats, large animals and plants)	Acetic acid	
Carbon dioxide (metabolite in rats, large animals)	Carbon dioxide	
Phosphonic acid (metabolite in rats, large animals and plants)	Phosphonic acid	

1.1 Absorption, distribution and excretion

The absorption, distribution and excretion of fosetyl-Al were studied in three separate toxicokinetic studies. All three followed separate protocols and are briefly described below. Summary information is presented in Tables 2 and 3.

In a toxicokinetic study, Cameron (2001) administered a single oral dose of [^{14}C]fosetyl-Al (batch no. CFQ 11651, specific activity 5.96 mBq/mg [57 mCi/mmol], radiopurity 99%) at a target dose level of 100 mg/kg body weight (bw) or 1000 mg/kg bw to Sprague Dawley rats (n = 4/sex per dose level). Urine, faeces and expired air were collected at 24-hour intervals up to 168 hours post dose. The animals were killed at 168 hours post dose, and selected organs, tissues and body fluids were retained for subsequent analysis.

In the rats administered 100 mg/kg bw, radioactivity was rapidly excreted with 71.2% and 71.9% of the administered dose recovered in 24 hours and total mean recoveries of 92.2% and 91.9% for male and female animals, respectively. The predominant route of excretion was via expired air (53.8% and 53.2% for male and female animals, respectively). Urinary and faecal excretion was lower, with mean recoveries of 25.9% and 27.2% in the urine of male and female animals and of 4.5% and 3.8% in faeces of male and female animals, respectively. The distribution of radioactivity was similar between male and female animals. The highest mean concentration of total radioactivity at 168 hours was in the renal fat and the adrenal gland. Lesser amounts were observed in the kidneys, skin and fat. The lowest mean concentrations were in plasma.

In the rats administered 1000 mg/kg bw, radioactivity was rapidly excreted with mean recoveries of 90.5% and 93.3% for male and female animals, respectively. The predominant route of excretion was via expired air (49.0% and 51.2% for male and female rats, respectively). Urinary excretion accounted for 25.4% and 29.1% of the dose, and faecal excretion accounted for 8.11% and 5.04% of the dose in male and female animals, respectively. The distribution of radioactivity was similar for male and female animals, and tissue levels were approximately 10-fold higher than in those animals treated with 100 mg/kg bw. A largely similar pattern of accumulation in tissues was observed as in animals treated with 100 mg/kg bw per day (renal fat, adrenal gland, skin, fat, ovaries, uterus, kidneys and thyroid), with lowest levels in plasma (Cameron, 2001).

In a toxicokinetic study, Savage (1982) administered a single oral dose of [^{14}C]fosetyl-Al (batch no. KWC 1053, specific activity 4.85 × 10^{8} Bq/mmol [13.1 mCi/mmol], radiopurity 98%) at 3000 mg/kg bw to Sprague Dawley rats (n = 5/sex). Urine and faeces were collected 24 hours after

dosing and at 24-hour intervals thereafter. Blood samples were collected from a tail vein approximately 0.25, 0.5, 1, 2, 4, 6 and 24 hours after dosing and at 24-hour increments thereafter until scheduled kill. Ethanol and carbon dioxide traps were renewed at 24-hour intervals up to 144 hours. Animals were killed seven days after dosing.

The primary routes of ^{14}C elimination were urine (35–57% of the dose) and exhaled air (59% of the dose). Faecal elimination contained 2–3% of the dose, and 3–4% of the dose remained in the tissues at the scheduled kill. The female rats consistently eliminated almost twice as much of the dose by the faecal route than the male animals (females 3.3%, males 1.9%). A single radiolabelled component was detected in urine; this was determined to be fosetyl-Al. Also detected was ^{14}C-labelled ethanol and this was quantified in 24-hour male and female urine samples.

Based on the results of the blood sampling, it appeared that absorption was essentially complete 10 hours after dosing. The mean elimination half-life was 184 and 129 hours for males and females, respectively. The mean elimination rate constant was 0.0042 and 0.0054 for males and females, respectively.

Tissues obtained at scheduled kill showed a generalized distribution of radioactivity. No single tissue contained less than 32 µg/g. Neither the parent compound nor possible metabolites were detected.

The data suggest that fosetyl-Al is almost completely absorbed and undergoes extensive metabolic transformation, the major end-product being carbon dioxide. The presence of labelled ethanol in the urine supports a metabolic route including acetaldehyde and acetate originating from the ethyl moiety of fosetyl-Al (Savage, 1982).

In a toxicokinetic study in rats, Unsworth (1976a) administered [^{14}C]fosetyl-Al (batch no. KWC 461, specific activity 5.0×10^8 Bq/mmol [13.48 mCi/mmol]) to Sprague Dawley rats (n = 3/sex) in seven daily oral doses. Urine, faeces and exhaled air were collected daily until 24 hours after the final dose. Tissue samples were assayed for radioactivity.

Each dose of [^{14}C]fosetyl-Al was extensively metabolized and excreted within 24 hours of administration. Radioactivity was excreted in expired air (60%), urine (26–27%) and faeces (2–3%). The carcass, skin and fur, and intestinal tract accounted for 3–5%, 2–3% and 1% of radioactivity, respectively (Table 2). Similar to the other toxicokinetic studies, the levels of radioactivity in the bodies of exposed rats were likely due to the incorporation of the radiolabel into naturally occurring molecules through biosynthetic pathways.

Table 2. Per cent recovery of radioactivity after administration of 14C-labelled fosetyl-Al in repeated-dose or single-dose studies

Tissue	% radioactivity							
	Repeated dose		Single dose					
	250 mg/kg bw per day; 7 days [a]		100 mg/kg bw [b]		1000 mg/kg bw [b]		3000 mg/kg bw [c]	
	Male	Female	Male	Female	Male	Female	Male	Female
Urine	26.4	27.3	25.9	27.2	25.4	29.1	37.4	34.5
Faeces	2.86	1.78	4.46	3.80	8.11	5.04	1.85	3.3
Cage wash	0.23	0.21	0.47	0.76	1.22	1.08	0.29	0.21
Tissues	4.95	3.96	0.97	0.90	0.90	1.01	0.99	1.17
Carcass	4.52	2.91	6.00	5.5	5.28	4.87	3.3	2.96
Carbon dioxide	59.8	60.0	53.8	53.2	49.0	51.7	59.1	57.9
Ethanol	0.02	0.03	NA	NA	NA	NA	0.23	0.05
GIT and contents	NA	NA	0.52	0.53	0.63	0.59	NA	NA
Total recovery	98.8	96.2	92.2	91.9	90.5	93.3	103.2	100.1

bw: body weight; NA: not analysed GIT: gastrointestinal tract

[a] Unsworth (1976a); [b] Cameron (2001); [c] Savage (1982).

In one fosetyl-Al toxicokinetic study radiolabelled phosphorus was used. In this study, Unsworth (1977a) administered [^{32}P]fosetyl-Al (batch no. KWC 565, specific activity 2.2 × 10^8 Bq/mmol [5.9 mCi/mmol]; purity 92%) orally to Sprague Dawley rats (n = 3/sex) for seven days as single doses of 111 mg/kg bw per day.

Urine and faeces were collected every 24 hours until 72 hours after the final dose. Tissue samples, urine, faeces and blood were assayed for radioactivity by liquid scintillation counting. Washings from the metabolic cages were also assayed for radioactivity.

There was no apparent sex difference in the excretion of radioactivity. At the end of the experiment, 94.5% and 93.2% of the radioactivity was recovered from male and female animals, respectively (Table 3). The major portion of the administered radioactivity was excreted in the faeces (54%) with a smaller amount in the urine (36–38%). Only minor amounts (1%) remained in the body at study end. The highest levels of radioactivity, 23–53 parts per million (ppm), were found in the spleen . The assessment of radioactivity in blood allowed estimation of the half-life. The elimination of radioactivity from the blood occurred in two stages. The initial stage involved rapid elimination with a half-life of 1–2 hours. The second stage was much slower, with a half-life of 40–75 hours. The slow phase allowed a gradual accumulation of the radioactivity in the blood over the period of dosing. The rapid phase after the final dose appeared to be slower (half-life of 3–5 h) than after the initial dose, presumably because the half-life of the rapid phase was modified by that of the slow phase of the previous dose.

There was no indication of bioaccumulation of the test article in any of the studies. Residual levels of radioactivity in tissues were consistent with the incorporation of the radiolabel into endogenous substances.

Table 3. Per cent recovery of radioactivity after administration of ^{32}P-labelled fosetyl-Al in a repeated-dose study [a]

Tissue	% radioactivity	
	Male	Female
Urine	37.9	36.2
Faeces	54.1	54.6
Cage wash	1.14	1.3
Blood samples	n.d.	0.01
Tissues	0.32	0.33
Carcass	1.05	0.82
Total recovery	94.5	93.2

bw: body weight; n.d.: not detected

[a] Repeated administration of 111 mg/kg bw per day for 7 days.

Source: Unsworth (1977a)

1.2 Biotransformation

A metabolism study on [^{14}C]fosetyl-Al was conducted using the samples obtained in the Unsworth (1976a) study. In this study, chromatographic investigations were performed using gas chromatography with a specific phosphorus detector following methylation of the phosphonate components. The data indicate that fosetyl-Al underwent extensive hydrolysis in vivo to give [^{14}C] ethanol and phosphonate. The phosphonate produced was excreted predominantly (equivalent to 73% of the administered compound) in the urine together with unchanged material (26–28% of the administered compound). The [^{14}C]ethanol that was produced was rapidly oxidized to $^{14}CO_2$ and was mainly excreted in the expired air (Unsworth, 1976b).

2. Toxicological studies

2.1 Acute toxicity

Studies on the acute toxicity, skin or eye irritation and skin sensitization potential of fosetyl-Al are summarized in Tables 4 and 5.

(a) Lethal doses

The acute oral toxicity of fosetyl-Al was studied in mice, rats and rabbits (median lethal dose [LD_{50}] > 2000 mg/kg bw) (Pasquet & Mazuret, 1977a; Ito & Kajiwara, 1979; Thouvenin, 1997a; Kumar, 2013a,b). The acute dermal toxicity was studied in rats (LD_{50} > 2000 mg/kg bw) (Thouvenin, 1997b; Kumar, 2012a). The acute inhalation toxicity was studied in rats (median lethal concentration [LC_{50}] > 5.11 mg/L) (Coombs & Clark, 1977; Blagden, 1997; Kumar, 2013c) (Table 4).

Common clinical signs in intoxicated animals treated at or above the LD_{50}/LC_{50} included sedation, emaciation, dyspnoea, prostration, depressive state, diarrhoea, abnormal gait, nasal bleeding, dacryohemorrhoea, gasping and loss of righting reflex. Gross pathology and histological findings included irritation of the digestive tract, peritonitis with ascites, peritoneal adhesions and lesions consistent with the route of administration (e.g. lesions in the gastrointestinal tract of animals exposed orally, the respiratory tract of animals treated by inhalational exposure and at the injection site and peritoneal cavity of animals treated by intraperitoneal injection).

Table 4. Summary of acute toxicity studies with fosetyl-Al

Route	Species	Strain	Sex	Purity	LD_{50}/LC_{50}	Reference
Oral	Rabbit	Fauve de Bourgogne	M + F [a]	Not stated	2.5 g/kg	Pasquet & Mazuret (1977a)
	Rat	CD (COBS)	M + F	Not stated	5.4 g/kg	Pasquet & Mazuret (1977a)
		SD	M + F	97%	> 7.08 g/kg	Thouvenin (1997a)
		SD	M + F	Not stated	11.3 g/kg M 10.6 g/kg F	Ito & Kajiwara (1979)
		Wistar	F	97.1%	> 2.0 g/kg	Kumar (2013a)
		ICR	M + F	Not stated	5.25 g/kg M 5.50 g/kg F	Ito & Kajiwara (1979)
	Mouse	Swiss Albino	F	97.1%	> 2.0 g/kg	Kumar (2013b)
Inhalational	Rat	SD	M + F	Not stated	> 1.73 mg/L	Coombs & Clark (1977)
		SD-CD	M + F	970 g/kg	> 5.11 ± 0.45 mg/L	Blagden (1997)
		Wistar	M + F	97.1%	> 1.24 mg/L	Kumar (2013c)
Intraperitoneal	Rat	SD	M + F	Not stated	1.27 g/kg M 1.15 g/kg F	Ito & Kajiwara (1979)
	Mouse	ICR	M + F	Not stated	0.940 g/kg M 1.09 g/kg F	Ito & Kajiwara (1979)
Subcutaneous	Mouse	ICR	M + F	Not stated	3.95 g/kg M 3.80 g/kg F	Ito & Kajiwara (1979)
	Rat	SD	M + F	Not stated	6.6 g/kg M 7.4 g/kg F	Ito & Kajiwara (1979)
Dermal	Rat	SD	M + F	97%	> 2.0 g/kg	Thouvenin (1997b)
		Wistar	M + F	97.1%	> 2.0 g/kg	Kumar (2012a)

F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

[a] Only two animals/sex.

(b) Dermal irritation

Dermal irritation was not observed in any of three studies (Table 5).

(c) Ocular irritation

Ocular irritation was observed in three studies (Table 5). In these studies, ocular irritation was characterized by moderate iridial and strong conjunctival changes in 4/6 treated animals and slight corneal opacity in all treated animals (Thouvenin, 1997d) when a recovery period was not included in the study design. A second study included a recovery period and observed conjunctival redness, chemosis and discharge in all treated animals, and corneal opacity in 2/3 animals (Kumar, 2012b). The third study did not characterize the clinical signs pertaining to ocular irritation, but did categorize the irritation as "mild" (Pasquet & Mazuret, 1981a).

(d) Dermal sensitization

There was no evidence in sensitization studies that fosetyl-Al induces dermal sensitization (Table 5).

Table 5. Skin or eye irritation and skin sensitization potential of fosetyl-Al

End-point	Species	Strain	Sex	Purity (%)	Result	Reference
Dermal irritation	Rabbit	Hy/Cr, derived from NZW	M	Not stated	Not irritating	Pasquet & Mazuret (1981b)
		NZW	M	97.1	Not irritating	Kumar (2013d)
		NZW	F	97	Not irritating	Thouvenin (1997c)
Ocular irritation	Rabbit	NZW	F	97	Severely irritating [a]	Thouvenin (1997d)
		Hy/Cr, derived from NZW	M	Not stated	"Mild" irritation [b]	Pasquet & Mazuret (1981a)
		NZW	M	97.1	Moderately irritating [c]	Kumar (2012b)
Sensitization	Guinea pig	Hartley/Dunkin	M	Not stated	Not sensitizing	Elliott & Seaber (1979)
		Hartley/Dunkin	M + F	Not stated	Not sensitizing	Manciaux (1998)
		Hartley/Dunkin	M + F	97.1	Not sensitizing	Kumar (2013e)

F: females; M: males; NZW: New Zealand White

[a] Moderate iridial and strong conjunctival changes in 4 of 6 tested animals. In addition, the test article induced slight to moderate corneal opacity in all treated animals. Ocular lesions were considered irreversible. Recovery period was not included in the study design.

[b] The study authors do not explicitly describe the signs of irritation.

[c] Conjunctival redness, chemosis and discharge observed in all treated rabbit. Two rabbits exhibited corneal opacity. Recovery period included in the study design.

2.2 Short-term studies of toxicity

(a) Oral administration

Three out of six studies of the short-term oral toxicity of fosetyl-Al were conducted according to GLP (Osborne, 1989; Dange, 1999; Yogesha, 2013).

Mouse

In an oral toxicity range-finding study, Goldenthal (1978) administered fosetyl-Al (purity 97.8%) to Charles River CD-1 mice (n = 10/sex per group) for six weeks at dose levels of 0, 5000, 10 000, 20 000, 30 000 or 40 000 ppm (equal to 0, 954, 2070, 4100, 5560 and 7390 mg/kg bw per day for males and 0,

1120, 2560, 4600, 6780 and 9360 mg/kg bw per day for females, respectively). Measured end-points included clinical signs (daily), body weights (weekly) and feed consumption (weekly).

There were no reported clinical signs or mortality in the treatment groups. There were no compound-related effects on body weight or feed consumption. No gross or histopathological examinations were performed.

The no-observed-adverse-effect level (NOAEL) was 40 000 ppm (equal to 7390 mg/kg bw per day), the highest concentration tested (Goldenthal, 1978).

Rat

In a 3-month oral toxicity study in rats, Coquet (1977a) administered fosetyl-Al (LS 74-783, batch nos FT 7793 and FT 794/795; purity 98.7–99.8%) to OFA (Sprague Dawley–derived) SPF rats (n = 15/sex per group) at dietary concentrations of 0, 1000, 5000 or 25 000 ppm (equal to 0, 75.2, 366 and 1920 mg/kg bw per day for males and 0, 98.0, 480 and 2500 mg/kg bw per day for females, respectively). Measured end-points included clinical signs (daily), body weights (weekly), feed and water consumption (weekly), ocular examination (weeks 0, 4, 8 and 13), haematology (weeks 4, 6, 8 and 12), bone marrow examination (scheduled kill), clinical chemistry (weeks 4, 8 and 12), urine analysis (weeks 4, 8 and 12), organ weights (at scheduled kill), histopathological analysis (scheduled kill) and evaluation of cerebral acetylcholinesterases (at scheduled kill).

There were no deaths of treated or control animals. There were no reported significant effects on clinical signs, feed consumption, water consumption, body weight gain, ocular examinations or urine analysis. Some effects on haematology and clinical chemistry were reported, but the changes were slight and transitory. The kidney weight (absolute and relative) in animals treated at the high dose was significantly elevated in males, but the magnitude was slight (< 10%). A decrease in thyroid weights in male animals treated at the low and mid doses was not seen at the high dose. The decrease in brain weights in low-dose females was not seen at the high dose. The only histopathological finding of note was extramedullary haematopoiesis in the spleens of rats treated at the high dose, but not the mid or low dose. Cerebral acetylcholinesterase was increased in males in all treatment groups but by less than 10%. The cerebral acetylcholinesterase values in females did not differ significantly from control.

The NOAEL was 25 000 ppm (equal to 1920 mg/kg bw per day), the highest concentration tested. The extramedullary haematopoiesis in the spleen was not considered adverse in isolation.

In a 13-week oral toxicity study, Osborne (1989) administered fosetyl-Al in the diet to four groups of Sprague Dawley rats (n = 70/sex per group) at 0, 8000, 30 000 or 50 000 ppm (equal to 0, 544, 2130 and 3500 mg/kg bw per day for males and 0, 648, 2400 and 4300 mg/kg bw per day for females, respectively). Subgroups ($n \leq 10$/sex per group) were killed after 2, 4, 8 and 13 weeks of treatment. An additional subgroup (n = 20/sex per group) was dosed for eight weeks; of these, 10 rats/sex per group were killed after eight weeks recovery and the remainder were killed after 16 weeks recovery. Up to 10 rats/sex per group were dosed for 13 weeks and then allowed to recover for 21 weeks; they were then killed. The end-points measured included clinical signs, body weight, feed and water intake, blood, urine, faeces and urinary calculi. Histopathology was limited to kidneys, ureters, urinary bladder and thyroids (plus parathyroid).

Ten high-dose males and two mid-dose males died or were euthanized in extremis. Clinical signs consisted of marked diuresis, red/brown staining of urine, abdominal distension, weakness, skin pallor and hypothermia. Marked diuresis and red/brown staining of abdominal fur were observed in high-dose animals, with similar but less severe effects at the mid dose. During the recovery period, only the abdominal fur staining persisted. Clinical signs were not seen in low-dose animals.

High-dose rats had reduced body weight and weight gain (decreased by 40–80%) that was not recouped during the recovery periods (Table 6). In mid-dose animals, similar but less severe effects on body weight were observed in males, but the body weight was recovered during the recovery period. No effects on body weight were observed in mid-dose females or low-dose animals of either sex. High-dose animals had consistently reduced feed intake throughout the dosing period (70% and 87% of control for male and female rats, respectively), while mid- and low-dose males had reduced feed intake only

initially. High-dose animals showed a marked increase in water consumption during treatment and recovery periods. Mid-dose animals showed a similar effect but only during the dosing period.

Changes in organ weights were observed in high-dose animals and were generally restricted to increased absolute and occasionally relative kidney weights. After 13 weeks of exposure to fosetyl-Al, males and females had an increase in absolute kidney weight of 30% and 40%, respectively, above controls, and an increase in relative kidney weight of 91% and 43%, respectively, above controls. Gross pathology showed urolithiasis and dilatation of kidney pelvis, ureters and urinary bladder. Urolithiasis and retained urine may have resulted in the increased relative and absolute kidney weights in high-dose animals.

Table 6. Body weights of rats in 13-week oral toxicity study of fosetyl-Al

Week	Body weight (g) per dose level and treatment													
	Controls		8000 ppm				30 000 ppm				50 000 ppm			
	M	F	M[a]	M[b]	F[a]	F[b]	M[a]	M[b]	F[a]	F[b]	M[a]	M[b]	F[a]	F[b]
0	200.8	146.0	199.8	–	145.5	–	198.4	–	146.2	–	197.2	–	145.1	–
4	380.4	222.4	378.0	–	223.2	–	344.8**	–	216.6	–	234.1**	–	181.5**	–
8	470.0	255.0	479.1	–	260.1	–	437.2**	–	253.4	–	287.1**	–	206.7**	–
16	560.5	287.3	558.1	573	285.2	291.2	528.6	530.9	288.7	289.1	466.6**	411.7**	264.0**	252.4**
24	623.0	318.4	615.7	636.3	322.0	330.7	611.6	619.9	315.6	320.4	541.3**	532*	312.1	293.1**
34	662.4	345.4	–	656.1	–	356.2	–	654.1	–	342.7	–	579.7**	–	320.1

F: female; M: male; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ *Source*: Osborne (1989)

[a] Groups administered treatment for eight weeks of ± recovery.

[b] Groups administered treatment for 13 weeks of ± recovery.

No effects were observed in haematology except minor effects in high-dose animals that may have been a result of marked diuresis. After 13 weeks of exposure to fosetyl-Al, blood urea nitrogen (BUN) levels in high-dose rats were 110% (male) and 44% (female) above control values and phosphorus levels were 38% (male and female) above control values; these increased BUN and phosphorous levels persisted during the recovery periods. Changes in other clinical chemistry parameters were restricted to a single sex (total protein − male; albumin − female) at the high dose and returned to control levels during the recovery period.

Uraemia occurred in all high-dose animals and persisted during treatment and recovery periods. Urine volume was increased and specific gravity and pH decreased in high-dose animals. Urine electrolytes were also reduced, except for calcium and aluminium, which increased (Table 7). These effects reversed when dosing ceased. Similar but less severe effects were observed in mid-dose animals.

Table 7. Urine analysis data in 13-week oral toxicity study of fosetyl-Al in rats

Study week	Measure per dose level							
	Controls		8000 ppm		30 000 ppm		50 000 ppm	
	M	F	M	F	M	F	M	F
Volume (mL)								
0	9.8±3.56	10.0±2.99	–	–	–	–	–	–
8	19.0±5.66	17.0±6.3	19.0±1.6	25.4±12.6	19.8±6.6	15.9±4.8	42.8±9.4**	28.7±5.46
13	19.1±5.13	14.9±4.07	15.9±5.3	16.7±8.3	26.0±7.9	18.2±8.9	43.6±5.3**	24.6±6.5
8±16	25.5±12.6	14.9±8.2	27.0±7.9	11.1±1.9	19.8±5.3	17.5±7.2	22.0±4.6	13.20±5.6
13±21	20.8±2.9	19.3±8.0	16.0±5.9	20.0±7.6	25.0±4.2	16.1±5.3	28.4±6.8	18.5±5.3
pH								
4	7.2±0.27	7.3±0.29	6.8±0.27	7.3±0.57	5.1±0.22**	5.3±0.27**	5.0±0.0**	5.2±0.45**
8	7.4±0.22	7.7±0.22	6.9±0.22*	7.0±0.35	5.8±0.45**	5.3±0.27**	5.0±0.00**	5.0±0.00**
13	7.2±0.27	7.7±0.22	6.7±0.27*	6.8±0.29*	6.0±0.35**	5.4±0.55**	5.0±0.00**	5.0±0.00**
8 + 8	6.8±0.27	6.2±0.27	6.9±0.22	6.9±0.96	6.9±0.22	7.3±0.91	6.5±0.00	6.4±0.65
13 + 21	7.1±0.22	6.9±1.24	6.8±0.27	6.7±0.76	7.2±0.87	6.6±0.65	6.8±0.27	6.6±0.65

Study week	Measure per dose level							
	Controls		8000 ppm		30 000 ppm		50 000 ppm	
	M	F	M	F	M	F	M	F
Calcium **(mg/dL)**								
0	13.3±5.43	10.0±5.15	–	–	–	–	–	–
8	10.6±12.9	14.6±7.1	10.7±1.6	12.9±3.5	18.4±17.6	41.7±18.1*	64.5±7.9**	73.7±18.9**
13	5.0±1.9	15.5±8.0	6.6±3.0	20.2±11.3	13.6±6.2**	45.3±21.5*	57.6±1.1**	70.8±12.1**
8 + 16	4.3±2.8	15.5±11.2	5.1±2.6	6.6±2.3	4.8±1.5	5.5±3.1	3.4±1.5	11.3±7.0
13 + 21	5.3±0.6	15.9±11.8	9.0±3.6	20.6±5.9	5.5±2.9	22.3±11.8	7.3±1.5	14.9±6.6
Sodium **(meq/L)**								
0	146.8±49.62	123.6±16.92	–	–	–	–	–	–
8	139.0±57.4	105.4±35.2	161.6±17.7	67.4±21.9	133.0±19.0	124±30.4	40.0±7.5**	45.6±8.3**
13	47.8±20.2	112.2±12.8	83.2±49.5	107.0±44.0	65.0±33.3	117.6±56.0	44.4±6.1	70.8±20.7
8 + 8	25.0±9.9	20.0±9.7	28.2±7.0	21.4±14.5	38.0±10.6	32.8±16.8	29.8±13.0	30.6±15.3
13 + 21	42.6±9.2	28.4±9.8	30.4±9.9	30.8±11.6	41.6±12.2	37.6±15.9	35.4±10.0	33.4±10.9
Potassium **(meq/L)**								
0	338.0±89.9	291.8±58.97	–	–	–	–	–	–
8	291.8±82.5	235.8±72.5	271.8±37.2	131.2±31.4**	201.0±37.3*	205.8±51.7	65.2±9.9**	69.2±20.4**
13	141.8±31.1	241.6±39.6	182.8±87.4	192.6±64.2	115.8±50.0	197.4±95.5	71.8±8.5	95.4±27.8**
8 + 16	82.8±29.3	70.0±29.7	77.8±22.7	76.4±14.0	100.6±26.7	67.4±27.2	83.5±13.5	90.2±41.6
13 + 21	107.2±19.5	8.30±30.6	116.2±26.1	62.8±14.4	94.0±13.8	83.0±24.0	88.8±20.0	37.4±14.6
Phosphorus **(mg/dL)**								
0	56.4±14.37	80.4±48.82	–	–	–	–	–	–
8	108.6±23.02	80.2±20.5	89.8±14.6	51.6±28.4	18.8±29.5	23.3±13.2	1.3±0.35**	1.3±0.35**
13	56.9±11.0	96.4±36.6	67.2±45.5	61.9±16.8	25.9±20.6	28.2±13.9	1.1±0.28**	1.5±0.58**
8 + 16	74.3±31.6	86.2±35.4	71.9±19.5	89.8±13.1	76.0±13.7	61.4±8.4	69.6±5.3	99.4±49.7
13 + 21	89.8±13.9	90.8±42.9	124.4±26.1*	77.5±16.9	80.8±14.7	86.9±26.5	72.4±11.4	95.2±21.4

eq: equivalent; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Osborne (1989)

Analysis of faeces of high-dose rats showed decreased calcium levels and increased phosphorus and aluminium levels; these returned to control levels during recovery. Such changes were occasionally seen in the mid-dose rats.

Marked urolithiasis was observed in the bladder, ureters and kidneys of high-dose animals and mid-dose males within two weeks of starting dosing (Table 8). Chemical analysis showed that in the calculi, approximately 23% of the content was phosphorus, 33% was calcium and less than 0.05% was aluminium and magnesium. The remaining content (44%) of these calculi was most likely oxalate, although due to the small size of the individual samples, it was not possible to analyse this constituent. The aluminium content of the calculi was negligible. Even after 21 weeks of recovery, small uroliths were still present in the folds of the urinary bladder and the kidneys.

Table 8. Histopathological data in 13-week oral toxicity study of fosetyl-Al in male and female rats

Organ / week	Findings per sex and dose level[a]							
	Male				Female			
	0 ppm	8000 ppm	30 000 ppm	50 000 ppm	0 ppm	8000 ppm	30 000 ppm	50 000 ppm
Urinary bladder								
Uroliths								
Week 2	0/10	0/10	5/10 (50%)	3/10 (33.3%)	0/10	0/10	0/10	0/10
Week 13	0/10	0/10	6/10 (60%)	6/6 (100%)	0/10	0/10	0/10	0/10
+ 21 weeks of recovery	1/10 (10%)	0/10	2/8 (33.3%)	3/9 (37.5%)	0/10	0/10	0/10	1/10 (10%)
Papillary hyperplasia								
Week 2	0/10	0/10	7/10 (70%)	8/10 (80%)	0/10	0/10	0/10	4/10 (40%)
Week 13	0/10	0/10	5/10 (50%)	6/6 (100%)	0/10	0/10	0/10	1/10 (10%)
+ 21 weeks of recovery	0/10	0/10	0/8	1/9 (11.1%)	0/10	0/10	0/10	1/10 (10%)
Transitional cell hyperplasia								
Week 2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Week 13	0/10	0/10	2/10 (20%)	0/6	0/10	0/10	0/10	0/10
+ 21 weeks of recovery	0/10	0/10	3/8 (37.5%)	2/9 (22.2%)	1/10 (10%)	0/10	0/10	1/10 (10%)
Kidney								
Uroliths								
Week 2	0/10	0/10	1/10 (10%)	4/10 (40%)	0/10	0/10	0/10	7/10 (70%)
Week 13	0/10	0/10	5/10 (50%)	6/6 (100%)	0/10	0/10	2/10 (20%)	9/10 (90%)
+ 21 weeks of recovery	0/10	0/10	0/8	2/9 (22.2%)	0/10	2/10 (20%)	1/10 (10%)	6/10 (60%)
Chronic interstitial nephritis								
Week 2	1/10 (10%)	1/10 (10%)	4/10 (40%)	8/10 (80%)	1/10 (10%)	0/10	0/10	5/10 (50%)
Week 13	0/10	0/10	2/10 (20%)	6/6 (100%)	0/10	0/10	1/10 (10%)	10/10 (100%)
+ 21 weeks of recovery	3/10 (33.3%)	3/10 (33.3%)	5/8 (62.5%)	9/9 (100%)	0/10	0/10	0/10	10/10 (100%)
Hydronephrosis								
Week 2	0/10	1/10 (10%)	3/10 (30%)	5/10 (50%)	0/10	2/10 (20%)	0/10	3/10 (30%)
Week 13	2/10 (20%)	0/10	3/10 (30%)	6/6 (100%)	0/10	0/10	1/10 (10%)	9/10 (90%)
+ 21 weeks of recovery	2/10 (20%)	0/10	4/8 (50%)	9/9 (100%)	1/10 (10%)	0/10	1/10 (10%)	9/10 (90%)

(*Continued on next page*)

Organ / week	Findings per sex and dose level [a]							
	Male				Female			
	0 ppm	8000 ppm	30 000 ppm	50 000 ppm	0 ppm	8000 ppm	30 000 ppm	50 000 ppm
Transitional cell hyperplasia								
Week 2	0/10	0/10	0/10	1/10 (10%)	0/10	0/10	0/10	3/10 (30%)
Week 13	0/10	0/10	0/10	6/6 (100%)	0/10	0/10	0/10	8/10 (80%)
+ 21 weeks of recovery	1/10 (10%)	0/10	1/8 (12.5%)	9/9 (100%)	1/10 (10%)	0/10	0/10	4/10 (40%)
Tubular dilatation								
Week 2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Week 13	0/10	0/10	0/10	2/6 (33.3%)	0/10	0/10	0/10	0/10
+ 21 weeks of recovery	0/10	0/10	1/8 (12.5%)	3/9 (33.3%)	0/10	0/10	0/10	1/10 (10%)
Ureters								
Dilatation								
Week 2	0/10	0/10	2/10 (20%)	9/10 (90%)	0/10	0/10	0/10	5/10 (50%)
Week 13	0/10	0/10	2/10 (20%)	6/6 (100%)	1/10 (10%)	0/10	1/10 (10%)	8/10 (80%)
+ 21 weeks of recovery	1/10 (10%)	1/9 (11.1%)	4/8 (50%)	6/9 (66.7%)	1/10 (10%)	1/10 (10%)	2/10 (20%)	7/10 (70%)
Transitional cell hyperplasia								
Week 2	0/10	0/10	0/10	4/10 (40%)	0/10	0/10	0/10	0/10
Week 13	0/10	0/10	0/10	0/6	0/10	0/10	0/10	0/10
+ 21 weeks of recovery	0/10	0/9	0/8	0/9	1/10 (10%)	0/10	0/10	0/10
Uroliths								
Week 2	0/10	0/10	0/10	6/10 (60%)	0/10	0/10	0/10	2/10 (20%)
Week 13	0/10	0/10	0/10	0/6	0/10	0/10	0/10	0/10
+ 21 weeks of recovery	0/10	0/9	0/8	0/9	0/10	0/10	0/10	0/10

ppm: parts per million

[a] Results presented as no. of animals with the finding/number of animals examined and, in parentheses, as a % of the animals examined.

Source: Osborne (1989)

Papillary hyperplasia was observed in the bladder epithelium of mid- and high-dose males treated for 2–13 weeks, but these findings decreased during recovery. Chronic interstitial nephritis, hydronephrosis, pyelitis, pyelonephritis, papillary necrosis, dilatation of the collecting tubules, and transitional cell hyperplasia of the kidney were seen in high-dose animals and mid-dose males from week 2 onwards, and persisted through recovery. Chronic interstitial nephritis is a common finding in male rats and was observed in control animals; however, this lesion in control animals was generally located throughout the kidney cortex, while the lesions in treated animals were located in the kidney pelvis. Dilation of ureters with occasional ureteritis or transitional cell hyperplasia was seen in these same groups. Cystitis or focal haemorrhage of the urinary bladder were also observed in a few animals (total incidence: cystitis

– three mid-dose males, two high-dose males, one high-dose female; haemorrhage – one mid-dose male (none in females)) and was considered directly related to physical irritation of the bladder epithelium by uroliths. The histopathological lesions in the kidneys and ureters were considered directly related to the urolithiasis occurring in the same groups.

One benign papilloma of the urinary bladder was found in a control female animal in the 13-week treatment/21-week recovery group and one benign transitional cell papilloma was found in a high-dose male that died in week 22 (after eight weeks of treatment followed by 16 weeks recovery). The data indicate that high doses of fosetyl-Al cause precipitation of calcium phosphate or calcium phosphonate uroliths in the urine that cause prolonged irritation of the transitional epithelium, leading to hyperplasia. The hyperplastic effects were not observed in animals exposed to levels of fosetyl-Al below 2300 mg/kg bw per day.

The NOAEL was 8000 ppm (equal to 544 mg/kg bw per day) based on histopathological changes in the kidney, impairment of calcium/phosphorous metabolism, calculi and hyperplasia in the urinary bladder observed at 30 000 ppm (equal to 2130 mg/kg bw per day) (Osborne, 1989).

In a 90-day oral toxicity study, Dange (1999) administered fosetyl-Al (batch no. 9810111; purity 981 g/kg) to Wistar AF rats RJ:WI=IOPS AF (n = 10/sex per dose level) in the diet at 0, 2000, 6000 or 20 000 ppm (equal to 0, 128, 383 and 1270 mg/kg bw per day for males and 0, 155, 455 and 1580 mg/kg bw per day for females, respectively). Measured end-points included clinical signs (daily); neurotoxicity assessment during acclimatization and on study week 12 (grasping reflex, righting reflex, corneal reflex, pupillary reflex, auditory startle reflex and head shaking reflex), body weight and feed consumption (weekly), ophthalmoscopy (acclimatization and on week 12), haematology (study days 86 or 87) and urine analysis (study days 91, 92 or 93). A full necropsy was performed on all animals at study end. An extensive range of tissues from control and top-dose animals plus liver, lung, thyroid and kidneys from the low- and mid-dose animals were examined histopathologically.

There were two mortalities among high-dose males for which a relation to treatment cannot be excluded. one moribund male was humanely euthanized on day 8 and one was found dead on day 22. The euthanized animal was observed to have been cold to the touch and pale and to have exhibited piloerection; bloody urine was found in the cage tray. The male found dead on study day 22 was observed to have been cold to the touch and to have exhibited piloerection, a swollen abdomen, reduced motor activity, polypnoea, tremors and an absence of grasping reflex prior to being found dead. Postmortem of the euthanized animal showed multiple red foci in the glandular stomach, dark red content in the duodenum, dark red kidneys and brown urine in the urinary bladder. The histopathology assessment showed liver congestion with hepatocellular basophilia. There was lymphocytolysis in various lymphoid organs with reduced haematopoiesis in bone marrow. Examination of the stomach revealed an erosion in the glandular region. Postmortem of the animal found dead on day 22 found fluid in the abdominal and thoracic cavities with multiple red foci in the glandular stomach, large kidneys, red appearance of the serosal side of the urinary bladder and dark and enlarged liver. The histopathological assessment of this animal showed autolysis of several organs. Incidental inflammation was noted in genital organs, the rectum, heart and meninges.

No consistent effects on body weight and no effects on feed consumption were observed. There were no effects observed in ophthalmoscopy. There were no differences between treated animals and controls in the neurotoxicity assessment. No significant effects were observed in haematology, except for some effects on mean platelet counts at all dietary levels in females. This effect on platelet counts was not considered biologically significant based on the observed variation of individual values and lack of other affected haematological parameters. Similarly, the only effects observed in clinical chemistry were lower mean total protein and albumin concentrations in females only at 6000 and 20 000 ppm. High-dose male animals had slightly decreased urinary pH, but no other parameters were affected and there were no significant effects seen in urine analysis of female rats in any dose group. Any changes in organ weights were isolated and did not demonstrate a dose response; they were therefore not considered biologically significant. Gross or histopathological examination found no treatment-related changes.

The NOAEL was 6000 ppm (equal to 383 mg/kg bw per day in male rats) based on mortality in male rats at 20 000 ppm (equal to 1270 mg/kg bw per day in male rats) (Dange, 1999).

In a 90-day oral toxicity study with a 28-day recovery period, Yogesha (2013) administered fosetyl-Al (batch no. 12020045; purity 97.1%) in the diet to Wistar HSD Han rats in four treatment groups and two recovery groups (n = 10/sex per dose level). The exposure levels were 0, 2000, 6000 or 20 000 ppm (equal to 0, 118, 363 and 1230 mg/kg bw per day for males and 0, 148, 446 and 1430 mg/kg bw per day for females, respectively). The recovery groups consisted of a second group of control and high-dose animals. Measured end-points included clinical signs, mortality, morbidity, body weights, ophthalmoscopy, feed consumption, functional observational battery (FOB; in last week of treatment and recovery periods), urine analysis, haematology, coagulation, clinical chemistry (at end of treatment) and macroscopic and microscopic pathology.

No treatment-related effects were observed in clinical signs, mortality or ophthalmoscopy, body weight, body weight gain or feed consumption. No test article-related effects were observed in clinical pathology, organ weights or histopathology. Compared to controls, urine volume increased in low-dose animals by 25%, but decreased in high-dose animals by about 33%. No correlates to renal toxicity were observed, such as increased relative or absolute kidney weight or histopathological lesions in the urinary system. Therefore, the finding of altered urinary volume was not considered significant in this study. Some sporadic effects were noted in the FOB, such as increased hindlimb strength and increased or decreased motor activity, but these effects were inconsistent, did not show evidence of dose response and were not correlated with other (unaffected) end-points to suggest a neurological effect.

The NOAEL was 20 000 ppm (equal to 1230 mg/kg bw per day), the highest concentration tested (Yogesha, 2013).

Dog

In a three-month oral toxicity study, Coquet (1977b) administered fosetyl-Al (batch no. DA 67; purity 99.7 ± 0.3%) to beagle dogs (n = 5/sex per group; aged 8–14 months at study initiation) in the diet at levels of 0, 2000, 10 000 or 50 000 ppm (equal to 0, 58, 272 and 1310 mg/kg bw per day for males and 0, 58, 272 and 1450 mg/kg bw per day for females, respectively). The dogs were housed individually. Measured end-points included feed consumption (daily), water consumption (daily), body weight (weekly), ophthalmoscopy (at weeks 0, 4, 8 and 12), retinographic examination (at week 12), rectal temperatures (every two weeks), haematology (at weeks 0, 4, 8 and 12), serum biochemistry (at weeks 0, 4, 8 and 12), cerebral acetylcholinesterase (at scheduled kill), urine analysis (at weeks 0, 4, 8 and 12), gross pathology and histopathology (including bone marrow).

No evidence of test article-related effects was reported. Some fluctuations in haematology, serum biochemistry and urine analysis were observed, but none showed consistency or evidence of a dose response.

The NOAEL was 50 000 ppm (equal to 1310 mg/kg bw per day), the highest concentration tested (Coquet, 1977b).

In a two-year chronic toxicity study, Spicer (1981b) administered fosetyl-Al (batch no. LS 74-783; purity 96.6%) to purebred beagle dogs (n = 6/sex per group) in the diet at 0, 10 000, 20 000 or 40 000 ppm for 24 months (equal to 0, 309, 609 and 1230 mg/kg bw per day for males and 0, 288, 632 and 1190 mg/kg bw per day for females, respectively). Measured end-points included clinical signs, body weights, feed consumption, ophthalmoscopy, haematology, clinical chemistry, urine analysis and acetylcholinesterase activities (brain, erythrocyte and plasma).

One low-dose male died, but this death was not considered related to treatment. The pathology report indicated the cause of death was gastric haemorrhage. No test article-related effects were noted on survival, feed consumption, ophthalmoscopy or physical examinations. There were no significant effects on body weight. No effects were noted in urine analysis.

Significant but sporadic effects in haematology (red blood cells, haemoglobin, haematocrit, platelets, methaemoglobin, segmented neutrophils) occurred without a consistent trend. Therefore, these changes were not considered toxicologically significant. Similarly, sporadic effects on clinical chemistry (glucose, serum glutamic–pyruvic transaminase [SGPT], serum glutamic oxaloacetic transaminase [SGOT], lactate dehydrogenase [LDH] and potassium in males; BUN, SGOT, total and direct bilirubin, and total protein in females) were without clear trends and not considered to be toxicologically significant.

There were no significant effects on organ weights or significant findings in gross pathology. However, testicular degeneration was noted in mid- and high-dose dogs. The incidence was 0/6, 0/6, 2/6 and 6/6 for control, low-, mid- and high-dose dogs. The lesions consisted of spermatocytic and/or spermatidic giant cells within the lumen of seminiferous tubules. In mid-dose dogs, the lesions were focal, involved one or both gonads, and were graded as "trace". In high-dose dogs, the lesions were more numerous (focal to multifocal in distribution) and bilateral but similar in severity as in the mid-dose dogs ("trace"). In epididymal ducts of high-dose males had small amounts of cellular debris from the testis and/or no sperm.

The pathology report also described a kidney lesion in female animals that increased in severity with dose group. Bilateral cytoplasmic vacuolation of the distal convoluted tubules in the inner cortex was observed in 5/6, 4/6, 6/6 and 6/6 of control, low-, mid-and high-dose animals. The lesion was considered "trace" in control and low-dose animals, trace to mild in all mid-dose animals, trace to mild in 5/6 high-dose animals and moderate for one high-dose animal. The pathologists considered this a reversible lesion. Due to the presence of the lesion in all treatment groups and lack of any correlation with toxicity in other measurements (urine analysis, etc.), this lesion was not considered toxicologically relevant.

The NOAEL was 10 000 ppm (equal to 309 mg/kg bw per day) based on the testicular degeneration observed at 20 000 ppm (equal to 609 mg/kg bw per day) (Spicer, 1981b).

(b) Dermal application

Both studies investigating the dermal toxicity of fosetyl-Al were conducted according to GLP.

Rat

In a 28-day dermal toxicity study, Weiler (1999) topically applied fosetyl-Al (lot no. OP 9850217; purity 98.1%) to the dorsal skin of Crl:CD (SD) IGS BR rats (n = 10/sex per group) at 1050 mg/kg bw per day. The vehicle was reverse osmosis water. The dose preparation was applied for 6–7 hours per day for 29 days. The test or control (reverse osmosis water) material was applied to the dorsal skin of each animal and held in contact with the skin by a gauze dressing. At the end of the contact period, the area was gently wiped with a disposable paper towel moistened with tap water. Measured end-points included cage-side observations (daily), expanded clinical observations (weekly), FOB (week 4), ophthalmoscopy (pre-exposure and at week 4), dermal irritation scoring (day 1 of treatment and weekly thereafter), body weights (weekly), feed consumption (weekly), blood sampling (day 30), macroscopic pathological assessment (at necropsy) and microscopic histopathological assessment (after necropsy). Urine analysis was not performed.

One treated male was found dead on day 2 and one treated female on day 19. Neither death was attributable to the test article. No test article-related clinical signs were reported during the weekly examinations or the FOB. There were no reported findings from the ophthalmoscopy assessment. The dermal irritation assessment indicated slight to moderate erythema, slight oedema and slight desquamation. There were no significant differences in body weights, but lower body weight gains for treated males were associated with lower feed consumption during the first week. There were no other significant findings related to body weight or body weight gain. In the haematological assessment, treated females had a slight increase in absolute neutrophil count (1.6 vs 0.6 × 10^3/μL) that may have resulted from acute inflammation of the treated skin. There were no effects on organ weights, organ-to-body weight ratios or organ-to-brain ratios. In the histological assessment, it was noted that the test article caused crusted areas of the skin and erosions, hyperkeratosis and acute inflammation.

Fosetyl-Al caused slight to moderate dermal irritation in some of the treated animals. The slightly higher neutrophil counts were likely correlated with the histological finding of acute inflammation of the treated skin. These lesions were limited to the site of dermal application and were not considered indicative of systemic toxicity.

The NOAEL was 1050 mg/kg bw per day, the highest tested dose (Weiler, 1999).

In a 21-day dermal toxicity study followed by a 14-day recovery period, Kumar CR (2013) applied fosetyl-Al to the clipped dorsolateral thoracic skin of Wistar rats once daily under semi-occlusive conditions for at least six hours per day for 21 days. There were four main groups and two recovery groups (n = 5 rats/sex per group). The dose groups were 100, 300 and 1000 mg/kg bw per day. After the contact period, the test patches were removed and the area was rinsed with water and dried with absorbent paper. A control group received vehicle (Milli-Q water) alone. Measured end-points included clinical signs, skin reactions, mortality and morbidity (daily); body weights and feed consumption; urine analysis, haematology, coagulation and clinical chemistry (at study end). Macroscopic and microscopic histopathological assessments were performed after study end.

No clinical signs, skin changes or deaths were reported. There were no significant effects on body weights, weight gain, feed consumption, haematology, clinical chemistry or urine analysis. There were no significant effects on organ weight (absolute or relative) or histopathology.

The NOAEL was 1000 mg/kg bw per day, the highest tested dose (Kumar CR, 2013).

2.3 Long-term studies of toxicity and carcinogenicity

The long-term studies did not comply with GLP, OECD test guidelines or other validated guidelines; the data were generated before the implementation these guidelines. However, the Meeting considered the studies adequate for the evaluation.

Mouse

In a 24-month carcinogenicity study, Spicer & Richter (1981) administered fosetyl-Al in the diet to Charles River CD-1 mice (n = 60/sex per group) at 0, 2500, 10 000 or 30 000 ppm (equal to 0, 352, 1410 and 3960 mg/kg bw per day for males and 0, 409, 1670 and 4550 mg/kg bw per day for females, respectively). The high-dose group were initially dosed with fosetyl-Al at 20 000 ppm, but this was increased to 30 000 ppm on study week 19. Measured end-points included clinical signs, body weights, feed consumption, haematology (n = 10/sex) at pretest, 12 months and study end, and urine analysis and clinical chemistry of all survivors at 24 months. Gross and histopathological examinations were performed on all mice.

No compound-related effects were noted on survival, feed consumption, body weight or clinical signs. Some changes were observed at the 12-month haematology assessment (decreased reticulocytes in high-dose males; decreased segmented neutrophils and increased lymphocytes in mid- and high-dose females), but it was considered that they lacked biological significance. Survival was more than 50% in males up to week 100 and in females up to week 96. No statistically or biologically significant differences in clinical chemistry or urine analysis were noted at any time point studied.

There were no treatment-related changes observed at necropsy or in histopathology.

There was no evidence of increased incidence of neoplasia or of earlier onset of neoplasia in treated animals.

The NOAEL for general toxicity and carcinogenicity was 30 000 ppm (equal to 3960 mg/kg bw per day), the highest tested concentration (Spicer & Richter, 1981).

Rat

In a 24-month carcinogenicity study, Spicer (1981a) administered fosetyl-Al in the diet to Charles River CD rats (n = 80/sex per group) at 0, 2000, 8000 or 30 000 ppm for 24 months (equal to 0, 88, 348 and 1370 mg/kg bw per day for males and 0, 117, 450 and 1790 mg/kg bw per day for females, respectively). The high dose was reduced from an initial level of 40 000 ppm to 30 000 ppm upon observing staining of the abdomen and red coloration of the urine at the higher dose. Measured end-points included clinical signs, body weight, feed consumption, ophthalmoscopy, blood and urine samples (taken at months 3, 6, 12, 18 and 24) and full necropsy and histopathology examinations.

Survival for 104 weeks was slightly lower for treated rats (males and females) than for controls; however, survival rates were within the normal bounds for a chronic rat study, and overall more than 25 animals in all groups survived to scheduled kill.

There were no toxicologically relevant effects on haematological parameters. Sporadic differences were considered incidental to treatment.

Serum biochemistry was not significantly affected by treatment, except for slightly elevated serum sodium from months 6 to 24 in males and months 6 to 28 in females; in all cases, the magnitude of the effect was less than 5% and there was no evidence of a dose response. It was therefore not considered toxicologically relevant. Other effects observed in clinical chemistry parameters were inconsistent across treatment groups and time points and were considered incidental to treatment.

Analysis of treated male rat urine found albumin. However, since albumin was also seen in the urine of control males, this was not considered to a test article-related effect. The urinary pH of male rats was significantly decreased compared to controls at months 3 and 12 (~10% decrease), but not at months 6, 18 and 24. Urinary pH of female rats in any treatment group or time point was unaffected. Sporadic significantly different values were seen for urine volume and specific gravity across treatment groups and time points, but this did not exhibit evidence of dose response. There were no consistent effects on organ weight attributable to fosetyl-Al exposure.

Macroscopic examination found "calculi/white flakes" in high-dose males (5/79) and females (3/79). In addition, a mass was found in the urinary bladder of 2/79 high-dose male rats but not female rats. There were no cases of "calculi/white flakes" or masses in control males or females.

Pathological lesions in two sites (adrenal medulla and the urinary system) underwent peer review as well as a blinded reassessment by an expert pathologist. The examining histopathologist did not consider the increased incidence of phaeochromocytoma in the adrenal medulla in mid- and high-dose males significant. In a peer review, the adrenal medulla slides underwent a blinded reanalysis by an external expert (King, 1984; Thompson, 1984). The adrenal medullary cell hyperplasia and phaeochromocytomas were grouped together for analysis. All three assessments reported similar values for the total combined hyperplasia + adenoma ± carcinoma (26–28% incidence in control compared to 28–33% incidence in high-dose male rats). Statistical analysis did not reveal any significant dose-related increases in any adrenal medulla lesions. Summary tables of the adrenal medulla histopathology assessments are presented in Appendix 1.

Based on the evidence summarized in the original study report and the pathology peer review, administration of a high-dose level of fosetyl-Al (greater than the limit dose of 1000 mg/kg bw per day) for 104 weeks did not produce carcinogenic effects in the adrenal medulla of rats.

There was increased incidence of epithelial hyperplasia and neoplasia in the urinary bladders and of hyperplasia and inflammation in the urinary tract of high-dose males (Table 9). Hyperplasia and inflammation of the transitional epithelium of the urinary tract were more common in high-dose male rats than controls or lower-dose groups. The lesions were characterized by an increase in thickness of the transitional epithelium forming folds and small "club formed" processes on the luminal surface. Acute or acute and chronic inflammation frequently accompanied the hyperplastic lesions, but in many cases, inflammation occurred without appreciable hyperplasia. Calculi, mineralized deposits and proteinaceous casts were occasionally seen; they were more frequently observed under gross examination, and their less frequent presence in histological sections suggests they were lost due to handling or processing. Cortical cysts and hydronephrosis were also more common in the kidneys of high-dose male rats reflecting obstruction or interference with urinary flow by proliferative processes or calculi.

Table 9. Nonneoplastic and neoplastic findings in kidney and bladder in a 24-month carcinogenicity study of fosetyl-Al in male and female rats

	Incidence per sex and dose level [a]							
	Males				**Females**			
Tissue	**0 ppm**	**2000 ppm**	**8000 ppm**	**30 000 ppm**	**0 ppm**	**2000 ppm**	**8000 ppm**	**30 000 ppm**
Nonneoplastic								
Kidney cortex								
Hyperplasia	5/78	7/80	4/81	4/79	1/76	0/78	1/78	2/79
Urolithiasis	8/78	10/80	17/81	7/79	27/76	27/78	19/78	5/79
Mineralization	4/78	4/80	7/81	3/79	1/76	1/78	1/78	3/79

Tissue	Incidence per sex and dose level [a]							
	Males				Females			
	0 ppm	2000 ppm	8000 ppm	30 000 ppm	0 ppm	2000 ppm	8000 ppm	30 000 ppm
Ectasis/tubules	74/78	69/80	71/81	74/79	49/76	50/78	49/78	56/79
Ectasis/Bowman's capsule	22/78	17/80	17/81	20/79	10/76	8/78	18/78	21/79
Cysts	60/78	53/80	59/81	57/79	25/76	26/78	28/78	54/79
Kidney medulla/pelvis								
Hyperplasia	13/78	8/80	15/81	21/79	6/76	10/78	2/78	18/79***
Urolithiasis	6/78	11/80	5/81	3/79	48/76	45/78	20/78	7/79***
Mineralization	2/78	3/80	0/81	2/79	10/76	8/78	2/78	0/79***
Ectasis/tubules	21/78	25/80	26/81	30/79	13/76	8/78	10/78	29/79***
Cystic tubules	2/78	1/80	4/81	2/79	2/76	3/78	0/78	2/79
Ectasis/hydronephrosis	6/78	4/80	13/81	16/79*	2/76	1/78	0/78	23/79***
Urinary bladder								
Hyperplasia	5/78	7/80	5/81	29/79***	1/76	7/78	3/78	11/79*
Urolithiasis	1/78	1/80	0/81	0/79	0/76	0/78	0/78	1/79
Mineralization	1/78	1/80	0/81	0/79	0/76	0/78	0/78	0/79
Neoplastic changes								
Kidney cortex								
Adenoma	2/78	3/80	2/81	–	–	1/78	–	1/79
Adenocarcinoma	2/78	2/80	1/81	2/79	–	–	1/78	3/79
Mesenchymal tumour	1/78	–	–	–	1/76	1/78	1/78	1/79
Lipoma/liposarcoma	-	–	1/81	1/79	–	–	1/78	–
Liposarcoma & reticulum cell	–	1/80	1/81	–	–	1/78	–	–
Lympholeukaemia	1/78	–	2/80	–	1/76	–	–	–
Myeloma	1/78	–	1/80	–	–	–	–	–
Kidney pelvis								
Papilloma	1/78	2/80	–	4/79	1/76	1/78	1/78	1/79
Papillosarcoma (transitional cell carcinoma)	1/78	1/80	–	–	–	–	–	6/79***
Urinary bladder								
Papilloma	1/78	1/80	1/81	5/79	–	2/78	–	1/79
Papillosarcoma (transitional cell carcinoma)	2/78	2/80	1/81	16/79***	–	1/78	–	5/79*
Leiomyosarcoma	1/78	–	1/81	1/79	1/76	1/78	–	–
Reticulum cell sarcoma	–	–	1/81	–	–	–	–	–
Lympholeukaemia	1/78	–	–	–	–	–	–	–

ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$, retrospective statistical analysis using Fisher exact test

[a] Results presented as number of animals with the finding number of animals examined.

Source: King (1985)

The urinary bladder slides were also peer reviewed and a blinded reanalysis was undertaken to assess whether the neoplastic effects noted in male rats were attributable to fosetyl-Al. The external expert conducting this re-examination noted the higher incidences of urinary bladder adenoma and carcinoma ("complex papillomatous carcinoma of the epithelium") in high-dose males, consistent with first pathology assessment (King, 1985; Thompson, 1985). Hyperplasia of the mucosal transitional cells and subacute lymphocytic inflammation of the submucosa of the urinary bladder were also observed in high-dose animals. The peer review concluded that there was a significant increase in proliferative lesions of transitional cell epithelium in the urinary tract of the high-dose females. The peer review also concluded that administration of fosetyl-Al produced a significant increase in several forms of inflammatory or degenerative deviations from the normal histological morphology of the kidneys in high-dose females (Table 10).

The increase in proliferative lesions of transitional cell epithelium in the urinary tract of the high-dose females identified during the peer review changed the interpretation of the original study. Previously, only a carcinogenic effect of fosetyl-Al in male (but not female) rats was identified. Ultimately, it was concluded that administration of a high-dose level of fosetyl-Al (greater than the limit dose of 1000 mg/kg bw per day) for 104 weeks increased the incidence of urinary bladder tumours in male and female rats.

The NOAEL for systemic toxicity was 8000 ppm (equal to 348 mg/kg bw per day) based on increased incidence of uroliths and mineralization in the urinary bladder and inflammation and hyperplasia in the urinary transitional epithelium at 30 000 ppm (1370 mg/kg bw per day).

The NOAEL for carcinogenicity was 8000 ppm (equal to 348 mg/kg bw per day) based on increased incidences of papilloma/papillosarcoma (transitional cell carcinoma) of the urinary bladder in both sexes and renal pelvis papillosarcoma (transitional cell carcinoma) in females at 30 000 ppm (1370 mg/kg bw per day) (Spicer, 1981a).

Table 10. Nonneoplastic findings in kidney and urinary bladder in a 24-month carcinogenicity study of fosetyl-Al in male and female rats as observed in blinded review

	Incidence per dose level [a]							
	Males				Females			
Tissue	0 ppm	2000 ppm	8000 ppm	30 000 ppm	0 ppm	2000 ppm	8000 ppm	30 000 ppm
Kidney cortex								
Interstitial fibrosis	58/80 (73%)	47/80 (59%)	53/81 (65%)	62/79 (77%)	13/79 (16%)	16/80 (20%)	8/80 (10%)	39/81 (48%)
Glomerular sclerosis	43/80 (54%)	33/80 (41%)	39/81 (48%)	38/79 (47%)	7/79 (8.9%)	11/80 (14%)	7/80 (8.8%)	23/81 (28%)
Tubular lumen, acute leukocytic inflammation	11/80 (14%)	20/80 (25%)	10/81 (12%)	13/79 (16%)	1/79 (1.3%)	5/80 (6.3%)	2/80 (2.5%)	14/81 (17%)
Ectasis of Bowman's space	19/80 (24%)	13/80 (16%)	14/81 (17%)	17/79 (21%)	8/79 (10%)	5/80 (6.3%)	12/80 (15%)	17/81 (21%)
Kidney medulla								
Cystic tubules	1/79 (1.3%)	0/78 (0%)	3/78 (3.8%)	2/79 (2.5%)	2/79 (2.5%)	2/78 (2.6%)	0/79 (0%)	2/79 (2.5%)
Tubular ectasis	20/79 (25%)	21/78 (26%)	24/78 (30%)	25/79 (31%)	11/79 (14%)	5/78 (6.4%)	8/79 (10%)	27/79 (34%)
Kidney pelvis								
Hydropelvis	0/78 (0%)	1/79 (1.3%)	1/79 (1.3%)	5/79 (6.3%)	0/73 (0%)	0/68 (0%)	0/70 (0%)	5/73 (6.8%)
Urinary bladder								
Epithelial hyperplasia	4/73 (5.5%)	6/75 (8.0%)	5/78 (6.4%)	24/78 (31%)	0/78 (0%)	5/79 (6.3%)	3/75 (4.0%)	9/78 (12%)

ppm: parts per million *Source*: Thompson (1985)

[a] Results presented as no. of animals with the finding/no. of animals examined and, in parentheses, as % of the animals examined.

2.4 Genotoxicity

Fosetyl-Al was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found in any assay (Table 11).

Table 11. Summary of genotoxicity studies with fosetyl-Al

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium*, *Escherichia coli* and *Saccharomyces cerevisiae*	125, 250, 500 or 1000 µg/plate ± S9; 0.1, 0.5, 5, 10, 50, 100 or 200 µg/mL ± S9; 250, 500 or 1000 µg/mL −S9 and 125, 250 or 500 µg/mL + S9, respectively	99.7	Negative	Bouanchaud & Cartier (1981)
Reverse mutation	*S. typhimurium* and *E. coli*	8, 40, 200, 1000 or 5000 µg/plate ± S9	97	Negative	Ballantyne (1997)
Reverse mutation	*S. typhimurium* and *E. coli*	50, 100, 158, 266 500, 707, 1581, 1880 or 5000 µg/plate ± S9	97.1	Negative	Suresh (2013)
"Inductest"	*E. coli*	1, 5, 10, 50, 100, 500, 1000 or 2000 µg/plate ± S9	> 95	Negative	Hofnung (1978)
Mammalian cell gene mutation	Mouse lymphoma L5178Y Cells (*tk* locus)	7.813, 15.625, 31.25, 62.5, 125 or 250 µg/mL ± S9	97	Negative	Fellows (1997)
DNA repair	*E. coli*	6.25, 12.5, 25, 50 or 100 µg/plate ± S9	99.7	Negative	Bouanchaud & Cartier (1981)
Chromosomal aberration	CHO cells	3, 10, 30 or 100 µg/mL ± S9	97.5	Negative	Mosesso & Nunziata (1982)
Chromosomal aberration	CHO cells	260, 823, 2600 µg/mL + S9 or 180, 570 or 1800 µg/mL − S9	97.1	Negative	Kavitha (2013)
HPRT assay	CHO cells	112, 227, 355, 567, 1121, 1416 or 3541 µg/mL ± S9	97.1	Negative	Indrani (2013)
In vivo					
Mouse micronucleus (gavage)	Swiss mice, M	1, 2 or 4 g/kg bw	95	Negative	Siou (1977)
Mouse micronucleus (gavage)	CD-1 mice, M + F	1063, 2125 or 4250 mg/kg bw	97	Negative	Marshall (1998)
Mouse micronucleus (gavage)	Swiss mice, M + F	500, 1000 or 2000 mg/kg bw	97.1	Negative	Rao (2013)

bw: body weight; CHO: Chinese hamster ovary; F: female; HPRT: hypoxanthine–guanine phosphoribosyltransferase; M: male; *tk*:: thymidine kinase

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a GLP-compliant continuous breeding study, Palmer et al. (1981) administered fosetyl-Al (batch no. DA 73; purity 97.3%) in the diet to SPF rats (CFY strain; $n = 25$/sex per group) through three generations at dose levels of 0, 6000, 12 000 or 24 000 ppm (equal to 0, 482, 954 and 1960 mg/kg bw per day in F_0 males and 0, 553, 1060 and 2130 mg/kg bw per day in F_0 females, respectively). In the first mating (F_0), animals were exposed from 90 days prior to mating, through mating, gestation and lactation. F_{1A} litters were reared to 21 days postnatal age, when they were killed, necropsied and examined for macroscopic changes. Ten days after weaning of F_{1A} litters, the F_0 parents were mated again, using different male and female pairings. A subgroup of pregnant dams ($n = 5$/group) were killed on gestation day 20 for teratological assessment (external examination, sectioning and staining), using the appearance of sperm in the vaginal smear to indicate gestation day 0. The remaining dams reared their young to postnatal day 21, when 25 males and 25 females from each group were selected to form the F_{1B} generation. P_0 animals and surplus F_{1B} pups were killed and examined macroscopically.

F_{1B} animals were maintained on their respective diets to at least postnatal day 90 and were then mated for 20 days. The resulting F_{2A} generation was reared to postnatal day 21, then killed and examined macroscopically. Approximately 10 days after the weaning the F_{2A} litters, the F_{1B} animals were remated for 20 days. A subgroup of pregnant females ($n = 10$/group) were killed for teratological examination. The remaining F_{1B} dams reared their litters until postnatal day 21, when 27 males and approximately 39 females from each group were selected (of these, 10/sex were reared on their diets for at least 91 days).

The remaining 12 males and 24 females were maintained on their diets for 91 days, after which two matings with rearing to postnatal day 21 were permitted. All F_{3A} pups were killed and discarded after macroscopic examination. Organs of F_{3B} pups ($n = 10$/sex per group) were weighed. Histopathological examination was initially restricted to control and high-dose groups. Unless indicated to the contrary, parent F_{2B} animals and remaining F_{3B} pups underwent macroscopic examination only.

Measurements in parental animals included clinical signs, urine analysis, feed and water consumption, body weight, mating performance, pregnancy rate, gestation length, necropsy (uteri of nonpregnant females immersed in 10% solution of ammonium sulfide to show implantation sites; testes of males that failed to impregnate females were weighed and preserved in 10% buffered formalin, histopathology was performed where indicated).

Litter measurements included the number of pups per litter, external abnormalities and survival to postnatal day 21. Pup body weight, litter weight, litter size, pup mortality and abnormalities were recorded on postnatal days 0, 4, 8, 12 and 21. Pups selected for rearing were selected by taking the pups closest to the median weaning weight of the respective sex within each litter. Brother/sister pairings were avoided; in addition, F_{2B} sisters were not mated to the same male. For F_{1B}, extra animals ($n = 2$/sex per group) were selected for replacement, if need be. Surplus pups were terminated on postnatal day 21 and examined internally and externally. Unmated F_{2B} and selected F_{3B} pups underwent macroscopic pathology. In F_{3B}, several organs were weighed (brain, heart, liver, kidneys, lungs, spleen, thymus). Tissues were examined microscopically. Of note, the urinary bladder was examined in all dose groups while other organs were examined only in the control and high-dose groups.

An interim teratology assessment was performed. On gestation day 20, selected F_0 and F_{1B} dams were killed and examined for embryo/fetal toxicity and macroscopic changes in female reproductive organs. Ovaries and uteri were examined immediately for corpora lutea, number of live young, embryonic/fetal deaths, litter weight, fetal abnormalities. Embryonic deaths were classified as early (only placenta visible) or late (both placental and embryonic remnants visible). Uteri or individual uterine horns without visible implantations were immersed in 10% ammonium sulfide. Pre- and postimplantation losses were assessed. Live young were examined externally and weighed. Half the pups in each litter were preserved for visceral abnormalities, and the remaining pups for macroscopic examination, sexing and skeletal examination.

For each dose group, the intake of test article was higher in F_{1B} and F_{2B} generations than for F_0 animals. There were 13 deaths among the treated male animals (one among controls), particularly clustered at the high dose for F_{1B} and F_{2B} generations. Necropsy of these animals revealed changes in the urinary tract (haemorrhage of the bladder wall, increased renal pelvic dilatation, interstitial nephritis and

papillary necrosis). A similarly clustered distribution of urinary tract changes was recorded at terminal examination of surviving animals. There were eight deaths among the treated female (zero among controls). The deaths were distributed evenly across groups and generations, and a definite association with urinary tract pathology was only observed with one F_0 female at the high dose. Urine analysis of a sample of control and high-dose males (n = 10/group) to clarify the relationship between death and urinary tract changes found no markedly abnormal individual or group mean values; however, the high-dose animals had statistically significant lower specific gravity and the presence of epithelial or polymorphonuclear cells in 4/10 tested animals compared to 0/10 controls.

There was a high incidence of animals with pathological changes in the urinary tract, particularly for F_{1B} and F_{2B} generations at the high dose (Table 12); these changes were associated with an increase in male but not female deaths in F_{1B} and F_{2B} high-dose animals. There were urinary tract effects in occasional adults and 1/10 weanling F_{3B} males at the mid dose. Treatment-related microscopic changes in the urinary tract were seen in the F_{3B} pups (minimal epithelial hyperplasia and/or hypertrophy of the transitional epithelium and/or desquamation of the epithelial cells; presence of crystalline or calcareous deposits) in 8/10 males and 8/10 females at 24 000 ppm and only one male at 12 000 ppm.

Body weight in male F_1 and F_2 high-dose rats was reduced in the premating period (by 8.9–12.8% of control).

In female rats, there were no consistent effects on body weight during gestation in any generation. During lactation, high-dose parental females consistently failed to reach a mid-lactation peak; they also had a marked weight loss from postnatal day 0 to 21. These effects did not occur in mid- or low-dose females. Decreased litter weight (14–35% lower), mean pup weights (13–32% lower) and mean pup weight gain were observed at the high dose in mid and late lactation. Lower litter and mean pup weights were seen in late lactation at the mid dose.

There were no effects on fertility or reproduction at any dose or in any generation (no effects on mating performance, pregnancy rate, duration of gestation, litter size). No significant effects were observed in the teratology cohort.

Table 12. Incidence of urinary tract lesions[a] in parental animals (survivors + decedents)

Dose	Incidence per dose level					
	Males			Females		
	F_0 (n = 25)	F_{1B} (n = 25)	F_{2B} (n = 27)	F_0 (n = 25)	F_{1B} (n = 25)	F_{2B} (n = 25)
0 ppm	1	0	1	0	1	0
6000 ppm	0	2	0	1	3	0
	N/S	N/S	N/S	N/S	N/S	
12 000 ppm	0	1	1	0	3	0
	N/S	N/S	N/S	N/S	N/S	
24 000 ppm	0	9**	5	4	10**	5
	N/S	P = 0.001 6	P = 0.192	P = 0.110	P = 0.004 6	P = 0.050 2

F_0: parental generation; F_{1B}: second litter of first filial generation; F_{2B}: second litter of second filial generation; N/S: nonsignificant; ppm: parts per million; **: $P < 0.01$; retrospective statistical analysis using Fisher exact test

[a] Observed lesions included haemorrhage of the bladder wall, increased renal pelvic dilatation, interstitial nephritis and papillary necrosis.

Source: Palmer et al. (1981)

To assign NOAELs and lowest-observed-adverse-effect levels (LOAELs), the means of the test article intake of F_0 rats during study weeks 1, 3, 6, 9 and 12 were used.

The NOAEL for reproductive toxicity was 24 000 ppm (equal to 1960 mg/kg bw per day), the highest concentration tested. The NOAEL for offspring toxicity was 6000 ppm (equal to 482 mg/kg bw per day for F_0 rats) based on decreased pup body weight and decreased litter weight at 12 000 ppm (1290 mg/kg bw per day). The NOAEL for parental toxicity was 6000 ppm (equal to 482 mg/kg bw per day) based on decreased body weight at 12 000 ppm (1290 mg/kg bw per day) (Palmer et al., 1981)

(b) Developmental toxicity

Rat

In a non-GLP prenatal toxicity study, James & Palmer (1977) administered fosetyl-Al (batch no. FR 794/795 FT; purity 99.8%) to CFY rats (n = 20/dose group) by gavage at 0, 500, 1000 or 4000 mg/kg bw per day from gestation day 6 to 15.

Rats were observed daily throughout the dosing period for mortality and clinical signs; body weight was measured on gestation days 1, 3, 6, 10, 14, 17 and 20. All rats were killed on gestation day 20. Macroscopic examination was performed with an emphasis on ovaries and uteri: number of corpora lutea; number and distribution of live young and early and/or late embryo/fetal deaths; number of live fetuses and their sex and weight. Half of the fetuses from each litter were examined for visceral abnormalities, and the other half for sexing and skeletal abnormalities.

Occasional deaths at 500 and 1000 mg/kg bw per day were attributed to dosing errors. At 4000 mg/kg bw per day, 5/20 rats died or were humanely euthanized on gestation day 9, 10 or 11; all had body weight loss and chromodacryorrhoea prior to death, and the postmortem found marked gastric dilatation and fluid retention. Among surviving animals, weight gain was retarded at 500 and 1000 mg/kg bw per day, and an almost complete suppression of body weight gain was observed for the first few days of dosing at 4000 mg/kg bw per day. Litter parameters were unaffected at 500 or 1000 mg/kg bw per day; at 4000 mg/kg bw per day, there was slightly higher postimplantation loss and slightly lower litter size and mean fetal weight (leading to a lower litter weight; Table 13). Embryo/fetal development was not affected at 500 or 1000 mg/kg bw per day. At 4000 mg/kg bw per day, there were incidences of major malformations (e.g. fused rib, hydrocephaly, transposed aortic arch), minor visceral and skeletal anomalies and sternebral variants. These effects were likely related to the maternal toxicity observed at this dose level.

Table 13. Maternal and litter observations in a prenatal toxicity study of fosetyl-Al in rats

Finding	Measure per dose level			
	0 mg/kg bw per day	**500 mg/kg bw per day**	**1000 mg/kg bw per day**	**4000 mg/kg bw per day**
Maternal observations				
Mortality [a]	0/20	1/20 [b]	2/20 [b]	5/20
Body weight gain (g)				
Day 6–10	23	21	18	31
Day 6–20	125	118	125	108
Pregnancy rate (%)	95	95	95	95
No. with viable young	19	18	17	14
No. of live young (per dam)	12.7	11.3	13.1	11.5
Caesarean section data				
No. of corpora lutea (per dam)	14.7	14.2	15.3	14.9
No. of implantations (per dam)	13.2	12.3	13.4	12.8
Mean preimplantation loss (%)	10.3	12.7	12.1	12.1
Mean postimplantation loss (%)	3.5	8.9	2.6	10.5
Embryonic deaths				
Early	0.4	0.6	0.4	0.5
Late	0.1	0.4	0.0	0.8
Total	0.5	0.9	0.4	1.3

(*Continued on next page*)

Finding		Measure per dose level			
		0 mg/kg bw per day	500 mg/kg bw per day	1000 mg/kg bw per day	4000 mg/kg bw per day
Litter observations					
Live young					
Males		5.4	5.8	5.1	5.3
Females		6.4	5.5	6.9	6.2
Total		12.7	11.3	13.1	11.5
Litter weight (g)		48.05	42.07	48.50	39.71*
Mean fetal weight (g)		3.79	3.74	3.72	3.46
Malformations [c]	per fetus	2/242 (0.8%)	1/204 (0.8%)	2/222 (0.8%)	5/161 (3.8%)
	per litter	2/19 (10.5%)	1/18 (5.6%)	2/17 (11.8%)	4/14 (28.6%)
Variations [c]					
Visceral	per fetus	8/119 (6.9%)	7/103 (6.6%)	8/108 (7.4%)	8/78 (13.0%)
	per litter	6/19 (31.6%)	5/18 (27.8%)	7/17 (41.2%)	3/14 (21.4%)
Skeletal	per fetus	9/121 (7.0%)	9/100 (12.6%)	14/112 (11.8%)	17/78 (22.4%)
	per litter	6/19 (31.6%)	6/18 (33.3%)	7/17 (41.2%)	9/14 (64.3%)
Skeletal variants [d]					
Extra ribs		24.6%	29.0%	42.1%	27.7%
Sternebrae extra ribs		23.8%	38.2%	8.9%	56.1%

bw: body weight; no.: number; *: $P < 0.05$ *Source*: James & Palmer (1977)

[a] Results presented as no. of animals with the finding / no. of animals examined.

[b] Dosing error.

[c] Results presented as no. of animals with the finding / no. of animals examined and in parentheses as % of the animals examined.

[d] Results expressed as % of the animals examined.

The NOAEL for maternal toxicity was 1000 mg/kg bw per day based on decreased body weight gain and mortality at 4000 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day based on lower litter weight, lower mean fetal weight, incidences of major malformations (e.g. fused rib, hydrocephaly, transposed aortic arch), minor visceral and skeletal anomalies and sternebral variants at 4000 mg/kg bw per day (James & Palmer, 1977).

Rabbit

In a non-GLP prenatal toxicity study, Pasquet & Le Bail (1976) administered fosetyl-Al (batch no. not stated; purity 99.8%) in a 10% solution of gum arabic to New Zealand White rabbits (n = 20/group) by gavage at dose levels of 150, 250 or 500 mg/kg bw per day from gestation day 6 to 16. The dose volume was 5 mL per kg body weight. Control animals received the vehicle. Body weight was recorded on gestation day 0, 6, 16 and 28. Feed intake was recorded daily from gestation day 0 to 27. Does were killed on gestation day 28. Live fetuses were weighted and examined externally. The fetal necropsy examined the thoracic organs, abdominal organs, eyes and brain. Skeletons were stained with Alizarin Red S to examine the bone segments.

No deaths were attributable to the test article; 15/88 does were excluded because of accidental death (gavage trauma or uterine and/or pulmonary disorder at time of scheduled kill). Feed consumption was decreased at the high dose, most markedly towards the end of treatment. Weight gain at the mid and high dose was lower than in controls. No effect on the pregnancy rate, litter resorption or total litter loss was observed. There was no effect on the number of implantation sites, fetal loss (resorptions and dead fetuses), numbers of live fetuses per litter, fetal weight or skeletal ossification. There were

no malformed fetuses in the low- or high-dose groups; three malformed young were observed in controls (with dilatation of the aortic arch and pulmonary artery; malrotated paw [talipes]) and two in the mid-dose group (with dilatation of the aortic arch plus dorsal spondylolithasis plus two fused ribs; cyclocephalus). The malformations were considered within the animal background levels (the study authors cite backgrounds of 0.29%, 0.16% and 0% for cardiovascular malformations, vertebral malformations and cyclocephalus, respectively).

The study report lacked many of the details needed to sufficiently evaluate and interpret the results.

The NOAEL for maternal toxicity was 125 mg/kg bw per day based on the reduced body weight and body weight gain at 250 and 500 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest tested dose (Pasquet & Le Bail, 1976).

In a GLP-compliant oral range-finding study of embryo/fetal development in rabbits, fosetyl-Al (batch no. 9810111; purity 981 g/kg) was administered to New Zealand White pregnant rabbits ($n = 5$ per group) by gavage (0.5% Methocel) at dose levels of 75, 125, 250, 500 or 1000 mg/kg bw per day from gestation day 4 to 28 (Thornton, 2000a). Measured end-points included clinical signs, body weight and feed consumption, and at necropsy, gravid uterine weight, number of corpora lutea and implantation data. Fetuses were removed, evaluated for external malformation and weighed.

All animals at 1000 mg/kg bw per day group were humanely euthanized on gestation day 10–13 due to severe decreases in maternal body weight and feed consumption. At 500 mg/kg bw per day, decreased body weight, body weight gain and feed consumption were observed (Table 14). At 250 mg/kg bw per day, there was some evidence of decreased body weight gains and feed consumption. At 250 and 500 mg/kg bw per day, there was a slight decrease in the mean number of live fetuses and a slight increase in the mean number of postimplantation losses. However, these findings may be incidental due to the small number of animals used ($n = 5$/group). There were no adverse effects at 75 or 125 mg/kg bw per day. Examination of live fetuses found spina bifida in one fetus exposed to 500 mg/kg bw per day. Analysis of historical data showed that this finding had been previously observed in this strain of rabbit; this finding was therefore considered spontaneous and not treatment related.

This range-finding study was unsuitable for determining points of departure (Thornton, 2000a).

Table 14. Maternal observations in pregnant rabbits in an oral range-finding study of fosetyl-Al

Parameter	Measure per dose level					
	0 mg/kg bw per day (Control)	**75 mg/kg bw per day**	**125 mg/kg bw per day**	**250 mg/kg bw per day**	**500 mg/kg bw per day**	**1000 mg/kg bw per day**
Mortality (dead or killed) [a]	**0/5**	**0/5**	**0/5**	**0/5**	**1/5**	**5/5**
Pregnancy rate (%)	80	100	100	100	75	–
Body weight gain (g) [b]						
Day 4–6	76 ± 38.1	65 ± 21.6	−4 ± 28.0	−10±63.5*	−39±43.5*	−155±66.3**
Day 4–29	517 ± 95.5	536 ± 110.0	440 ± 110.6	358 ± 73.6	312 ± 101	–
Feed consumption (g) [b]						
Day 4–6	45 ± 1.5	46 ± 5.9	43 ± 3.9	42 ± 13.8	33 ± 4.0	23 ± 5.6**
Day 4–29	39 ± 3.7	40 ± 3.3	37 ± 1.4	39 ± 4.8	32 ± 6.7	–
Caesarean section data						
No. of pregnant dams	4	5	5	5	3	–
No. of dams with viable fetuses	4	5	5	5	3	–
Corpora lutea (no./rabbit) [b]	11.8 ± 1.5	11.2 ± 0.84	13.2 ± 2.86	12.2 ± 3.11	11.0 ± 4.36	–
Implantation sites (no./rabbit) [b]	2.0 ± 1.2	1.6 ± 0.9	2.6 ± 2.7	3.2 ± 2.8	1.7 ± 2.1	

(*Continued on next page*)

Parameter	Measure per dose level					
	0 mg/kg bw per day (Control)	75 mg/kg bw per day	125 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1000 mg/kg bw per day
Preimplantation losses (% per rabbit)	2.0	1.6	2.6	3.2	1.7	–
Postimplantation losses (% implants per rabbit)	0	2	2.5	14.5	12.5	
Live fetuses (per rabbit)	9.8	9.4	10.4	7.6	8.0	0
Dead fetuses (% implants per rabbit)	0	0	0	4	6.9	0
Early resorptions (% implants per rabbit)	0	2	2.5	6.5	2.8	0
Late resorptions (% implants per rabbit)	0	0	0	8	2.8	0
Fetal body weight (g)	41.4	45.4	41.7	43.5	42.9	–

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

[a] Results presented as the no. of animals with the finding / no. of animals examined.

[b] Results presented as group mean ± standard deviation.

Source: Thornton (2000a)

A GLP-compliant embryo/fetal toxicity study was conducted in New Zealand White rabbits. Thornton (2000b) administered fosetyl-Al (batch no. 9810111, purity 98.1%) in 0.5% Methocel by gavage to pregnant rabbits (n = 25/group) at 0, 50, 100 and 300 mg/kg bw per day from gestation day 4 to 28 (doses based on the Thornton, 2000a, range-finding study). Measured end-points included clinical signs, body weight, feed consumption, macroscopic findings at necropsy (gravid uterine weight, number of corpora lutea and uterine implantation data). Fetuses were evaluated for malformations and weighed. All fetuses were examined viscerally for malformations and variations, and sexed. The skeletons were stained with Alizarin Red S and Alcian Blue.

Sporadic deaths were observed during this study, with one high-dose death possibly related to fosetyl-Al treatment (Table 15). One low-dose rabbit that had an apparent abortion (the fetus was found under the cage) was humanely euthanized on gestation day 27. As abortions were not seen in other treatment groups and spontaneous abortions are common among rabbits under stress, this death was not considered treatment related. One mid-dose rabbit was found dead on gestation day 11. Postmortem of this rabbit revealed possible gavage trauma: the lungs were discoloured, and the lungs and trachea filled with fluids. Six high-dose rabbits were also found dead; five of these rabbits (two that died on gestation day 6, and the others on gestation days 9, 12 and 18) showed possible gavage trauma, described as discoloured lungs and fluid-filling lungs and trachea. The death of one high-dose rabbit, on gestation day 27, did not appear to be related to gavage trauma. Postmortem of this rabbit revealed discoloured lung foci and haemorrhagic kidneys. The kidneys had bilateral, multiple haemorrhages 0.1–0.2 cm in diameter. The right kidney had extreme discoloration (red) at the medulla/cortex region.

Fused sternebrae were observed in all treatment groups, but without an apparent dose response (there was a single incidence in each treatment group). Examination of the dead fetus exposed at 50 mg/kg bw per day found external malformations (gastroschisis, hindlimb hyperflexion and agenesis of the tail), which could potentially be incidental because there were no such findings at higher dose levels. None of the few malformations occurring in the high-dose group (hydrocephaly, ribs fused or branched, sternebrae fused; one fetus each) were considered related to treatment.

Table 15. Maternal observations in pregnant rabbits following oral administration of fosetyl-Al from gestation day 4 to 28

Parameter	Measure per dose level			
	0 mg/kg bw per day (Control)	50 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
Mortality (gestation day of mortality)[a]	0/25	1/25 (27)	1/25 (11)	6/25 (6, 6, 9, 12, 18, 27)
Mean body weight gain (g)[b]				
GD 0–29	501 ± 229.4	531 ± 189.9	552 ± 209.6	476 ± 208.6
GD 4–6	76 ± 53	78 ± 46	75 ± 54	55 ± 105
GD 6–9	56 ± 50	87 ± 37	43 ± 46	61 ± 63
Feed consumption (g/kg bw per day)[b]				
GD 4–6	43 ± 10.5	47 ± 5.7	45 ± 6.3	44 ± 13.6
GD 27–29	21 ± 8.2	26 ± 10.6	31 ± 9.1**	29 ± 7.5**
GD 4–29	39 ± 7.5	40 ± 7.6	41 ± 7.0	38 ± 6.1
Pregnancy rate	88.0	95.8	95.8	94.7
Caesarean section data				
No. of pregnancies	22	23	23	19
No. of dams with viable fetuses	22	23	23	18[c]
Corpora lutea (no./rabbit)	10.9	11.8	10.7	11.6
Implantation sites (no./rabbit)	8.7	9.5	8.5	8.7
Preimplantation loss (% per rabbit)	20.2	18.8	22.1	24.6
Postimplantation loss (% implants per rabbit)	4.4	8.2	2.1	6.3
Live fetuses				
Total no.	183	200	190	148
No. of males (total)	98	109	91	77
No. of females (total)	85	91	99	71
Early resorptions (% of implants per rabbit)	1.9	0.9	1.4	3.9
Late resorptions (% of implants per rabbit)	2.5	6.8	0.7	2.4
Fetal body weight (g)[b]	45.3 ± 3.91	44.7 ± 3.44	45.6 ± 4.33	43.8 ± 4.77

bw: body weight; GD: gestation day; no.: number; **: $P < 0.01$

[a] Results presented as the no. of animals with the finding / no. of animals examined.

[b] Results presented as group mean ± standard deviation.

[c] One pregnant rabbit was found dead on GD 27.

Source: Thornton (2000b)

A reanalysis of the incidence of fetal findings by the sponsor found a statistically significant increase in the incidence of distended ureters in high-dose fetuses (Table 16). Distended (dilated) ureter was found in a single fetus per litter and was often observed bilaterally. This effect occurred in the absence of maternal toxicity, suggesting that it may be a direct effect of the test article on the fetus. In an external review solicited by the sponsor, a developmental toxicology expert acknowledged that the effect may be related to the test article, but emphasized that distended ureters are considered a developmental variation. By definition, a variation is "an alteration that represents a retardation in development, a transitory alteration, or a permanent alteration not believed to adversely affect survival, growth, or development" (French, 2017). The developmental toxicology expert concluded that the NOAEL for fosetyl-Al in this study remained at 300 mg/kg bw per day since the observation of distended ureters was a variation and by definition should not be used to set an adverse effect level. The expert stated that this finding may instead be used to set a no-observed-effect level of 100 mg/kg bw per day.

Table 16. Results of reanalysis of incidences of fetal abnormalities in pregnant rabbits following oral administration of fosetyl-Al from gestation day 4 to 28

	Measure per dose level[b]							
	0 mg/kg bw per day (Control)		50 mg/kg bw per day		100 mg/kg bw per day		300 mg/kg bw per day	
Parameter[a]	Fetal incidence	Litter incidence	Fetal incidence	Litter incidence	Fetal incidence	Litter incidence	Fetal incidence	Litter incidence
Malformations								
All malformations	1/183	1/22 (4.5%)	4/201	4 /23 (17.4%)	1/190	1 (4.3%)	3/148	3/18 (16.7%)
Variations, visceral								
Ureter, distended (dilated)	4/183	4/22 (18.2%)	4/201	4/23 (17.4%)	3/190	3 (13.0%)	10/148	10/18 * (55.6%)
Renal papilla, absent	5/183	4/22	4/201	4/23	4/190	4	5/148	5/18

bw: body weight; *Source*: Thornton (2000b)

*: $P < 0.05$, retrospective statistical analysis, Chi-square analysis ($\alpha = 0.05$) followed by Fisher exact test. Several fetuses carried multiple abnormalities.

[a] Malformations and variations were grouped into total incidences, except for noTable visceral variations relevant in the context of fosetyl-Al toxicity. The listing of all malformations and variations is available as supplemental information (Supplemental Table 2).

[b] Results presented as no. of animals with the finding / no. of animals examined and, in parentheses, as % of the animals examined.

Upon review of the study data, the peer review by the developmental toxicology expert and the preceding range-finding study (Thornton, 2000a) data as well as the test article effects described in other studies, the Meeting concluded that the NOAEL for maternal toxicity was 100 mg/kg bw per day based on one maternal death on gestation day 27 at 300 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day based on increased incidence of distended (dilated) ureters in fetuses at 300 mg/kg bw per day. No malformations related to fosetyl-Al exposure were observed (Thornton 2000b).

The death of the high-dose rabbit on gestation day 27 cannot be discounted as not treatment related because there were similar findings in the urinary tract (haemorrhage) in rats exposed to high doses of fosetyl-Al (Palmer et al., 1981; Osborne, 1989). In addition, excessive maternal toxicity was observed in the preceding range-finding study in rabbits (Thornton, 2000a). In that study, the entire high-dose (1000 mg/kg bw per day) cohort was either humanely euthanized (4/5) or found dead (1/5) prior to the end of the study (gestation day 10–13); only one of these rabbits showed evidence of gavage trauma. The limited investigations conducted in the range-finding study prevent understanding the cause of the death of the high-dose rabbits. However, of note is the fact that the rabbits exhibited such severe toxicity at doses much lower than those that elicited severe toxicity in pregnant rats (4000 mg/kg bw per day; Palmer et al., 1981); the 1000 mg/kg bw per day dose did not elicit any sign of severe maternal toxicity in pregnant rats. The data suggest that rabbits may be more sensitive to the effects of fosetyl-Al exposure.

Increased incidence of distended (dilated) ureter cannot be discounted due to the similarity of this effects with the effect of fosetyl-Al observed in other studies. Fetal ureters may become distended or dilated because of urinary stasis caused by blockage. In repeated-dose toxicity studies in rats, urinary stasis, hydronephrosis and dilated ureters were observed after the formation of calcium phosphonate or calcium phosphate uroliths (Spicer, 1981a; Osborne, 1989). In the context of the dataset associated with fosetyl-Al, the increased incidence of distended (dilated) ureters in high-dose group fetuses was considered adverse.

2.6 Special studies

(a) Neurotoxicity

In an acute delayed neurotoxicity study, Jones (1982) administered fosetyl-Al (batch no. DA 203; purity 97.5%) by gavage to domestic hens (n = 10) at 2000 mg/kg bw. Two control groups (n = 10/group) were included: a positive control (750 mg/kg bw triorthocresyl phosphate [TOCP]) and a negative control (vehicle: 0.5% weight per volume [w/v] sodium carboxymethylcellulose). Measured end-points included clinical signs, evaluation of neurotoxicity (examination daily while hens were moving freely in an open pen – animals received a qualitative scoring), body weight and feed consumption. At necropsy, the entire spinal cord was removed, as were both sciatic nerves and the proximal part of peroneal and tibial nerves from each leg, and the brain was removed intact. Histology was performed on the brain, spinal cord and sciatic, peroneal and tibial nerves.

No clinical signs of toxicity or significant effects on body weight or feed consumption were observed with the vehicle control or fosetyl-Al groups. No significant treatment-related findings were found in the histological examination of the hens treated with fosetyl-Al.

Clinical signs consistent with cholinergic poisoning were observed with the positive controls: 6/9 surviving positive control hens developed signs of delayed neurotoxicity, identified by ataxia, between 9 and 18 days after TOCP treatment. Axonopathy was observed in several sites, notably in the cervical cord and peripheral nerve.

There was no evidence that fosetyl-Al induces neurotoxicity under the conditions of this study (Jones, 1982).

Although no stand-alone neurotoxicity assessments in rodents or dogs were performed, functional end-points were included in the short-term toxicity studies, including acetylcholinesterase assays; none demonstrated evidence of neurotoxicity due to exposure to fosetyl-Al). There was no evidence in any of the repeated-dose toxicity studies in rodents or dogs that fosetyl-Al induces neurotoxicity.

The committee concluded that fosetyl-Al does not induce delayed neuropathy.

(b) Mechanistic studies

In a four-week dietary mechanistic study, Kalifat et al. (1981) administered fosetyl-Al to CD rats (n = 5/sex per group) at 0, 10 000, 20 000 or 40 000 ppm. The animals were observed daily. Body weight, feed intake, clinical chemistry and urine analysis measurements were taken weekly. Urine analysis end-points included 24-hour volume, calcium levels and phosphorous levels. Clinical chemistry measurements included calcium levels and phosphorous levels. The amount of calcium and phosphorous in faeces was determined on a weekly basis. At the end of the treatment period, the principal organs were examined macroscopically, and the thyroid, parathyroids and kidneys were examined microscopically.

No deaths or clinical signs attributable to the test article were observed at any concentration. There was one accidental death of a female rat (at 20 000 ppm) during blood sampling. There was no significant difference in feed intake across groups. Body weight gain was similar to controls except for mid-dose males, which had a decrease of 7% after two weeks of treatment. Serum calcium and phosphorous levels of the treated animals were similar to the controls. Increases in phosphorous levels were seen in high-dose female rats, but these were within the range for control females in the study. The volume of urine at 24 hours was similar across treatment groups. In high-dose animals, an increase in calciuria was especially apparent in male animals (4–6 × control values); the highest value was obtained two weeks after treatment and a decrease was observed at the end of treatment. The increase in calciuria was dose dependent: mid-dose males had a value 2–3 times that of control for the first three weeks. In these mid-dose males, the calciuria was equivalent to control at the end of the treatment period. No significant difference was observed between control and low-dose animals.

A similar trend with phosphorous levels in urine was not observed.

Faecal weight did not vary significantly across groups. High-dose males had a slight increase in faecal calcium levels, but this increase was within the range of values for controls. At the highest concentration, there was a significant increase in phosphorous levels in the faeces from week 2 of treatment onwards. In female rats, the increase was slight and not generally considered to be significant.

The mid-dose males showed an increase in faecal phosphorous after 3 and 4 weeks of treatment, but the value was within the range of the control.

All male rats except two mid-dose animals had signs of vacuolar degeneration of the epithelial cells of the renal tubules. The number of affected cells increased with concentration of fosetyl-Al in a dose–responsive manner. The vacuoles appeared to contain hyaline coloured with methyl green. Cell necrosis and desquamation inside the lumen were rarely observed. No other histopathological findings were reported by the study authors.

In summary, high-dose male rats had an increase in calciuria that was less pronounced at the end of treatment; a decrease in urinary phosphorous; an increase in faecal phosphorus; and vacuolar degeneration in the renal tubules. At the mid dose, the changes were similar but less pronounced, and the effects at the low dose were not significantly different from controls. These findings support the conclusion that administration of a high dose of fosetyl-Al causes an increase in calcium in urine as well as renal toxicity.

Mechanism of action

Key event 1
High doses of phosphonate lead to increased calcium concentration in urine. This is noted after one week of treatment in the mechanistic study (Kalifat et al., 1981) and persists for 13 weeks (Osborne, 1989). The mechanism by which this occurs has not been investigated.

Key event 2
High calcium in the urine eventually exceeds calcium phosphonate or calcium phosphate solubility, resulting in bladder calculi composed of calcium and phosphorus. This is seen after two weeks of fosetyl-Al treatment in male rats at 30 000 and 50 000 ppm. Uroliths in the kidney were noted after two weeks of treatment of high-dose rats of both sexes. After 13 weeks, mid-dose animals of both sexes also showed kidney urolithiasis (Osborne, 1989).

Key event 3
The presence of calculi and uroliths causes a chronic mechanical irritation and damage of the urothelium and the transitional cell epithelium. The resulting urothelial repair activity is a proliferative stimulus that leads to papillary and transitional cell hyperplasia. This is a well known phenomenon in rodent bladder carcinogenesis and can be observed with chemically induced calculi as well as with foreign objects implanted into the urinary bladder (reviewed by Clayson, Fishbein & Cohen, 1995). Evidence of the persistent presence of uroliths was observed by Osborne (1989). In this study, uroliths were observed histopathologically 21 weeks after the cessation of exposure.

Applying the International Programme on Chemical Safety (IPCS) Human Relevance Framework to fosetyl-Al

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

> **Yes**: the development of uroliths in the urinary tract by high doses of fosetyl-Al could be clearly demonstrated in a time- and dose-dependent fashion. The development of urothelial neoplasia in rodents in response to bladder calculi is well established. A genotoxic MOA can be excluded due to the unambiguously negative genotoxicity database for fosetyl-Al. The urolithiasis is not consistent across studies and was only seen in a small number of animals with the bladder tumours; however, it is possible that uroliths may have been lost during the processing of histopathological samples.

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

> **No**: urinary bladder calculi, irrespective of composition, cause irritation and cell proliferation in humans. There is some epidemiological evidence that urinary tract cancer in humans is associated with a history of calculi in the bladder. The risk in humans may not be as great as that in rodents because the calculi are usually voided spontaneously or removed by surgical procedures. Thus, although there are quantitative differences in the carcinogenic response to calculi between species, the effect is not species-specific. However, calculus

formation is dependent on attainment in the urine of critically high concentrations of the constituent chemicals which form the calculus. The carcinogenic effects are also dependent on reaching a threshold concentration for calculus formation (IARC, 1999).

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

Yes: healthy humans have very low concentrations of urinary protein and much lower urinary osmolalities than rodents, two of the critical parameters required for the formation of cytotoxic calcium phosphate-containing precipitate (IARC, 1999).

Furthermore, the LOAEL for eliciting Key event 1 (increased urinary calcium) and Key event 2 (calculus formation) is 30 000 ppm (Osborne, 1989). Likewise, the substance intake at the LOAEL for bladder tumours in the two-year rat study (Spicer, 1981a) was greater than 4500 mg/kg bw per day during the first two weeks of exposure when increased calcium excretion was already detectable in the Kalifat et al. (1981) study. These doses are much higher than the practical limit dose of 1000 mg/kg bw per day described in the relevant OECD Test Guideline 453.

In summary, fosetyl-Al at high doses (>1000 mg/kg bw per day) results in precipitation of calcium phosphonate or calcium phosphate and formation of uroliths in the urine. These uroliths cause sustained irritation, inflammation and cytotoxicity to urothelial cells and induce regenerative urothelial hyperplasia that progresses to transitional cell carcinomas, particularly in the bladder and kidney. Furthermore, this carcinogenic MOA only occurs at doses high enough to cause calcium phosphate or calcium phosphonate precipitation in urine. Human exposure levels will not approach the solubility limit of calcium phosphate or calcium phosphonate. Therefore, fosetyl-Al is unlikely to pose a carcinogenic risk to humans from the diet.

(c) Studies on metabolites

The main metabolite of fosetyl-Al, phosphonic acid, was tested in biochemical studies, short-term toxicity studies, a chronic toxicity study in rats and genotoxicity studies as either the free acid or the sodium or potassium salts.

In a toxicokinetic study, sodium [^{32}P]phosphonate (specific activity of 5.92×10^6 Bq/mmol per L [0.16 mCi/mM]; purity not stated) was added to nonradioactive material and dissolved in water. Unsworth (1977b) administered the test article orally for seven days as single doses of 111 mg/kg bw per day to Sprague Dawley rats (n = 3/sex). Urine and faeces were collected every 24 hours until 72 hours after the final dose. Blood samples were taken from each animal at regular intervals during the 24 hours after the first and last doses as well as at 24-hour intervals after the first dose for the duration of the study. Tissue samples, urine, faeces and blood were assayed for radioactivity using liquid scintillation counting. Washings from the metabowl were also assayed for radioactivity.

Labelled phosphonate was excreted predominately in urine (59–65%) and faeces (30–32%), with 1–2% of the administered dose remaining in the body at study end. The highest amounts of radioactivity were found in the spleen in the tissue samples; the lowest levels were found in the fat. These residues may arise from radiolabelled phosphorous incorporated into endogenous metabolic chemicals. The maximum level (C_{max}) in blood was reached 1–2.5 hours after the ingestion of the labelled material. The half-life in blood was estimated to be 1–3 hours. The radioactivity level in blood after the final dose showed an apparent half-life between 2 and 12 hours, and was likely influenced by the radioactivity remaining in the animal. The data indicate that elimination of radioactivity was at least a two-stage process, with a rapid first stage and a slower second stage. The data do not support calculation of an elimination rate for the second phase because the decline in the levels of radioactivity for the first 72 hours after final dose was too small. The residual radioactivity in blood will arise from the parent compound as well as from endogenous incorporation of the labelled material (Unsworth, 1977b).

In a follow-up study on the metabolism of sodium [^{32}P]phosphonate, Unsworth (1978) examined urine and extracts from various samples from the Unsworth (1977b) study by thin-layer chromatography and by gas–liquid chromatography to identify metabolites. Thin-layer chromatography of urine and

faeces showed that there was a single major radioactivity component that corresponded to the parent radiolabel. Labelled phosphonate was also found in the tissue extracts after methylation and analysis by gas–liquid chromatography. The majority (59–65%) of labelled substance was excreted unchanged in urine, and 30–32% of the labelled substance was excreted in faeces. Up to 35% of the radiolabel excreted in faeces was identified as [^{32}P]phosphate; the remainder of the radiolabel in faeces was associated with [^{32}P]phosphonate. Overall conversion to phosphate was around 10% of the administered dose.

Acute toxicity

Phosphonic acid or its sodium or potassium salts exhibited low acute oral toxicity in rats and mice ($LD_{50} \geq 1600$ mg/kg bw), inhalation toxicity in rats ($LC_{50} > 6.14$ mg/L) and dermal toxicity in rabbits ($LD_{50} > 2000$ mg/kg bw) (Pasquet & Mazuret, 1977b; Kieran, Punler & Walker, 1994; Wilson, 1995) (see Table 17).

Table 17. Summary of acute toxicity studies with phosphonic acid and its sodium or potassium salts

Test article	Route	Species	Strain	Sex	Purity (%)	LD_{50}/LC_{50}	Reference
Phosphonic acid	Oral	Rat	CD (COBS)	M + F	Not stated	3.0 g/kg bw	Pasquet & Mazuret (1977b)
Phosphonic acid	Oral	Mouse	OF1 (SPF)	M + F	Not stated	1.6 g/kg bw	Pasquet & Mazuret (1977b)
Sodium phosphite	Oral	Rat	CD (COBS)	M + F	Not stated	5.3 g/kg bw	Pasquet & Mazuret (1977b)
Sodium phosphite	Oral	Mouse	OF1 (SPF)	M + F	Not stated	2.4 g/kg bw	Pasquet & Mazuret (1977b)
Potassium phosphite	Oral	Rat	SD	M + F	Not stated	3.6 (range 3.1–4.2) g/kg bw	Wilson (1995)
Potassium phosphite	Inhalation	Rat	SD	M + F	Not stated	> 6.14 mg/L	Kieran, Punler & Walker (1994)
Potassium phosphite	Dermal	Rabbit	NZW	M + F	Not stated	> 2.0 g/kg bw	Wilson (1994a)

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

Phosphonic acid was not irritating to the skin of rabbits (Wilson, 1994b) and was slightly irritating to the eyes of rabbits (Wilson, 1994c) (Table 18).

In all studies, phosphonic acid or its salts had LD_{50} or LC_{50} values in the same order of magnitude as observed with fosetyl-Al. The observed effects in decedents were consistent with gastrointestinal tract irritancy.

Table 18. Dermal and ocular irritation of potassium phosphonate

End-point	Species	Strain	Sex	Purity (%)	Result	Reference
Skin irritation	Rabbit	NZW	M	Not stated	Not irritating	Wilson (1994b)
Eye irritation	Rabbit	NZW	M	Not stated	Slightly irritating[a]	Wilson (1994c)

M: male

[a] Slight conjunctival redness in 4/6 treated eyes with slight to moderate discharge in all treated eyes one hour after instillation. A full recovery was established 24 hours after instillation.

Short-term toxicity

In an oral toxicity study, Ganter (1978) administered monosodium phosphonate (anhydrous NaH_2PO_3: 69.3%; water: 27.7%; batch no. DA88) to CD-COBS rats (n = 15/sex per group) for three months at dose levels of 0, 2500, 5000 or 25 000 ppm (equal to 0, 100, 200 and 1200 mg/kg bw per day for males and 0, 100, 300 and 1600 mg/kg bw per day for females, respectively, expressed as phosphonic acid). Two control groups were used, one was fed the normal diet only, and the other was fed sodium chloride mixed with feed at a concentration equivalent to the theoretical sodium concentration of the highest treatment group (about 5500 ppm). Measured end-points included daily "general examination"; feed/water intake and body weights; haematology (haemoglobin, microhaematocrit, red and white blood cell counts, differential leukocyte count); clinical chemistry (serum electrolytes, glucose, urea, bromsulfophthalein [BSP], transaminases [aspartate aminotransferase and SGPT], alkaline phosphatase); urine analysis (volume and pH, electrolytes, glucose, albumin, urobilin, bile salts, urinary sediment); bone calcium and phosphate; and macroscopic and microscopic pathology.

Two rats were found dead, but the deaths were not attributed to treatment. The authors noted that a large number of rats had excessive incisor growth that impacted their ability to eat. Overall, the authors reported that high-dose rats had increased water intake, diarrhoea, lower urinary pH, increased urine sodium and calcium. However, it was noted that the difference in water intake was relative to the water control group, and there was no significant difference between the water intake of the high-dose phosphonate group and the sodium chloride control group. Mid-dose rats showed only an occasional increase in urine sodium and calcium. Low-dose rats did not have any effects attributable to treatment.

The NOAEL was 5000 ppm (equivalent to 200 mg/kg bw per day) based on diarrhoea, increased water consumption, lower urinary pH, and urinary sodium and calcium excretion at 25 000 ppm (equivalent to 1200 mg/kg bw per day) (Ganter, 1978).

Long-term studies of toxicity and carcinogenicity

In a 27-month chronic toxicity and carcinogenicity study, Spicer (1981c) administered monosodium phosphonate in the diet to Charles River CD rats (n = 60/sex per dose level) to provide dose levels of 2000, 8000 or 32 000 ppm (expressed as anhydrous salt; the product containing 27% water). These exposure levels were equal to phosphonic acid intakes of 0, 83.9, 348 and 1480 mg/kg bw per day for males and 0, 104, 434 and 1820 mg/kg bw per day for females, respectively. Measured end-points included clinical signs, body weight, feed consumption, ophthalmoscopy, haematology and clinical chemistry. An interim kill at 12 months was conducted for 10 rats/sex per group.

Survival was decreased at 24 months for all male treatment groups compared to the control groups, due mainly to increased mortality in the 12–19 month period. Overall, more than 25 animals surviving to scheduled kill in each group. A decrease in body weight was observed in high-dose animals from week 13 onward. Feed efficiency was decreased, particularly during the second year of the study. The only notable clinical finding was that high-dose males had soft stools.

Haematology and clinical chemistry effects lacked consistency and progression and were not considered toxicologically relevant. Slight but significant decreases in red blood cells, haemoglobin and haematocrit were seen at 12 months but not at other intervals in the mid- and high-dose males. There was a tendency towards reduced pH in males (significant at months 6, 12, 18 and 24), but no other urine analysis effects were noted.

Relative organ weights for liver, kidney and heart for males and kidney and heart for high-dose females were increased. At 12 months, relative kidney weights were found to be statistically significant, while at 27 months both relative and absolute kidney weights were significantly elevated, possibly related to hydronephrosis and urinary stasis (Table 19). The study authors considered the incidence of chronic nephritis within the expected range for this strain and species. No evidence of increased neoplasia in treated animals was observed. There were no reports of increased urolithiasis.

The NOAEL for chronic toxicity of monosodium phosphonate was 8000 ppm (equal to 348 mg/kg bw per day sodium phosphonate or 274 mg/kg per day expressed as phosphonic acid) based on soft stools, decreased body weight, decreased feed efficiency, decreased urine pH and increased relative

kidney weight observed at 32 000 ppm (equal to 1480 mg/kg bw per day sodium phosphonate or 1160 mg/kg bw per day expressed as phosphonic acid).

There was no evidence of increased incidence of neoplasia as a result of exposure to fosetyl-Al (Spicer, 1981c).

Table 19. Renal findings in 27-month chronic toxicity and carcinogenicity study of dietary monosodium phosphonate in rats

Treatment length / sex	Measure per dose per sex				
	Body weight (g)	Kidney weight (g)	Kidney : body (%)	Chronic nephritis [a,b]	Hydronephrosis [a,b]
12 months					
Controls 0 ppm					
Male	716	4.34	0.61	4/12 (33%)	0/12 (0%)
Female	417	2.71	0.66	1/11 (9%)	0/11 (0%)
Phosphonate 32 000 ppm					
Male	691	5.14	0.75*	9/13 (69%)	1/13 (8%)
Female	360	2.95	0.83*	2/12 (17%)	0/12 (0%)
27 months					
Controls 0 ppm					
Male	747	5.73	0.79	40/48 (83%)	2/48 (4%)
Female	470	3.34	0.72	12/49 (25%)	2/49 (4%)
Phosphonate 32 000 ppm					
Male	633	2.11*	1.15*	43/47 (91%)	6/47 (13%)
Female	424	3.92*	0.95*	37/48 (77%)	4/48 (8%)

ppm: parts per million; *: $P < 0.05$ *Source*: Spicer (1981c)

[a] Includes scheduled and unscheduled deaths.

[b] Results presented as no. of animals with the finding / no. of animals examined and, in parentheses, as a % of the animals examined.

Genotoxicity

Phosphonic acid or its sodium salt tested negative for genotoxicity in bacteriophage induction, bacterial reverse mutation and in vivo mouse micronucleus assays (Siou, 1977; Hofnung, 1978; Willington & Riach, 1994) (Table 20).

Table 20. Summary of genotoxicity of fosetyl metabolites

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vivo						
Monosodium phosphite	Mouse micronucleus	Swiss mice, M	1, 2 or 4 g/kg bw	97	Negative	Siou (1977)
In vitro						
Phosphonic acid	Reverse mutation	*Salmonella typhimurium*	33, 100, 333, 1000, 3333 or 10 000 µg/plate ± S9	65.26	Negative	Willington & Riach (1994)
Phosphonic acid	Bacteriophage induction	*Escherichia coli*	1, 5, 10, 50, 100, 500, 1000 or 2000 µg/plate ± S9	Not stated	Negative	Hofnung (1978)

bw: body weight; M: males; S9: 9000 × *g* supernatant fraction from rat liver homogenate

3. Observations in humans

No human poisoning cases or epidemiological studies on fosetyl metabolites have been published.

The sponsor provided some workplace monitoring information. Occupational medicinal surveillance of personnel employed in the production and handling of fosetyl-Al at Production Site 1 (n = 55, since 2004) includes physical and neurological examinations, heart rate, blood pressure, haematology, liver enzymes, thorax X-ray, electrocardiogram and sonography. At Production Site 2 (n = 180, since 2007), the surveillance also included spirometry, measurement of serum cholinesterase and lipid profile, as well as urine analysis. At both sites, medical examinations were conducted at least annually. No pathological results related to the handling of fosetyl-Al have been reported at either site. No complaints requiring medical visits have occurred.

Comments

Biochemical aspects

Following oral administration to rats at doses of 100–3000 mg/kg bw, ^{14}C-labelled fosetyl-Al was almost completely absorbed and almost completely eliminated in urine, faeces and exhaled air within 24 hours. There was no evidence of accumulation. Fosetyl-Al is metabolized to ethanol, acetic acid, carbon dioxide and phosphonic acid (referred to as phosphonate) (Cameron, 2001; Savage, 1982; Unsworth, 1976a,b, 1977a). The phosphonate is excreted predominantly in the urine (equivalent to 73% of the administered compound) together with unchanged material (26–28% of the administered compound) (Unsworth, 1976b).

Toxicological data

The acute toxicity of fosetyl-Al was studied after oral administration in mice, rats and rabbits (LD_{50} > 2000 mg/kg bw) and dermal administration in rats (LD_{50} > 2000 mg/kg bw) (Pasquet & Mazuret, 1977a; Ito & Kajiwara, 1979; Thouvenin, 1997a,b; Kumar, 2012a; Kumar, 2013a,b) and after inhalation in rats (LC_{50} > 5.11 mg/L) (Coombs & Clark, 1977; Blagden, 1997; Kumar, 2013c). Fosetyl-Al was not irritating to the skin of rabbits (Pasquet & Mazuret, 1981a; Thouvenin, 1997c; Kumar, 2013d) but produced moderate to severe ocular irritation in rabbits (Thouvenin, 1997d; Kumar, 2012b). Fosetyl-Al was not sensitizing in guinea-pigs (Elliott & Seaber, 1979; Manciaux, 1998; Kumar, 2013e).

In short-term studies of toxicity in different species, the most notable effects were seen in the bladder, ureters and kidneys of rats exposed to high doses of fosetyl-Al. In contrast, fosetyl-Al exhibited low toxicity in rat oral toxicity studies at doses less than the limit dose (1000 mg/kg bw per day).

In the first available short-term study, rats were administered fosetyl-Al at dietary concentrations of 0, 1000, 5000 or 25 000 ppm for three months (equal to 0, 75.2, 366 and 1920 mg/kg bw per day for males or 0, 98.0, 480 and 2500 mg/kg bw per day for females, respectively). A slight increase in the incidence of extramedullary haematopoiesis was observed in the spleen of high-dose rats, but there were no corresponding effects on haematology or spleen weight changes. The NOAEL was 25 000 ppm (equal to 1920 mg/kg bw per day) (Coquet, 1977a).

Another short-term dietary toxicity study in rats examined the effects of fosetyl-Al at 0, 8000, 30 000 and 50 000 ppm (equal to intakes of 0, 544, 2130 and 3500 mg/kg bw per day for males and 0, 648, 2400 and 4300 mg/kg bw per day for females, respectively) after exposure durations of 2–13 weeks and recovery periods of 8–16 weeks. The NOAEL was 8000 ppm (equal to 544 mg/kg bw per day) based on histopathological changes in the kidney, impairment of calcium/phosphorous metabolism, calculi and hyperplasia in the urinary bladder observed at 30 000 ppm (equal to 2130 mg/kg bw per day) (Osborne, 1989).

In a subsequent 90-day toxicity study, rats were administered fosetyl-Al in the diet at 0, 2000, 6000 or 20 000 ppm (equal to 0, 128, 383 or 1270 mg/kg bw per day for males and 0, 155, 455 or 1580 mg/kg bw per day for females, respectively). There were two treatment-related deaths at 20 000 ppm (equal to 1270 mg/kg bw per day). The NOAEL was 6000 ppm (equal to 383 mg/kg bw per day) (Dange, 1999).

In the final 90-day toxicity study, rats were administered 0, 2000, 6000 or 20 000 ppm fosetyl-Al in the diet (equal to 0, 118, 363 and 1230 mg/kg bw day for males and 0, 148, 446 and 1430 mg/kg bw per day

for females, respectively). No treatment-related effects were observed. The NOAEL was 20 000 ppm (equal to 1230 mg/kg bw per day), the highest dose tested (Yogesha, 2013).

In a three-month study of oral toxicity in dogs, fosetyl-Al was administered in the diet at 0, 2000, 10 000 or 50 000 ppm (equal to 0, 58, 274 and 1310 mg/kg bw per day for males and 0, 58, 272 and 1450 mg/kg bw per day for females, respectively). No treatment-related effects were observed. The NOAEL was 50 000 ppm (equal to 1310 mg/kg bw per day), the highest dose tested (Coquet, 1977b).

In a two-year toxicity study in dogs, fosetyl-Al was administered in the diet at 0, 10 000, 20 000 or 40 000 ppm (equal to 0, 309, 609 and 1230 mg/kg bw per day for males or 0, 288, 632 and 1190 mg/kg bw per day for females, respectively). The NOAEL was 10 000 ppm (equal to 309 mg/kg bw per day) based on testicular degeneration observed at 20 000 ppm (equal to 609 mg/kg bw per day) (Spicer, 1981b). No other treatment-related effects were observed.

In a 24-month carcinogenicity study, mice were administered fosetyl-Al in the diet at 0, 2500, 10 000 or 30 000 ppm (equal to 0, 352, 1410 and 3960 mg/kg bw per day for males and 0, 409, 1670 and 4550 mg/kg bw per day for females, respectively). There were no treatment-related effects on the incidence of neoplasia or on systemic toxicity end-points. The NOAEL for general toxicity and carcinogenicity was 30 000 ppm (equal to 3960 mg/kg bw per day), the highest dose tested (Spicer & Richter, 1981).

In a 24-month carcinogenicity study in rats, fosetyl-Al was administered in the diet at 0, 2000, 8000 or 30 000 ppm (equal to 0, 88, 348 and 1370 mg/kg bw per day for males and 0, 117, 450 and 1790 mg/kg bw per day for females, respectively). Fosetyl-Al exposure resulted in increased incidence of uroliths and mineralization in the urinary bladder, and inflammation, hyperplasia and neoplasia (transitional cell carcinoma) in the urinary transitional epithelium at doses of 30 000 ppm (equal to 1370 mg/kg bw per day). The NOAEL was 8000 ppm (equal to 348 mg/kg bw per day) (Spicer, 1981a).

The Meeting concluded that fosetyl-Al is carcinogenic in rats but not in mice.

Fosetyl-Al 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 fosetyl-Al is unlikely to be genotoxic.

The carcinogenicity in rats was related to the exceedingly high dose administered, resulting in precipitation in the urinary tract. The Meeting concluded that the toxicological data and physical chemistry associated with fosetyl-Al support the following MOA: Fosetyl-Al at high doses (> 1000 mg/kg bw per day) results in precipitation of calcium phosphonate or calcium phosphate and formation of uroliths in the urine. These uroliths cause sustained irritation, inflammation and cytotoxicity to urothelial cells and induce regenerative urothelial hyperplasia that progresses to transitional cell carcinomas, particularly in the bladder and kidney. Furthermore, this carcinogenic MOA only occurs at doses high enough to cause calcium phosphate or calcium phosphonate precipitation in urine. Human exposure levels will not approach the solubility limit of calcium phosphate or calcium phosphonate. Therefore, fosetyl-Al is unlikely to pose a carcinogenic risk to humans from the diet.

In a three-generation study of reproductive toxicity in rats, fosetyl-Al was administered in the diet at 0, 6000, 12 000 or 24 000 ppm (equal to 0, 482, 954 and 1960 mg/kg bw per day for males and 0, 553, 1060 and 2130 mg/kg bw per day for females, respectively). The NOAEL for parental toxicity was 6000 ppm (equal to 482 mg/kg bw per day) based on reduced body weights at 12 000 ppm (equal to 954 mg/kg bw per day). The NOAEL for offspring toxicity was 6000 ppm (equal to 482 mg/kg bw per day) based on reduced pup weight during lactation at 12 000 ppm (equal to 954 mg/kg bw per day). The NOAEL for reproductive effects was 24 000 ppm (equal to 1960 mg/kg bw per day), the highest dose tested (Palmer et al., 1981).

In a study of developmental toxicity, rats were dosed by oral gavage at 0, 500, 1000 or 4000 mg/kg bw per day on gestation days 6–15. The NOAEL for maternal toxicity was 1000 mg/kg bw per day based on mortality and markedly reduced body weight gain at 4000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day based on reduced litter weight, lower mean fetal weight, increased incidence of major malformations (fused rib, hydrocephaly, transposed aortic arch) and minor visceral and skeletal anomalies at 4000 mg/kg bw per day. The fetal effects were seen at a dose associated with maternal lethality that is above the recommended limit dose for this study type; thus, the effects are considered not indicative of specific developmental toxicity (James & Palmer, 1977).

In a rabbit developmental toxicity study, animals were dosed by gavage at 0, 50, 100 or 300 mg/kg bw per day. The NOAEL for maternal toxicity was 100 mg/kg bw per day based on one maternal death on gestation day 27 at 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day based on increased incidence of distended (dilated) ureters in fetuses at 300 mg/kg bw per day. No malformations were observed (Thornton 2000b).

The Meeting concluded that fosetyl-Al was not teratogenic in rabbits; fosetyl-Al was teratogenic in the rat only when tested at excessive dose levels that induced severe maternal toxicity.

Fosetyl-Al did not produce delayed neuropathy in an acute study in hens dosed at 2000 mg/kg bw (Jones, 1982).

None of the neurological end-points included in the short-term toxicity studies, including an acetylcholinesterase assay, demonstrated evidence of neurotoxicity.

The Meeting concluded that fosetyl-Al was not neurotoxic and did not induce delayed neuropathy.

Toxicological data on metabolites and/or degradates

The main metabolite of fosetyl-Al, phosphonic acid, was tested in biochemical studies, short-term toxicity studies, a chronic toxicity study in rats and genotoxicity studies as either the free acid or the sodium or potassium salts.

Sodium [^{32}P]phosphonate was extensively absorbed and rapidly excreted, largely unchanged (Unsworth, 1977b, 1978). The majority (59–65%) of labelled substance was excreted unchanged in urine, and 30–32% of the labelled substance was excreted in faeces. Up to 35% of the radiolabel excreted in faeces was identified as ^{32}P-labelled phosphate; the remainder of the radiolabel in faeces was associated with ^{32}P-labelled phosphonate. Overall conversion to phosphate was around 10% of the administered dose.

Phosphonic acid or its sodium or potassium salts exhibited low acute oral toxicity in rats and mice ($LD_{50} \geq 1600$ mg/kg bw), inhalation toxicity in rats ($LC_{50} > 6.14$ mg/L) and dermal toxicity in rabbits ($LD_{50} > 2000$ mg/kg bw) (Pasquet & Mazuret, 1977b; Kieran, Punlerv& Walker, 1994; Wilson, 1995). Phosphonic acid was not irritating to the skin of rabbits (Wilson, 1994b) and was slightly irritating to the eyes of rabbits (Wilson, 1994c).

In a three-month toxicity study in rats, monosodium phosphonate was administered in the diet at 0, 2500, 5000 or 25 000 ppm (equal to 0, 100, 200 and 1200 mg/kg bw per day for males and 0, 100, 300 and 1600 mg/kg bw per day for females, respectively, expressed as phosphonic acid). Diarrhoea, increased water consumption, lower urinary pH and increased urinary sodium and calcium excretion were observed at 25 000 ppm (equal to 1200 mg/kg bw per day phosphonic acid). The NOAEL was 5000 ppm (equal to 200 mg/kg bw per day phosphonic acid) (Ganter, 1978).

In a 27-month chronic toxicity and carcinogenicity study in rats, monosodium phosphonate was administered in the diet at 0, 2000, 8000 or 32 000 ppm (equal to 0, 83.9, 348 and 1480 mg/kg bw per day for males and 0, 104, 434 and 1820 mg/kg bw per day for females, respectively). Soft stools, decreased body weight, decreased feed efficiency, decreased urine pH and increased relative kidney weight were observed at 32 000 ppm (equal to 1480 mg/kg bw per day sodium phosphonate or 1160 mg/kg bw per day when expressed as phosphonic acid). The NOAEL was 8000 ppm (equal to 348 mg/kg bw per day sodium phosphonate or 274 mg/kg per day when expressed as phosphonic acid) (Spicer, 1981c). There was no evidence of increased neoplasia in treated animals.

Phosphonic acid or its sodium salt tested negative for genotoxicity in bacteriophage induction, bacterial reversevmutation and in vivo mouse micronucleus assays (Siou, 1977; Hofnung, 1978; Willington & Riach, 1994).

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisoning in humans was available.

The Meeting concluded that the existing database on fosetyl-Al 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 fosetyl-Al of 0–1 mg/kg bw based on the NOAEL of 100 mg/kg bw per day for maternal and embryo/fetal toxicity from the rabbit developmental toxicity study. A safety factor of 100 was applied.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for fosetyl-Al in view of its low acute oral toxicity and the absence of embryo/fetal toxicity and any other toxicological effects that would be likely to be elicited by a single dose. An increase in malformations in a rat developmental study was seen at 4000 mg/kg bw per day, with a NOAEL of 1000 mg/kg bw per day, which is above the trigger level used by JMPR for ARfDs. No effects were seen at the beginning of dosing in the rabbit developmental study and the effects on fetuses (dilated ureter) are considered unrelated to a single dose.

Phosphonic acid, the major metabolite, is toxicologically similar to the parent and was considered to be covered by the ADI of fosetyl-Al.

Levels potentially relevant to risk assessment of fosetyl-Al

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a]	Toxicity	30 000 ppm, equal to 3960 mg/kg bw per day [b]	–
		Carcinogenicity	30 000 ppm, equal to 3960 mg/kg bw per day [b]	–
Rat	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	8000 ppm, equal to 348 mg/kg bw per day	30 000 ppm, equal to 1370 mg/kg bw per day
		Carcinogenicity	8000 ppm, equal to 348 mg/kg bw per day	30 000 ppm, equal to 1370 mg/kg bw per day
	Three-generation study of reproductive toxicity [a]	Reproductive toxicity	24 000 ppm, equal to 1960 mg/kg bw per day [b]	–
		Parental toxicity	6000 ppm, equal to 482 mg/kg bw per day	12 000 ppm, equal to 954 mg/kg bw per day
		Offspring toxicity	6000 ppm, equal to 482 mg/kg bw per day	12 000 ppm, equal to 954 mg/kg bw per day
	Developmental toxicity study [c]	Maternal toxicity	1000 mg/kg bw per day	4000 mg/kg bw per day
		Embryo/fetal toxicity	1000 mg/kg bw per day	4000 mg/kg bw per day
Rabbit	Developmental toxicity study [c]	Maternal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
		Embryo/fetal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
Dog	Three-month [a]	Toxicity	50 000 ppm, equal to 1310 mg/kg bw per day [b]	–
	Two-year [a]	Toxicity	10 000 ppm, equal to 309 mg/kg bw per day	20 000 ppm, equal to 609 mg/kg bw per day

[a] Dietary administration.

[b] Highest dose tested.

[c] Gavage administration.

AccepTable daily intake (ADI; applies to fosetyl-Al and phosphonic acid)

0–1 mg/kg bw per day fosetyl-Al

Estimate of acute reference dose (ARfD)

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 fosetyl-Al

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; > 90%
Dermal absorption	No data
Distribution	Widespread: highest concentrations in fat, adrenals, skin, fur, testes and kidneys
Potential for accumulation	Low
Rate and extent of excretion	Rapid: Largely complete by 24 h; primarily via urine
Metabolism in animals	Converted to ethanol, acetic acid, carbon dioxide and phosphonic acid
Toxicologically significant compounds in animals, plants and the environment	Fosetyl-Al, phosphonic acid
Acute toxicity	
Rat, LD_{50}, oral	5250 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.11 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Moderately irritating
Guinea-pig, dermal sensitization	Non-sensitizing (Magnusson–Kligman)
Repeated-dose studies of toxicity	
Target/critical effect	Urinary bladder and kidney/histopathological changes, impairment of calcium/phosphorous metabolism, calculi and hyperplasia
Lowest relevant oral NOAEL	544 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Urinary bladder/hyperplasia and neoplasia of the urinary transitional epithelium
Lowest relevant NOAEL	348 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats but not in mice [a]
Genotoxicity	Unlikely to be genotoxic [a]
Reproductive toxicity	
Target/critical effect	No reproductive effect
Lowest relevant parental NOAEL	482 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	482 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	954 mg/kg bw per day (rat)

(*Continued on next page*)

Developmental toxicity	
Target/critical effect	Ureter/dilated ureter
Lowest relevant maternal NOAEL	100 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	100 mg/kg bw per day (rabbit)
Other toxicological studies	
Phosphonic acid and its sodium or potassium salts	Excreted in urine (59–65%) and faeces (30–32%)
	Oral $LD_{50} \geq 2400$ mg/kg bw (rat), 1600 mg/kg bw (mouse)
	90-day rat: NOAEL 200 mg/kg bw per day, expressed as phosphonic acid
	27-month chronic toxicity rat: NOAEL 274 mg/kg bw per day, expressed as phosphonic acid
	Negative in genetic toxicity assays
	No evidence of carcinogenicity
Human data	None identified

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

Compound		Value	Study	Safety factor
Fosetyl-Al [a]	ADI	0–1 mg/kg bw	Rabbit developmental toxicity	100
	ARfD	Not necessary		

[a] Applies to fosetyl-Al and phosphonic acid, expressed as fosetyl-Al.

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Appendix 1: Histopathology reviews of adrenal medulla slides from Spicer (1981a)

A summary of pathology scoring of adrenal medulla slides from the Spicer (1981a) study is shown in Table A1. The historic control data provided by the sponsor evidenced a mean of 8.9% (range: 0–23.9%) benign phaeochromocytoma and a mean of 0.2% (range: 0–3.8%) malignant phaeochromocytoma between the years 1975 and 1985.

Table A1 Adrenal medullary changes in males

	Findings per dose level[a]			
	Controls	**2000 ppm**	**8000 ppm**	**30 000 ppm**
Hyperplasia				
1st pathologist	16/80	11/79	10/81	9/81
2nd pathologist	5/80	3/80	5/80	4/80
3rd pathologist	15/78	14/74	13/72	16/79
Adenoma ± carcinoma				
1st pathologist	6/80	7/79	16/81	18/81
2nd pathologist	17/80	15/79	19/81	21/81
3rd pathologist	6/78	5/74	10/72	6/79
Total combined				
1st pathologist	22/80	18/79	26/81	27/81
2nd pathologist	22/80	18/79	24/81	25/81
3rd pathologist	21/78	19/74	23/72	22/79

ppm: parts per million; *Source*: Spicer (1981a)

1st pathologist = Dr Kovatch; 2nd pathologist = Dr Richter; 3rd pathologist = Dr Thompson

[a] Results presented as no. of animals with the finding / no. of animals examined.

Table A2. Neoplastic findings in the adrenal medulla of male rats following a 104-week dietary exposure to fosetylAl, as noted in initial and blinded pathology report

	No. per dose level[a]			
Report / neoplastic finding	**0 ppm**	**2000 ppm**	**8000 ppm**	**30 000 ppm**
Initial pathology report				
Combined (A + C)	6/80	7/79	16/81*	18/81*
Adenoma (A)	5/80	7/79	15/81	16/81*
Carcinoma (C)	1/80	0/79	1/81	2/81
Hyperplasia (H)	16/80	11/79	9/81	7/81
All combined (A + C + H)	22/80	18/79	25/81	25/81
Blinded pathology review				
Combined (A + C)	6/78	5/74	10/72	6/79
Adenoma (A)	6/78	5/74	10/72	6/79
Carcinoma (C)	0/78	0/74	0/72	0/79
Hyperplasia (H)	15/78	14/74	13/72	16/79
All combined (A + C + H)	21/78	19/74	23/72	22/79

no.: number; ppm: parts per million; *Source*: Spicer (1981a)

*: $P < 0.05$, significantly different from control (statistical analysis was presented only for the first diagnosis)

[a] Results presented as no. of animals with the finding / no. of animals examined.

Appendix 2: Supplemental tables

The incidence of specific abnormalities observed in James & Palmer (1977), according to the current nomenclature (www.devtox.org), was provided by the sponsor. The incidences of mild to moderate subcutaneous oedema and small interparietal bone were significantly increased in the high-dose group. Frequently, several abnormalities occurred in a single pup, so that more abnormalities appeared to occur in the high dose group, whereas the proportion of pups with abnormalities was not different from controls.

Supplemental Table 1 Incidence of specific malformations and variations (number / % of pups affected) [a]

		Dose (mg/kg bw per day)							
		0		500		1000		4000	
		n	%	*n*	%	*n*	%	*n*	%
Malformations									
No. of pups examined		242	100.0%	204	100.0%	222	100.0%	161	100.0%
No. of litters examined		19	100.0%	18	100.0%	17	100.0%	14	100.0%
Hind limb, malrotated,	per foetus	1	0.4%	–	–	–	–	–	–
	per litter	1	5.3%	–	–	–	–	–	–
Sternebra, fused,	per foetus	1	0.4%	1	0.5%	–	–	–	–
	per litter	1	5.3%	–	–	–	–	–	–
Thoracic centrum, bipartite ossification,	per foetus	1	0.4%	–	–	–	–	1	0.6%
	per litter	1	5.3%	–	–	–	–	1	7.1%
Thoracic centrum, dumbbell-shaped,	per foetus	1	0.4%	–	–	–	–	1	0.6%
	per litter	1	0.4%	–	–	–	–	1	7.1%
Thoracic arch, fused,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Thoracic arch, misaligned,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Lumbar centrum, fused,	per foetus	–	–	1	0.5%	–	–	–	–
	per litter	–	–	1	5.6%	–	–	–	–
Rib, fused,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Rib, branched,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Sternum, split,	per foetus	1	0.4%	–	–	–	–	–	–
	per litter	1	5.3%	–	–	–	–	–	–
Digit, supernumerary,	per foetus	–	–	1	0.5%	–	–	–	–
	per litter	–	–	1	5.6%	–	–	–	–
Abdomen, haemorrhage,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Ventricular septum, defect,	per foetus	–	–	–	–	2	0.9%	–	–
	per litter	–	–	–	–	2	11.8%	–	–

		Dose (mg/kg bw per day)							
		0		500		1000		4000	
		n	%	*n*	%	*n*	%	*n*	%
Kidney, malpositioned,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Hydrocephaly, internal, moderate,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Renal pelvis, increased cavitation, marked,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Subcutaneous oedema,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Azygous vein, transposed,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Aortic arch, transposed,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Ductus arteriosus, transposed,	per foetus	–	–	–	–	–	–	1	0.6%
	per litter	–	–	–	–	–	–	1	7.1%
Variations, visceral									
No. of pups examined		119	100.0%	103	100.0%	108	100.0%	78	100.0%
No. of litters examined		19	100.0%	18	100.0%	17	100.0%	14	100.0%
Testis, malpositioned,	per foetus	5	4.2%	2	1.9%	5	4.6%	2	2.6%
	per litter	3	15.8%	2	11.1%	5	29.4%	2	14.3%
Renal pelvis, increased cavitation,	per foetus	1	0.8%	2	1.9%	2	1.9%	–	–
	per litter	1	5.3%	1	5.6%	1	5.9%	–	–
Abdomen, haemorrhage,	per foetus	2	1.7%	1	1.0%	–	–	–	–
	per litter	2	10.5%	1	5.6%	–	–	–	–
Medulla oblongata, subarachnoid haemorrhage,	per foetus	1	0.8%	–	–	–	–	–	–
	per litter	1	5.3%	–	–	–	–	–	–
Anterior chamber, haemorrhage, mild,	per foetus	1	0.8%	–	–	–	–	–	–
	per litter	1	5.3%	–	–	–	–	–	–
Subcutaneous oedema, mild to moderate,	per foetus	–	–	1	1.0%	–	–	6	7.7%
								***	$p = 0.000\,14$
	per litter	–	–	1	5.6%	–	–	2	14.3%
								N/S	$p = 0.12$
Thorax, internal haemorrhage, moderate,	per foetus	–	–	–	–	1	0.9%	–	–
	per litter	–	–	–	–	1	5.9%	–	–

(*Continued on next page*)

		Dose (mg/kg bw per day)							
		0		500		1000		4000	
		n	%	*n*	%	*n*	%	*n*	%
Variations, skeletal									
No. of pups examined		120	100.0%	100	100.0%	112	100.0%	78	100.0%
No. of litters examined		19	100.0%	18	100.0%	17	100.0%	14	100.0%
Thoracic centrum, bipartite ossification,	per foetus	3	2.5%	1	1.0%	–	–	4	5.1%
	per litter	2	10.5%	2	11.1%	–	–	2	14.3%
Thoracic centrum, dumbbell-shaped,	per foetus	4	3.3%	1	1.0%	1	0.9%	3	3.8%
	per litter	3	15.8%	1	5.6%	1	5.9%	3	21.4%
Thoracic centrum, misshapen,	per foetus	–	–	–	–	–	–	1	1.3%
	per litter	–	–	–	–	–	–	1	7.1%
Presacral vertebrae, supernumerary,	per foetus	3	2.5%	–	–	10	8.9%	–	–
						**	$p = 0.0018$		
	per litter	3	15.8%	–	–	3	17.6%	–	–
		–	–	–	–	N/S	$p = 0.20$	–	–
Pelvic girdle, asymmetry attachment, slight,	per foetus	1	0.8%	2	2.0%	2	1.8%	2	2.6%
	per litter	1	5.3%	2	11.1%	2	11.8%	1	7.1%
General ossification, mild retardation,	per foetus	–	–	4	4.0%	–	–	–	–
				*	p=0.041				
	per litter	–	–	2	11.1%	–	–	–	–
		–	–	N/S	$p = 0.23$	–	–	–	–
Interparietal, small,	per foetus	–	–	–	–	0	0.0%	3	3.8%
								*	p=0.031
	per litter		–		–		–	2	14.3%
								*	p=0.040
Occipital, small,	per foetus	–	–	–	–	–	–	6	7.7%
								***	p=4E-05
	per litter		–		–		–	3	21.4%
								***	p=0.007
Parietal, small,	per foetus	–	–	–	–	–	–	1	1.3%
	per litter	–	–	–	–	–	–	1	7.1%
Rib, supernumerary,	per foetus	–	–	–	–	–	–	1	1.3%
	per litter	–	–	–	–	–	–	1	7.1%
Rib, short,	per foetus	–	–	–	–	–	–	1	1.3%
	per litter	–	–	–	–	–	–	1	7.1%

Statistically significant difference from controls: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in Fisher exact test; retrospective statistical analysis. Chi-square analysis ($\alpha = 0.05$) followed by Fisher exact test.

[a] Several pups carried multiple abnormalities. Therefore. the sum of specific abnormalities (this table) is higher than the combined incidence of pups diagnosed with any abnormality.

Source: James & Palmer (1977)

Supplemental Table 2: Incidences of fetal abnormalities as categorized for malformations and variations by the sponsor (Thornton 2000b)

	Controls		50 mg/kg per day		100 mg/kg per day		300 mg/kg per day	
No. of pups examined	**183**		**201**		**190**		**148**	
No. of litters examined	**22**		**23**		**23**		**18**	
	Fetal incidence	**Litter incidence**	**Fetal incidence**	**Litter incidence**	**Fetal incidence**	**Litter incidence**	**Fetal incidence**	**Litter incidence**
Malformations								
Trunk, spina bifida	0	0	1	1	0	0	0	0
Trunk, gastroschisis	1	1	1	1	0	0	0	0
Tail, absent	0	0	1	1	0	0	0	0
Head, domed	1	0	0	0	0	0	0	0
Face, proboscis	1	1	0	0	0	0	0	0
Eye, open	1	1	0	0	0	0	0	0
Hindlimb, hyperflexion	0	0	1	1	0	0	0	0
Head, hydrocephalus	0	0	0	0	0	0	1	1
Heart/great vessels, anomaly	1	1	1	1	0	0	0	0
Sternebra, fused	0	0	1	1	1	1	1	1
Ribs, fused/branched	0	0	0	0	0	0	1	1
External malformations	1	1	2	2	0	0	0	0
Visceral malformations	1	1	1	1	0	0	1	1
Skeletal malformations	0	0	1	1	1	1	2	2
All malformations	1	1 (4.5%)	4	4 (17.4%)	1	1 (4.3%)	3	3 (16.7%)
Variations, visceral								
Iris, surrounded by haemorrhagic ring	11	8	7	6	9	7	5	5
Heart/great vessels, alteration	7	6	6	5	6	5	5	5
Ureter, distended	4 [a]	4 (18.2%)	4	4 (17.4%)	3	3 (13.0%)	10	10* (55.6%)
Renal papilla, absent	5	4	4	4	4	4	5	5
Gall bladder, absent	2	2	0	0	0	0	0	0
Gall bladder, small	0	0	0	0	2	1	0	0
Variations, skeletal								
Hyoid lesser horn, bent	1	1	0	0	0	0	1	1
Hyoid body/lesser horn, unossified	4	4	1	1	2	2	0	0
Ossification, reduced	0	0	1	1	0	0	0	0
Presacral vertebrae, 25	0	0	1	1	0	0	0	0
Presacral vertebrae, 27	22	12	26	13	25	15	15	6
Rib, 13th rudimentary	17	11	21	9	10	8	17	11
Rib, 13th full	67	22	80	19	65	19	48	14
Pubis, unossified	0	0	2	1	1	1	1	1

Statistically significant difference from controls: $*P < 0.05$; retrospective statistical analysis per request of the RMS. Chi-square analysis (α=0.05) followed by Fisher exact test.

[a] This finding did not occur in control rabbit foetuses within the last 28 developmental toxicity studies in NZW rabbits conducted at Huntingdon Life Sciences.

Source: James & Palmer (1977)

Appendix 2: References

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ISOPROTHIOLANE

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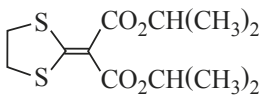
Explanation

Isoprothiolane is the International Organization for Standardization–approved name for diisopropyl 1,3-dithiolan-2-ylidenemalonate (International Union of Pure and Applied Chemistry name), with the Chemical Abstract Service number 50512-35-1. Isoprothiolane is a fungicide used on rice crops. Isoprothiolane belongs to the family of dicarboxylic acids and derivatives, organic compounds containing two carboxylic acid groups, which act by inhibition of phospholipid biosynthesis. Isoprothiolane 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 evaluated in this monograph were performed by laboratories that were certified for good laboratory practice (GLP) and that complied, where appropriate, with the relevant Organisation for Economic Co-operation and Development (OECD) Test Guidelines or similar guidelines of the European Union or United States Environmental Protection Agency (USEPA), unless otherwise indicated.

The chemical structure of isoprothiolane is shown in Fig. 1.

Figure 1. Chemical structure of isoprothiolane



Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

An oral study was conducted to determine the absorption, distribution, metabolism and excretion (ADME) of isoprothiolane. The study was conducted in accordance with the corresponding most recent guidelines of the Japanese Ministry of Agriculture, Forestry, and Fisheries (JMAFF), OECD, USEPA and European Union, and GLP compliance and quality assurance statements were provided. Male and female Sprague Dawley rats (seven weeks of age; weight not specified) received a single oral dose of 5 or 500 mg/kg of [dithiolane-4,5-^{14}C]isoprothiolane (lot no. CP-3018; radiochemical purity > 96%) suspended in olive oil as a vehicle. ADME of radiolabelled isoprothiolane was examined according to the dose groups outlined in Table 1.

Table 1. Doses of radiolabelled isoprothiolane, blood sampling times and other test parameters in absorption, distribution, metabolism and excretion (ADME) experiments

Study/test group name	Dose of radiolabelled isoprothiolane (mg/kg bw)	No. of animals/ dose group	Sex	Sampling time (hours post dose)
Absorption study				
DML	5	4	M	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
DFL	5	4	F	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
DMH	500	4	M	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
DFH	500	4	F	Blood: 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 168
Distribution, metabolism and excretion study (low dose)				
DML-1	5	4	M	Organs/tissues: 6
DML-2	5	4	M	Organs/tissues: 24
DML-3	5	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
DFL-1	5	4	F	Organs/tissues: 6
DFL-2	5	4	F	Organs/tissues: 24
DFL-3	5	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
Distribution, metabolism and excretion study (high dose)				
DMH-1	500	4	M	Organs/tissues: 9
DMH-2	500	4	M	Organs/tissues: 24
DMH-3	500	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

(*Continued on next page*)

Study/test group name	Dose of radiolabelled isoprothiolane (mg/kg bw)	No. of animals/ dose group	Sex	Sampling time (hours post dose)
DFH-1	500	4	F	Organs/tissues: 9
DFH-2	500	4	F	Organs/tissues: 24
DFH-3	500	4	F	Expired air, urine, faeces: 24, 48, 72, 96, 120, 144, 168 Organs/tissues: 168

bw: body weight; F: females; M: males; no.: number *Source:* Yoshizane (2007)

DFL: [dithiolane-4,5-^{14}C]isoprothiolane / female / low-dose study; DMH: [dithiolane-4,5-^{14}C]isoprothiolane / male / high-dose study; DFH: [dithiolane-4,5-^{14}C]isoprothiolane / female / high-dose study; DML: [dithiolane-4,5-^{14}C]isoprothiolane / male / low-dose study

Absorption

Blood samples were drawn from the retro-orbital plexus at 1, 3, 6, 9, 12, 24 hours and every 24 hours thereafter until 168 hours post dose. Plasma samples were obtained by centrifugation of the blood samples. Radioactivity concentration in blood and plasma samples was quantified by liquid scintillation counting.

Concentration and kinetic parameters of the radiolabel in blood and plasma are shown in Table 2. In males and females in the low-dose group (5 mg/kg body weight [bw]), radioactivity in blood and plasma reached a maximum (C_{max}) at 6 hours post dose. In males and females in the high-dose group (500 mg/kg bw), the kinetic profile of radiolabelled isoprothiolane in blood and plasma was comparable to that for the corresponding low dose groups except that time to reach maximum concentration (T_{max}) was slightly longer, at 9–12 hours post dose, which suggests that the absorption rate of the chemical was comparable at both doses.

Table 2. Concentration and kinetic parameters of radiolabel following a single oral administration of [dithiolane-4,5-^{14}C]isoprothiolane to male and female rats

Time (hours post dose)	Radioactivity concentrations per administered dose (µg equiv./g) [a]							
	Males				Females			
	5 mg/kg bw		500 mg/kg bw		5 mg/kg bw		500 mg/kg bw	
	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
1	0.686	1.082	38.9	64.0	1.141	1.808	38.6	61.5
3	1.754	2.767	70.2	108.6	1.999	3.132	66.9	108.4
6	2.120	3.240	122.2	186.0	2.149	3.389	134.4	200.4
9	2.042	3.127	132.5	209.0	2.015	3.098	152.0	227.3
12	1.894	2.950	133.1	207.9	1.929	2.906	161.3	233.4
24	1.124	1.695	69.9	111.5	1.102	1.667	111.5	178.3
48	0.915	0.890	60.2	64.2	0.875	0.926	82.8	109.5
72	0.781	0.660	47.9	42.1	0.727	0.703	65.7	79.2
96	0.593	0.577	34.4	33.9	0.522	0.569	46.3	59.4
120	0.529	0.412	29.3	23.5	0.481	0.394	36.1	34.6
144	0.482	0.327	27.2	17.7	0.415	0.314	30.4	21.3
168	0.505	0.253	27.3	13.1	0.424	0.232	30.4	19.5
T_{max} (h)	6	6	12	9	6	6	12	12
C_{max} (µg equiv./g)	2.120	3.240	133.1	209.0	2.149	3.389	161.3	233.4
$t_{½}$ (day) T_{max} – 48 h	1.36	0.89	1.47	0.92	1.28	0.91	1.64	1.35
48–168 h	5.27	2.68	4.17	2.23	4.47	2.49	3.24	1.89
AUC (µg equiv·h/g)	137.60	152.11	8 356.0	9 629.3	131.09	154.32	10 940.0	14 137.1

AUC: area under the concentration–time curve; C_{max}: maximum or peak concentration in blood or plasma; eq: equivalents; T_{max}: time to reach maximum concentration (C_{max}) bw: body weight;

[a] Mean of four rats per group. *Source:* Yoshizane (2007)

Distribution

[Dithiolane-4,5-^{14}C]isoprothiolane-dosed rats were terminated at 6 (T_{max}), 24 and 168 hours post dose (low-dose group) and 9 (T_{max}), 24 and 168 hours post dose (high-dose group). These times were determined based on the blood and plasma radioactivity profile.

Radioactivity in the organs and tissues was quantified by liquid scintillation counting. Distribution of radioactivity in major organs and tissues of male and female rats is shown in Table 3 and Table 4.

Table 3. Distribution of radioactivity following a single oral administration of [dithiolane-4,5-^{14}C] isoprothiolane to male rats

	Radioactivity concentration (µg equiv./g) per administered dose[a]					
	5 mg/kg bw			500 mg/kg bw		
Organs/tissues	6 h	24 h	168 h	9 h	24 h	168 h
Blood	1.861	1.107	0.397	134.6	65.3	21.9
Plasma	2.174	1.717	0.135	237.8	102.6	14.7
Brain	0.294 (0.05)	0.210 (0.04)	0.154 (0.03)	41.4 (0.08)	13.0 (0.02)	9.6 (0.02)
Pituitary	0.833 (<0.01)	0.976 (<0.01)	0.296 (<0.01)	44.8 (<0.01)	43.7 (<0.01)	23.5 (<0.01)
Eyeball	0.337 (<0.01)	0.253 (<0.01)	0.178 (<0.01)	32.3 (<0.01)	14.4 (<0.01)	10.3 (<0.01)
Thyroid	0.759 (<0.01)	0.714 (<0.01)	0.318 (<0.01)	84.7 (<0.01)	40.6 (<0.01)	22.4 (<0.01)
Salivary gland	1.925 (0.11)	0.927 (0.05)	0.337 (0.02)	102.9 (0.05)	56.5 (0.03)	20.5 (<0.01)
Heart	0.579 (0.04)	0.537 (0.04)	0.333 (0.03)	64.6 (0.05)	34.6 (0.03)	23.1 (0.02)
Lung	1.310 (0.15)	0.895 (0.10)	0.403 (0.05)	153.3 (0.17)	56.0 (0.06)	25.3 (0.03)
Thymus	1.003 (0.06)	1.127 (0.06)	0.386 (0.02)	59.8 (0.03)	44.0 (0.02)	20.1 (<0.01)
Liver	7.713 (5.96)	4.537 (4.94)	1.780 (1.73)	408.1 (3.31)	327.3 (3.57)	114.2 (1.24)
Kidney	3.141 (0.51)	1.996 (0.31)	0.663 (0.11)	219.7 (0.37)	119.6 (0.19)	44.2 (0.07)
Adrenal	1.343 (<0.01)	1.339 (<0.01)	0.471 (<0.01)	148.5 (<0.01)	89.0 (<0.01)	43.8 (<0.01)
Spleen	1.282 (0.07)	1.089 (0.05)	0.395 (0.02)	68.2 (0.03)	44.7 (0.02)	24.2 (0.01)
Pancreas	1.855 (0.10)	0.853 (0.05)	0.364 (0.02)	148.3 (0.08)	87.5 (0.05)	30.6 (0.01)
Stomach	2.235 (0.34)	0.865 (0.13)	0.342 (0.05)	1742.8 (2.70)	244.5 (0.39)	22.9 (0.04)
Small intestine	2.833 (1.67)	1.783 (1.02)	0.304 (0.16)	390.7 (2.21)	134.1 (0.75)	18.4 (0.11)
Large intestine	3.391 (0.86)	1.043 (0.26)	0.275 (0.07)	176.6 (0.46)	76.6 (0.19)	16.7 (0.05)
Bladder	0.871 (<0.01)	0.564 (<0.01)	0.364 (<0.01)	83.7 (<0.01)	52.0 (<0.01)	30.2 (<0.01)
Testis	0.526 (0.13)	0.442 (0.11)	0.253 (0.07)	51.9 (0.13)	29.0 (0.07)	17.7 (0.05)
Prostate	1.126 (0.02)	0.863 (0.02)	0.290 (<0.01)	71.9 (0.02)	56.1 (<0.01)	18.1 (<0.01)
Fat	0.250	0.298	0.336	453.2	63.7	25.4
Muscle	0.336	0.345	0.261	41.6	16.5	15.0
Skin	1.282	1.824	1.164	121.2	78.1	56.8
Hair	0.359	0.551	2.380	67.9	36.0	121.6
Bone marrow	2.515	2.998	0.393	134.2	127.1	64.5
Gastrointestinal content	2.207	2.245	0.921	93.7	87.5	55.3

bw: body weight; equiv.: equivalents *Source*: Yoshizane (2007)

[a] Mean of four rats per group. Numbers in parenthesis are percentage of dosed radioactivity (%).

Table 4. Distribution of radioactivity following a single oral administration of [dithiolane-4,5-^{14}C] isoprothiolane to female rats

Organs/tissues	Radioactivity concentration (µg equiv./g) per administered dose[a]					
	5 mg/kg bw			500 mg/kg bw		
	6 h	24 h	168 h	9 h	24 h	168 h
Blood	1.948	0.972	0.313	104.5	86.3	22.6
Plasma	3.490	1.537	0.133	194.0	149.9	18.1
Brain	0.338 (0.11)	0.208 (0.05)	0.150 (0.03)	35.6 (0.12)	19.5 (0.05)	11.1 (0.03)
Pituitary	0.978 (<0.01)	1.020 (<0.01)	0.232 (<0.01)	40.5 (<0.01)	46.5 (<0.01)	19.2 (<0.01)
Eyeball	0.330 (<0.01)	0.220 (<0.01)	0.158 (<0.01)	24.5 (<0.01)	18.8 (<0.01)	10.8 (<0.01)
Thyroid	0.951 (<0.01)	0.650 (<0.01)	0.245 (<0.01)	68.9 (<0.01)	45.5 (<0.01)	22.3 (<0.01)
Salivary gland	1.869 (0.09)	0.769 (0.04)	0.266 (0.02)	88.8 (0.04)	81.1 (0.04)	21.9 (0.01)
Heart	0.643 (0.05)	0.432 (0.03)	0.273 (0.02)	50.5 (0.04)	34.7 (0.03)	24.4 (0.02)
Lung	1.443 (0.16)	0.833 (0.10)	0.305 (0.04)	84.1 (0.10)	67.8 (0.07)	26.8 (0.03)
Thymus	0.930 (0.06)	0.942 (0.05)	0.319 (0.02)	43.8 (0.02)	50.8 (0.03)	21.7 (<0.01)
Liver	8.030 (5.67)	4.567 (4.72)	1.526 (1.35)	353.0 (2.65)	464.8 (4.61)	128.5 (1.29)
Kidney	3.354 (0.56)	1.918 (0.34)	0.593 (0.10)	173.2 (0.29)	139.5 (0.24)	41.5 (0.08)
Adrenal	1.403 (0.01)[b]	1.105 (<0.01)	0.444 (<0.01)	124.4 (<0.01)	121.7 (0.01)	65.3 (<0.01)
Spleen	0.999 (0.05)	0.864 (0.04)	0.311 (0.01)	59.1 (0.03)	58.7 (0.03)	27.5 (0.01)
Pancreas	1.508 (0.08)	0.737 (0.04)	0.363 (0.02)	151.8 (0.09)	122.1 (0.09)	33.5 (0.02)
Stomach	5.071 (0.78)	0.775 (0.13)	0.261 (0.04)	1 516.2 (2.67)	606.6 (1.09)	22.2 (0.04)
Small intestine	3.168 (1.69)	1.506 (0.94)	0.274 (0.16)	465.4 (2.39)	190.6 (1.14)	22.2 (0.14)
Large intestine	3.174 (0.85)	0.878 (0.24)	0.240 (0.07)	142.9 (0.39)	113.8 (0.33)	17.2 (0.05)
Bladder	1.061 (<0.01)	0.570 (<0.01)	0.285 (<0.01)	107.4 (0.01)	52.3 (<0.01)	31.8 (<0.01)
Ovary	1.290[b] (0.02[b])	1.012 (0.02)	0.255 (<0.01)	101.7 (0.02)	66.7 (<0.01)	22.6 (<0.01)
Uterus	0.814 (0.03)	0.507 (0.02)	0.241 (<0.01)	74.0 (0.03)	64.9 (0.03)	22.0 (0.01)
Fat	0.400	0.453	0.450	449.6	85.5	29.6
Muscle	0.331	0.304	0.217	35.5	19.3	14.6
Skin	0.728	0.792	0.635	83.5	46.7	35.4
Hair/fur	0.340	1.271	0.311	35.2	38.9	14.1
Bone marrow	2.122	2.345	0.337	92.1	148.6	97.0
Gastrointestinal content	2.166	1.844	0.965	73.0	87.7	58.2

bw: body weight; equiv.: equivalents; *Source*: Yoshizane (2007)

[a] Mean of four rats per group. Numbers in parenthesis are percentage of dosed radioactivity (%).

[b] Mean of three rats.

Regardless of sex and dose administered, at around T_{max}, that is, 6 or 9 hours post dose, the highest radioactivity was found in the liver, kidney and gastrointestinal tract. Other than these organs,

radioactivity was distributed in all organs and tissues to a fairly equal extent. At 24 or 168 hours post dose, radioactivity in almost all organs and tissues was eliminated; at 168 hours post dose, hair/fur and skin contained considerably higher levels of radioactivity than other tissues. Thus, characterization of the metabolites in these tissues was performed.

Excretion

Excreta (urine, faeces and expired air) of [dithiolane-4,5-^{14}C]isoprothiolane-dosed rats were periodically collected until 168 hours post dose, and radioanalysed by liquid scintillation counting. Cumulative excretion of radioactivity in males is summarized in Table 5 and in females is summarized in Table 6.

Table 5. Excretion of radioactivity following a single oral administration of [dithiolane-4,5-^{14}C]isoprothiolane to male rats

Time (hours post dose)	Cumulative excretion (% of dosed radioactivity) per administered dose[a]					
	5 mg/kg bw (DML-3)			500 mg/kg bw (DMH-3)		
	Urine	Faeces	Expired air	Urine	Faeces	Expired air
0–24	31.26	11.72	23.87	38.63	3.41	15.58
0–48	32.44	12.32	26.77	51.54	5.80	24.89
0–72	33.13	12.56	28.54	52.29	6.12	26.76
0–96	33.52	12.72	29.58	52.70	6.31	27.76
0–120	33.83	12.86	30.32	52.98	6.43	28.39
0–144	34.04	12.99	30.94	53.18	6.55	28.84
0–168	34.27	13.11	31.40	53.29	6.63	29.22
Cage wash	**0.21**			**0.08**		
Carcass	**11.69**			**8.05**		
Total	**90.68**			**97.27**		

[a] Mean of 4 rats/dose. bw: body weight;
DMH: [dithiolane-4,5-^{14}C]isoprothiolane / female / high-dose study;
DML: [dithiolane-4,5-14C]isoprothiolane / male / low-dose study

Source: Yoshizane (2007)

Table 6. Excretion of radioactivity following a single oral administration of[dithiolane-4,5-^{14}C] isoprothiolane to female rats

Time (hours post dose)	Cumulative excretion (% of dosed radioactivity) per administered dose[a]					
	5 mg/kg bw (DFL-3)			500 mg/kg bw (DFH-3)		
	Urine	Faeces	Expired air	Urine	Faeces	Expired air
0–24	20.55	21.58	23.48	28.74	5.63	15.40
0−48	21.99	22.35	26.15	41.95	9.09	26.61
0−72	22.62	22.60	27.61	44.27	9.68	30.02
0−96	23.02	22.75	28.56	44.87	9.94	31.40
0−120	23.31	22.86	29.34	45.25	10.09	32.28
0−144	23.55	22.97	29.93	45.50	10.21	32.89
0−168	23.71	23.09	30.41	45.67	10.28	33.36
Cage wash	**0.41**			**0.11**		
Carcass	**9.45**			**7.50**		
Total	**87.07**			**96.92**		

[a] Mean of four rats/dose. bw: body weight;
DFH: [dithiolane-4,5-14C]isoprothiolane / female / high-dose study;
DFL: [dithiolane-4,5-14C]isoprothiolane / female / low-dose study;
DMH: [dithiolane-4,5-14C]isoprothiolane / female / high-dose study;
DML: [dithiolane-4,5-14C]isoprothiolane / male / low-dose study

Source: Yoshizane (2007)

In all dose groups, dosed radioactivity was quantitatively recovered in excreta and residual carcass. As shown in Tables 5 and 6, following oral dosing, radioactivity was excreted mainly in urine and expired air. Radioactivity in expired air was confirmed to be $^{14}CO_2$ by $BaCO_3$ precipitation method. In both low and high dose groups, excretion of radioactivity was rapid and essentially complete by 24 and 48 hours post dose, respectively, with only very small amounts excreted thereafter. However, even after 168 hours post dose, approximately 10% of dosed radioactivity was still retained in residual carcass in both sexes and dose groups. Significant amounts of radioactivity (~30% of dosed radioactivity) was excreted into expired air as CO_2, which suggests that one route of metabolism of isoprothiolane involved complete mineralization of the dithiolane ring moiety (Yoshizane, 2007).

1.2 Biotransformation

As described in section 1.1, regardless of sex and administered dose, excretion of dosed radioactivity was almost complete within 72 hours post dose. Excreta obtained in this period were analysed for metabolites. In addition, liver, fur and skin at 6 or 9 (T_{max}), 24 and 168 hours post dose were further analysed for metabolites.

As shown in Table 7, regardless of sex and administered dose, the glucuronic acid conjugate of the monoester (C) was the most prominent metabolite in excreta (urine), accounting for 8.15% and 19.85% (males) and 5.77% and 15.43% (females) of dosed radioactivity in low and high dose groups, respectively. Other metabolites detected in significant quantities were the unconjugated monoester (C) and U9. Chemical structural analysis suggests that U9 was the intermediate metabolite to CO_2 resulting from cleavage of the dithiolane ring moiety. In addition to unconjugated monoester (C) in faeces, unchanged isoprothiolane (A) and 4-hydroxyisoprothiolane (B) were also detected.

In liver analysed at T_{max}, didehydroisoprothiolane (E) was detected in addition to the metabolites found in urine and faeces. In contrast, at 168 hours post dose, only polar metabolites localized to the periphery of the thin-layer chromatography origin were detected, even after development with polar solvent systems. As shown in Table 8, residual radioactivity in fur and skin existed mostly in an unextractable fraction. Characterization of the residual radioactivity in fur at 168 hours post dose suggests that the unextractable radioactivity consists mostly of the keratin fraction. Levels of radioactivity in the keratin fraction from skin were too low to enable their characterization. However, because epidermis consists mainly of keratin, it was considered that radioactivity in skin was incorporated into keratin, as in fur. From the results of metabolite analysis and characterization in liver, fur and skin, isoprothiolane appears to be metabolized to amino acid(s), incorporated into keratin.

Table 7. Excretion of metabolites following a single oral administration of [dithiolane-4,5-^{14}C] isoprothiolane to male and female rats

Metabolites	Metabolites excreted (% of dosed radioactivity) per sex per dose administered [a]							
	Males				Females			
	5 mg/kg bw		500 mg/kg bw		5 mg/kg bw		500 mg/kg bw	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Isoprothiolane (A)	ND	4.79	ND	0.09	ND	6.42	ND	0.06
4-Hydroxyisoprothiolane (B)	ND	0.24	ND	0.19	ND	0.57	ND	0.39
Monoester (C)	1.41	0.19	5.98	0.53	1.06	0.45	4.79	1.29
Monosulfoxide (D)	ND	ND	ND	ND	ND	ND	ND	ND
Didehydroisoprothiolane (E)	ND	ND	ND	ND	ND	ND	ND	ND
Monoester (C) glucuronide	8.15	ND	19.85	ND	5.77	ND	15.43	ND
U9	4.57	ND	7.79	ND	2.82	ND	6.52	ND
Polar metabolites	5.60	1.14	7.32	0.92	5.15	2.76	7.50	1.35
TLC origin	8.85	3.47	6.58	2.58	4.79	6.80	6.82	3.90
Sum of others	4.22[b]	0.76[b]	4.53[c]	0.55[c]	2.71[d]	1.98[d]	2.76[e]	1.19[e]
Unextractable	0.33	1.98	0.25	1.25	0.31	3.61	0.45	1.49
Total	33.13	12.56	52.30	6.12	22.62	22.60	44.27	9.68

(*Table footnotes on next page*)

bw: body weight; ND: not detected *Source*: Yoshizane (2007)

[a] Mean of 4 rats. Excreta obtained until 72 hours post dose respectively from 5 and 500 mg/kg dosed group were analysed.

[b] No individual component exceeded 2.20% and 0.55% of dose in urine and faeces, respectively.

[c] No individual component exceeded 1.82% and 0.46% of dose in urine and faeces, respectively.

[d] No individual component exceeded 1.43% and 2.38% of dosed in urine and faeces, respectively.

[e] No individual component exceeded 0.86% and 1.01% of dosed in urine and faeces, respectively.

Table 8. Characterization of radioactivity in fur and skin at 168 hours following a single oral administration of [dithiolane-4,5-^{14}C]isoprothiolane to male and female rats

	Radioactivity concentration (µg equiv./g) [a]							
	Fur				**Skin**			
	Males		**Females**		**Males**		**Females**	
	5 mg/ kg bw	**500 mg/ kg bw**	**5 mg/kg bw**	**500 mg/ kg bw**	**5 mg/kg bw**	**500 mg/ kg bw**	**5 mg/kg bw**	**500 mg/ kg bw**
Diethylether extractable	0.187 (7.86)	11.4 (9.37)	0.054 (17.40)	2.8 (20.04)	0.067 (5.74)	6.5 (11.52)	0.031 (4.84)	0.0 (0.00)
Tris-HCl extractable	0.037 (1.58)	3.9 (3.21)	0.025 (8.01)	2.9 (20.56)	0.109 (9.34)	9.2 (16.12)	0.095 (14.99)	7.4 (20.94)
Unextractable	2.155 (90.57)	106.3 (87.42)	0.232 (74.59)	8.4 (59.40)	0.988 (84.92)	41.1 (72.36)	0.509 (80.17)	28.0 (79.06)
Keratin fraction	1.697 (71.33)	84.0 (69.13)	0.192 (61.71)	6.3 (45.00)	–	–	–	–
Others	0.458 (19.24)	22.2 (18.29)	0.040 (12.89)	2.0 (14.40)	–	–	–	–
Total	2.380 (100)	121.6 (100)	0.311 (100)	14.1 (100)	1.164 (100)	56.8 (100)	0.635 (100)	35.4 (100)

bw: body weight; equiv.: equivalents *Source*: Yoshizane (2007)

[a] Values in parentheses represent per cent (%) of total radioactivity.

As shown in Table 7, orally dosed isoprothiolane was found to be metabolized by hydroxylation and hydrolysis and also by cleavage of the dithiolane ring, the molecular backbone of isoprothiolane, and mineralized to CO_2.

The disposition of [dithiolane-4,5-^{14}C]isoprothiolane in male and female rats is summarized as follows:

- Rapid and high absorption rate of the radioactivity from the gastrointestinal tract.
- Highest concentrations of radioactivity in organs and tissues around T_{max} were detected mainly in blood and plasma. Radioactivity distribution was higher in liver, kidney and gastrointestinal tract than other organs and tissues.
- Gradual decline of radioactivity concentrations in organs and tissues.
  Radioactivity concentrations in fur and skin were rather constant or increased over time.
- Rapid excretion of radioactivity, mainly into urine and expired air.
- Various metabolic routes, including hydroxylation and hydrolysis, cleavage of the dithiolane ring, mineralization to CO_2 and other low molecular weight metabolites.
- Conversion of dithiolane ring moiety of the chemical towards a component or components of keratin.
- No significant sex- or dose-related differences in metabolic fate.

Based on the results described above, the proposed metabolic pathways of [dithiolane-4,5- ^{14}C] isoprothiolane in rats are shown in in Fig. 2. Compounds formed by the metabolism of isoprothiolane in plants and animals are shown in Table 9.

Figure.2. Proposed metabolic pathways of [dithiolane-4,5-^{14}C]isoprothiolane in rats

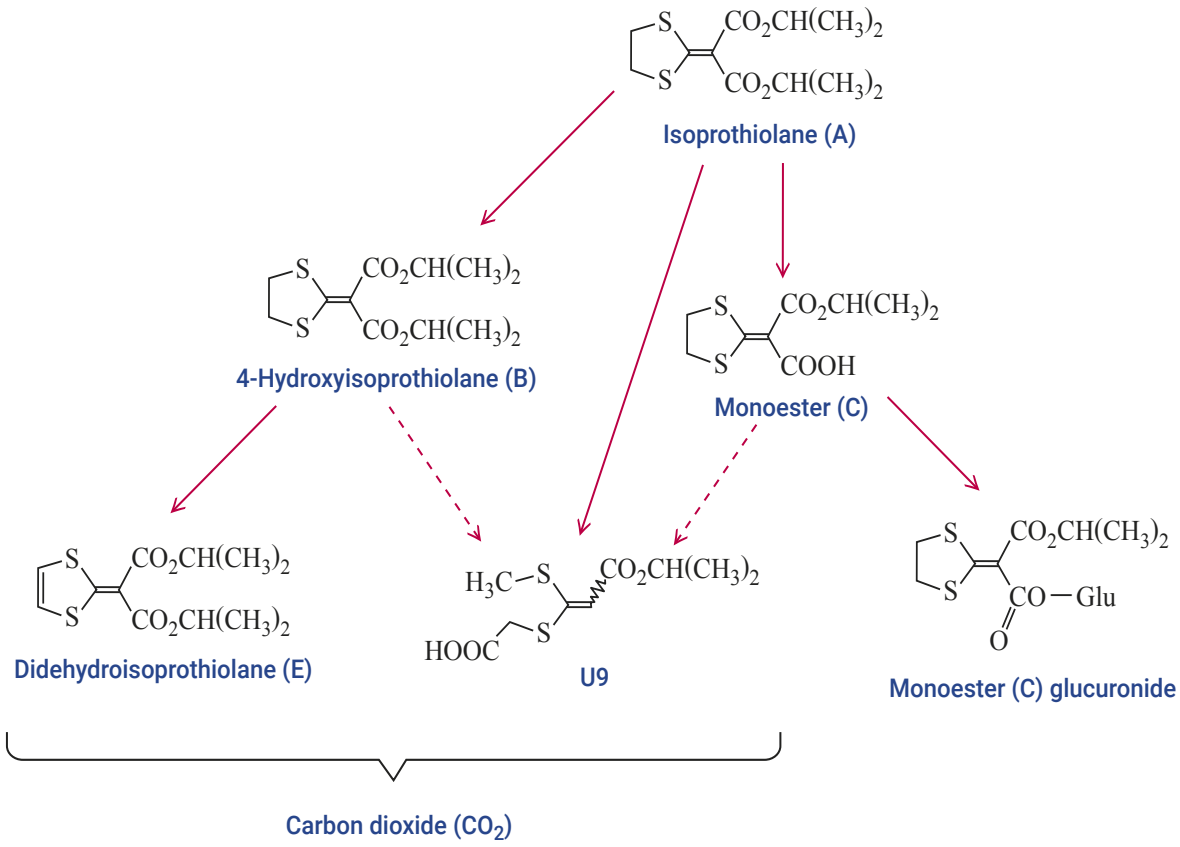
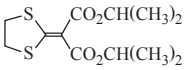
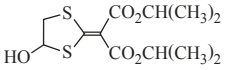
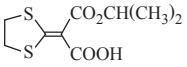
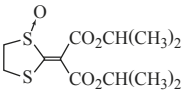
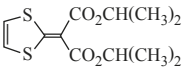
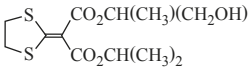
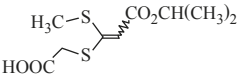
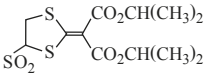
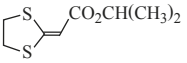
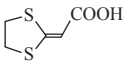


Table 9. Degradation compounds from metabolism of isoprothiolane in plants and animals

Name	Chemical name	Structure	Compound found in:
Isoprothiolane (parent)	Diisopropyl 1,3-dithiolan-2-ylidenemalonate		Rat Mouse Goat fat: 0.7%, 0.002 mg/kg Rice grain: 16–32%, 0.03–0.06 mg/kg Rotational crops: ND–29%; ND–0.93 mg/kg
4-hydroxy isoprothiolane (M-3)	Diisopropyl 4-hydroxy-1,3-dithiolan-2-ylidenemalonate		Rat Mouse Goat fat: 0.7–1.0%, 0.002–0.003 mg/kg Rice grain: 0.1–0.5%, < 0.01 mg/kg Rotational crops: ND–4.3%; ND– 0.22 mg/kg

Name	Chemical name	Structure	Compound found in:
Isoprothiolane monoester (M-2)	Monoisopropyl 1,3-dithiolan-2-ylidenemalonate		Rat (including glucuronide) Mouse Goat offal[a]: 18–44%, 0.21–0.44 mg/kg Goat muscle: 31–33%, 0.031–0.038 mg/kg Goat fat: 12–24%, 0.036–0.052 mg/kg Rice grain: < 0.1–0.2%, < 0.01 mg/kg Rotational crops: ND–7.7%; ND– 0.13 mg/kg
Isoprothiolane monosulfoxide (M-1)	Diisopropyl 1-oxo-1,3-dithiolan-2-ylidenemalonate		Mouse (including glucuronide) Rice grain: 0.1–0.3%, < 0.01 mg/kg Rotational crops: ND–3.9%; ND–0.309 mg/kg
Didehydroisoprothiolane	Diisopropyl 1,3-dithiol-2-ylidenemalonate		Rat Rotational crops: ND–1.8%; ND–0.10 mg/kg
Hydroxyl-isopropyl	1-Hydroxypropan-2-yl isopropyl 1,3-dithiolan-2-ylidenemalonate		Rotational crops: ND–8.1%; ND–0.46 mg/kg
U9	–		Rat
	–		Mouse
Isoprothiolane – demonoisopropoxy-carbonyl derivative (M-4)	–		Mouse
			Mouse (intermediate)
Isoprothiolane-dediisopropoxy-carbonyl derivative (M-5)			Mouse

bw: body weight; ND: not determined *Source:* Harris (2017)

[a] Including glucuronide conjugate (RT-19).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with isoprothiolane administered orally, dermally or by inhalation are summarized in Table 10.

The results of dermal and eye irritation and skin sensitization studies with isoprothiolane are summarized in Table 11.

Table 10. Summary of acute toxicity studies with isoprothiolane

Species	Strain	Sex	Route	GLP	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	Donryu	M + F	Oral [a]	No [b]	98	1190 (1610–880) (M) 1340 (1880–960) (F)	Miyazaki (1970)
Rat	Crl:CD[SD]	F	Oral [c]	Yes [d]	98.2	300–2 000	Oda (2010a)
Rat	Donryu	M + F	Dermal	No [b]	98	> 10 250 (M) > 10 250 (F)	Miyazaki (1970)
Rat	Crl:CD[SD]	M + F	Dermal	Yes [d]	98.2	> 2000	Oda (2010b)
Rat	Albino rat	M + F	Inhalation [e]	–	97.4	–	Normand (2011)
Rat	Sprague Dawley Crl:CD (SD)	M + F	Inhalation [f]		40.4 (w/w)	> 5.746 (MMAD: 2.8 and 2.32 µm)	Pouline (2010)

bw: body weight; F: females; M: males; GLP: good laboratory practice; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; MMAD: mass median aerodynamic diameter; w/w: weight per weight

[a] Several hours after administration of the test material, depression of spontaneous movement and crouching were observed. Ocular haemorrhages, rhinorrhoea, salivation, incontinentia urinae and diarrhoea were noted markedly at high-dose levels. Animals died 1–5 days after administration. Most of these dead animals were found clinging to the wire of the cages with their anterior limbs and their noses thrust into meshes of the cages. Convulsion of posterior limbs was observed prior to death.

[b] Conducted before GLP was mandatory.

[c] Signs of toxicity observed at 2000 mg/kg bw were a decrease in spontaneous movement from 4 hours after administration; prone position and pale skin from 2 days after administration; and death after 2–3 days after administration. The signs of toxicity disappeared by 2 days after administration in surviving animals. There were no abnormalities in clinical signs at 300 mg/kg dose. Two animals at 2000 mg/kg bw died, but as there were no deaths at 300 mg/kg, the LD_{50} value was estimated to be between 300 and 2000 mg/kg bw.

[d] Quality assurance (QA) statement provided.

[e] During this study (as determined in the previous technical validation), most of the particle MMADs were > 4 µm and therefore considered unrespirable. The study was therefore not assessed.

[f] The animals were exposed to an average aerosol concentration at 5.746 mg/L of isoprothiolane 40EC for 4 hours and tolerated the exposure without any mortality. The LC_{50} of isoprothiolane 40EC was calculated to be > 5.746 mg/L, which corresponds to 2.321 mg/L of isoprothiolane for both sexes.

Table 11. Summary of dermal and eye irritation and skin sensitization studies with isoprothiolane

Species	Strain	Sex	End-points	GLP	Purity (%)	Result	Reference
Rabbit	Albino	M	Primary dermal irritation	No [a]	Not given	Not irritating	Suzuki (1984a)
Rabbit	Albino	M	Primary eye irritation	No [a]	Not given	Reversible irritating effect	Suzuki (1984b)
Rabbit	Japanese White	F	Acute eye irritation	Yes [b]	98.2	Mildly irritating effect, reduced by washing	Suzuki (2010a)
Guinea Pig	Hartley White	F	Skin sensitization (Maximization test)	Yes [b]	98.2	Has skin sensitization potential	Suzuki (2010b)

F: females M: males GLP: good laboratory practice;

[a] Conducted before GLP was mandatory. [b] Quality assurance (QA) statement provided.

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a dose range-finding study conducted to select dose levels for a subsequent carcinogenicity study, isoprothiolane (lot no. DBADME; purity 97.5%) was administered to ICR SPF mice (Crj:CD-1; 6/sex per group; four weeks of age; males: 18–22 g, females: 17–21 g) at dietary concentrations of 0, 100, 500, 2500, 5000 or 10 000 ppm (equivalent to 0, 15, 75, 375, 750 and 1500 mg/kg bw per day) for a period of 28 days (four weeks).

At 10 000 ppm, both sexes demonstrated feed aversion to the test diet, leading to markedly decreased feed consumption. Body weights were persistently lower than in the control groups; in females, body weights were lower than at treatment initiation throughout the treatment period. Both sexes at this dose level had increases in plasma glutamic–pyruvic transaminase (GPT; alanine transaminase [ALT]) and liver weights as well as enlarged and dark-coloured liver. Males also had decreased urinary specific gravity, protein and urobilinogen, and increased relative weight of the adrenal. Females had decreases in haematocrit, haemoglobin concentration, erythrocyte count, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, which are suggestive of anaemia, and a decrease in leukocyte count and increases in plasma glutamic oxaloacetic transaminase (GOT, or aspartate transaminase [AST]) and blood urea nitrogen (BUN).

At 5000 ppm, both sexes demonstrated feed aversion to test diets at week 1, and body weight of females was lower than at treatment initiation. Both sexes had increases in plasma GOT (AST), plasma total cholesterol and liver weights. Enlarged and dark-coloured liver was noted at necropsy. Males also had decreases in urinary specific gravity and urobilinogen. Females had decreases in haemoglobin concentration, erythrocyte counts and mean corpuscular haemoglobin concentration, which are suggestive of anaemia.

At 2500 ppm, both sexes demonstrated feed aversion to test diets on week 1, and increases in liver weights. Plasma GPT (ALT) was increased compared with controls, although this difference was not statistically significant. Males also had a decrease in urinary urobilinogen.

At 500 and 100 ppm, no treatment- or dose-related changes were observed in either sex.

As isoprothiolane at a dietary concentration greater than 2500 ppm induced toxic changes caused by feed aversion and treatment, the highest dose level recommended in a subsequent carcinogenicity study was 5000 ppm.

The no-observed-adverse-effect level (NOAEL) was 500 ppm (equivalent to 75 mg/kg bw per day) based on the increase in liver weight and a decrease in urinary urobilinogen in males at 2500 ppm (equivalent to 375 mg/kg bw per day) (Yoshida, 1986).

In a subacute toxicity study carried out before GLP was mandatory, isoprothiolane (lot no. not stated; purity > 99%) was administered to ICR SPF mice (10/sex per group; age not stated; males: 28 ± 2 g, females: 24 ± 2 g) at dietary concentrations of 0, 20, 100, 300, 900 or 2700 ppm (equal to 0, 3.32, 14.77, 47.99, 132.31 and 472.29 mg/kg bw per day for males and 0, 2.81, 14.27, 47.18, 140.26 and 444.35 mg/kg bw per day for females, respectively) for a period of 112–115 days.

There were no differences in feed consumption between the control and treated animals and no treatment-related effects on body weight.

Significant differences were noted in organ weights, GOT (AST) and GPT (ALT) activities, specific gravity of whole blood, haemoglobin, haematocrit and red blood cell (RBC) count. Marked increases in liver weight were noted in both sexes at 2700 ppm compared to control. A marked decrease in ovary weight was noted in animals at 900 and 2700 ppm compared to controls. Though these weight changes could be treatment related, the changes were considered to be mild because of an absence of concomitant specific histopathological findings. Although significant differences in weights of the kidneys, spleen, brain, heart and adrenals were noted between the treatment and control groups, these changes were probably not treatment related because of the absence of any histopathological changes and a lack of dose dependency.

GPT (ALT) activities at 100, 300 and 2700 ppm and GOT (AST) activities at 300 ppm were somewhat higher than in the control group. However, these changes were noted only in males and were neither consistent nor dose dependent, and therefore considered not treatment related.

The specific gravity of the whole blood, haemoglobin, haematocrit and RBC count in males at 100 and 900 ppm were higher than the control. However, these changes were probably not treatment related as they were found only in males, were not dose dependent and were within normal values.

Residue analysis found detectable levels of isoprothiolane, mainly in the liver and kidney, with traces in fat and/or muscle, at 900 and 2700 ppm.

The NOAEL was 900 ppm (equal to 140 mg/kg bw per day), based on 40% decreases in ovarian weight at 2700 ppm (equal to 444 mg/kg bw per day). The toxicological significance of the effects on the ovary was equivocal because of the absence of specific histopathological findings at 2700 ppm (equal to 444 mg/kg bw per day) (Miyazaki, 1972)

Rat

In a dose range-finding study, isoprothiolane (lot no. DBBCFG; purity 97.1%) was administered to Sprague Dawley (Crj:CD) rats (6/sex per group; five weeks of age at study initiation; males: 140 g, females: 113 g) at dietary concentrations of 0, 200, 1000 or 5000 ppm (equal to 0, 15.6, 75.2 and 350 mg/kg bw per day for males and 0, 16, 79.3 and 360 mg/kg bw per day for females, respectively) for a period of one month (31 days).

At 5000 ppm, retardation of body weight gain was observed in both sexes throughout the treatment period. However, it was not clear whether the change was an adverse effect of isoprothiolane treatment because a reduction in feed efficiency was not observed. Feed consumption was reduced throughout the treatment period in both sexes at 5000 ppm. The reduction at week 1 was particularly remarkable, suggesting that the reduction in feed consumption was due to feed aversion. Aversion to the test diet was also observed in high-dose animals in the 28-day oral toxicity study in mice by Yoshida (1986). The retardation of body weight gain at 200 ppm was considered to be incidental as it was not correlated with the dose levels.

In urine analysis, a reduction in specific gravity was noted in females at 1000 ppm or higher, and increases in ketones and urobilinogen were observed in males at 5000 ppm, suggesting a treatment-related effect. Other statistically significant changes were considered to be incidental as they were not correlated with the dose levels.

In haematology, decreases in haematocrit, haemoglobin and erythrocyte count were observed in both sexes at 5000 ppm, suggesting treatment-related anaemic changes. The decreased haemoglobin level in males at 200 ppm was considered to be incidental as it was not correlated with the dose levels.

Blood biochemistry examinations found increases in total cholesterol in both sexes at 1000 ppm or higher; these increases were considered to be a toxic effect of treatment with the test substance. At 5000 ppm, males had increases in albumin, calcium, total protein and albumin/globulin ratio together with the increased albumin; females had a decrease in GOT (AST). However, the toxicological significances of these changes are unclear. Other statistically significant changes in the treated groups were considered incidental as they were not correlated with the dose levels.

At necropsy, increases in the incidences of darkening and enlargement of the liver were observed in both sexes at 5000 ppm. This was considered to be a toxic effect of treatment. In addition, pale-coloured kidneys were observed in half of the male animals in this group, and the effect was suspected of being treatment related. The enlargement of the thyroid in males at 1000 ppm was considered incidental as it was not correlated with the dose levels.

Increases in liver weight were observed in both sexes at 1000 ppm or higher. This was considered to be a toxic effect caused by treatment with isoprothiolane. Taking into account these organ weight changes and the gross pathological findings described above, the liver was recognized as a main target organ. In females at 1000 ppm or higher, increases in relative kidney weight were observed, suggesting a toxic effect on the kidney by treatment with the test substance. Other organ weight changes, observed in both sexes at 5000 ppm, were interpreted as secondary changes due to the low body weights noted.

Aside from the slight and clear toxic changes observed at 1000 and 5000 ppm, respectively, feed aversion was noted at 5000 ppm. Therefore, a dietary level of between 1000 and 5000 ppm would be recommended as the highest dose level in a subsequent combined chronic toxicity and carcinogenicity study with isoprothiolane in Sprague Dawley rats.

The NOAEL was 200 ppm (equal to 15.6 mg/kg bw per day) based on increases in liver weight, a reduction in specific gravity of urine (females only) and increases in total cholesterol at 1000 ppm (equal to 75.2 mg/kg bw per day) (Inui, 1987).

In another doserange-finding study for a combined chronic toxicity and carcinogenicity study in rats, isoprothiolane (lot no. DBBCFG; purity 97.1%) was administered to SPF Fischer rats (F344/DuCrj; 6/sex per group; four weeks at receipt; males and females: 47–55 g) in feed at dietary concentrations of 0, 200, 500, 1000, 3000 and 5000 ppm (equal to 0, 15.4, 38.3, 77.9, 255 and 357 mg/kg bw per day for males and 0, 16.0, 39.3, 76.5, 225 and 350 mg/kg bw per day for females, respectively) for a period of 31 days.

There were no abnormal signs or deaths in any dose group of either sex. Body weight gain was retarded in males at 3000 ppm and males and females at 5000 ppm. Females at 5000 ppm had decreased feed efficiency. Unpalatability of test diets was apparent at 3000 ppm or higher.

Total cholesterol in males at 1000 ppm or higher and females at 3000 ppm or higher was increased. An increase in BUN was observed in females at 3000 ppm or higher. In males, a decrease in creatinine was observed at 1000 ppm or higher and in glucose at 3000 ppm or higher. At necropsy, increases in the incidence of hepatic enlargement were observed in males at 3000 ppm or higher. Males at 5000 ppm had smaller body size.

While absolute liver weights were increased in males at 500 ppm or higher and females at 1000 ppm or higher, relative liver weights were increased in males at 200 ppm or higher and females at 500 ppm or higher. Relative kidney weights were increased in males at 200 ppm or higher and females at 500 ppm or higher; absolute kidney weights were increased in males at 1000 and 3000 ppm and females at 500 ppm.

In summary, it was evident that isoprothiolane mainly targeted the liver, with treatment at all concentrations having an effect on hepatic parameters, although the degree of changes was relatively slight. An effect on liver weight was observed even at the lowest dose level, 200 ppm.

No NOAEL could be identified, as effects were observed at all doses. The low-observed-adverse-effect level (LOAEL) of isoprothiolane was less than 200 ppm (equal to 15.4 mg/kg bw per day) in this one-month oral toxicity study in Fischer rats (F344/DuCrj) (Yoshida, 1987).

In a non-GLP compliant subacute toxicity study, isoprothiolane (lot no. not stated; purity > 99%) was administered to SD rats (10/sex per group; age not stated; males and females: 110 ± 10 g) at dietary concentrations of 0, 20, 100, 300, 900 or 2700 ppm (equal to 0, 1.17, 5.92, 17.28, 53.04 and 157.61 mg/kg bw per day for males and 0, 0.69, 7.27, 21.56, 61.67 and 182.48 mg/kg bw per day for females, respectively) for a period of 112–115 days.

Feed consumption in female rats at 2700 ppm was somewhat less than that of controls. Body weight gain and body weight were significantly lower in female rats at 2700 ppm.

There were statistically significant differences between treatment and control groups in organ weights, RBC count, GOT (AST) and GPT (ALT) activities, haematocrit and RBC count. Compared to controls, RBC counts in females at 900 and 2700 ppm were increased and haematocrit at 2700 ppm was slightly decreased. Although these results suggest an increase in number of smaller RBCs, it is difficult to determine if the changes were treatment-related; in addition, the effect was marginal and seen only in females.

Compared to the control group, decreased activities of GPT (ALT), GOT (AST) and alkaline phosphatase (ALP) were noted in the groups treated at 300 ppm and higher. However, these changes were apparent only in males. Moreover, the values in the control group were slightly elevated from the norm and the values in the treated groups were within the normal range. Based on these data and the knowledge that any changes in liver function were a decrease, these effects were not considered toxicologically relevant.

Relative liver weight was increased in both sexes at 2700 ppm. As there were no associated histopathological or clinical chemistry changes, these effects may be due to an adaptive response due to an augmentation of the drug-metabolizing activity of the liver. Significant increases in the relative weight of the kidney, spleen and brain were noted at higher doses relative to controls. However, as there was an absence of any other changes, including specific pathological changes, these effects may not be treatment related and were considered not toxicologically significant.

Residues of isoprothiolane were detectable in liver and fat only in animals at 2700 ppm.

According to these data, the body weight and liver weight changes appear to be readily influenced by isoprothiolane.

The NOAEL was 300 ppm (equal to 17.28 mg/kg bw per day for males and 21.56 mg/kg bw per day for females) based on increased RBC of animals at 900 ppm (equal to 53.04 mg/kg bw per day for males and 61.67 mg/kg bw per day for females) The toxicological significance of this effect is equivocal. (Miyazaki, 1972).

In a 90-day dietary study, isoprothiolane (lot no. 002120; purity 98.2%) was administered to SD rats (Crl:CD (SD); 12/sex per group; five weeks of age at study start; males 124–140 g (mean: 131.3 g), females 107–134 g (mean: 120.1 g) via feed at dietary concentrations of 0 (control group), 50, 300 and 3000 ppm (equal to 0, 3.4, 20.9 and 200.9 mg/kg bw per day for males and 0, 4.0, 23.4 and 223.2 mg/kg bw per day for females, respectively) for 90 days.

The general appearance of all animals was observed twice a day, in the morning and afternoon, from day 1 to 91, and once in the morning on the day after day 91 (scheduled kill). Detailed clinical observations were performed before treatment initiation and once a week during the treatment. A functional observational battery was performed once in week 13. Body weight and feed consumption were measured twice a week on the first week of treatment and once a week thereafter. At week 13, all control and high-dose animals underwent ophthalmological examination, while urine analysis was performed for 5 rats/sex per dose group. All animals were autopsied after blood sampling following fasting overnight, and haematological, blood chemistry and histopathological examination were performed and organs weighed.

There were no treatment-related changes in general appearance, clinical signs or functional observational battery in any group. Body weight gain was significantly reduced, or tended to be reduced (nonsignificantly), and there was a decrease in feed consumption in males and females at 3000 ppm from treatment day 4 throughout the dosing period. Information was not available on body weight 24 hours after commencement of dosing. Ophthalmological examination found no treatment-related changes in males or females at 3000 ppm. Urine analysis found no changes in any group.

Haematological examination found significantly reduced RBC count, haematocrit value and haemoglobin concentration, significant shortening of prothrombin time, significantly increased reticulocyte count and significant prolongation of activated partial thromboplastin time in female rats at 3000 ppm (Table 12).

Table 12. Haematological findings in female rats in 90-day repeated-dose oral toxicity test with isoprothiolane

Dose level (ppm)		Measure per dose level [a]					
		RBC (× 10^4/µL)	Hct (%)	Hb (g/dL)	Reticulocyte (%)	Prothrombin time (s)	APTT (s)
0 (Control)	Mean	861.3	46.26	16.49	23.4	17.43	20.08
	SD	39.4	2.48	0.67	4.8	0.75	1.59
50	Mean	870.7	45.70	16.01	24.3	16.88	20.55
	SD	23.3	1.79	0.43	3.6	0.40	1.48
300	Mean	861.6	45.30	16.16	24.3	17.13	20.26
	SD	33.8	1.72	0.62	3.6	0.59	1.15

(*Continued on next page*)

Dose level (ppm)		Measure per dose level[a]					
		RBC (× 10^4/µL)	Hct (%)	Hb (g/dL)	Reticulocyte (%)	Prothrombin time (s)	APTT (s)
3000	Mean	816.5*	43.59*	15.76*	34.9#	15.92**	22.33**
	SD	49.5	2.45	0.77	8.9	0.62	2.20

APTT: activated partial thromboplastin time; Hct: haematocrit; Hb: haemoglobin; RBC: red blood cell; SD: standard deviation; *: $P \leq 0.05$ (Dunnett test); **: $P \leq 0.01$ (Dunnett test); #: $P \leq 0.01$ (Mann–Whitney *U* test)

[a] Mean of 12 animals.

Source: Sunaga (2006)

Significantly increased γ-glutamyltransferase (GGT) activity was seen in males at 300 ppm. At 3000 ppm, GGT activity and total cholesterol levels were significantly increased in males and females, and total protein, albumin and ALT were significantly increased in males (Table 13).

Table 13. Blood chemistry findings in male and female rats in 90-day repeated-dose oral toxicity tests with isoprothiolane

Dose level (ppm)		Measure per dose level[a]											
		Total protein (g/dL)		Albumin (g/dL)		AST (IU/L)		ALT (IU/L)		γ-GGT (IU/L)		Total cholesterol	
		M	F	M	F	M	F	M	F	M	F	M	F
0 (Control)	Mean	5.91	6.31	2.881	3.454	74.8	96.8	32.8	34.5	0.63	0.73	61.6	74.1
	SD	0.16	0.28	0.098	0.220	14.8	23.0	6.2	8.8	0.18	0.26	14.6	10.3
50	Mean	5.78	6.32	2.809	3.495	88.8	85.2	34.4	30.5	0.65	0.77	62.2	76.0
	SD	0.28	0.39	0.134	0.270	36.9	26.0	8.4	13.8	0.13	0.32	19.1	14.6
300	Mean	6.08	6.64	2.971	3.733	102.3	96.8	54.2	33.5	0.93#	0.74	76.8	80.2
	SD	0.21	0.48	0.170	0.342	35.6	41.5	42.8	15.3	0.43	0.38	20.3	14.1
3000	Mean	6.48**	6.74	3.190**	3.721	139.2	83.1	121.1#	35.3	6.22##	1.31**	111.3**	97.7**
	SD	0.35	0.58	0.192	0.362	129.0	35.2	173.0	19.2	6.31	0.43	23.6	22.0

ALT: alanine transaminase; AST: aspartate transaminase; γ-GGT: γ-glutamyltransferase; *Source*: Sunaga (2006)
IU: International Units; ppm: parts per million; SD: standard deviation; M: males; F: females;
*: $P \leq 0.05$ (Dunnett test); **: $P \leq 0.01$ (Dunnett test);
#: $P \leq 0.01$ (Mann–Whitney *U* test); ##: $P \leq 0.05$ (Mann–Whitney *U* test)

[a] Twelve rats/sex per dose.

At 300 ppm and above, there was a significant increase in the relative weights of the liver and kidneys in male rats. These increases at 300 ppm were relatively modest, 10% for liver and 7% for kidneys. In female rats, relative weights of the liver and kidneys were significantly increased at 3000 ppm. Histopathological examination found centrilobular hepatocyte hypertrophy in males and females and haemosiderin deposition in the spleen was noted in females at 3000 ppm.

The NOAEL was 50 ppm (equal to 3.4 mg/kg bw per day) based on increased relative weights of liver and kidneys and increased GGT activity observed in male rats at 300 ppm (Sunaga, 2006).

Dog

Isoprothiolane (lot no. DBBCFG, purity 97.09%) was administered to beagle dogs (4/sex per group; 4–4.5 months old at study start; males 5.1–8.7 kg , females 5.0–7.6 kg) via gelatine capsules at concentrations of 0, 2.0, 10.0 or 50.0 mg/kg bw per day for a minimum of 52 weeks. Clinical signs, body weight and feed consumption were routinely monitored, and ophthalmology and laboratory investigations were performed at intervals and at study termination.

There were no deaths or clinical signs in any dose group of either sex. Treatment at up to 50 mg/kg bw for 52 weeks had no effect on body weight or body weight gain in males. In females, body weight gain was reduced compared to controls, in a dose-related manner. The reduction was statistically significant ($P < 0.05$) at the highest dose. Overall body weight gains per administered dose level are shown in Table 14.

There were no obvious treatment-attributable changes in feed consumption. No ophthalmological changes were observed during the treatment. No adverse haematological changes were observed in any dose groups. Evaluation of the clinical chemistry results found no obvious treatment-related changes during the study except for increased ALP levels in dogs at 50 mg/kg bw per day. Although individual animals sometimes had values within the normal range for this enzyme, the group means of high-dose males and females were consistently elevated (3.3-fold in males [$P < 0.05$] and 1.6-fold in females) compared to controls throughout the duration of treatment (from 12 weeks, when first measurements were made). There were no treatment related changes in urine analysis.

Table 14. Overall body weight gain in male and female dogs in one-year oral study with isoprothiolane

Dose (mg/kg bw per day)	Body weight gain ± SD (kg) [a]	
	Males	Females
Control	3.80 ± 0.698	4.13 ± 0.714
2	4.88 ± 0.950	3.38 ± 0.850
10	4.22 ± 1.652	3.33 ± 0.613
50	3.78 ± 0.754	2.70 ± 0.906*

bw: body weight; SD: standard deviation; *: $P < 0.05$

[a] Mean of four dogs/sex per dose group.

Organ weights were within normal ranges. Relative liver weight was slightly higher (17%, $P < 0.05$) in males at 50 mg/kg bw per day. While relative values in other dose groups were within normal limits, this higher value at 50 mg/kg bw per day may be related to the increased ALP activity in the high-dose dogs. The absolute and relative thyroid/parathyroid weights were increased at the highest dose, significantly so in females (by 37% and 72%, respectively).

There were no lesions that could be attributable to isoprothiolane administration. Gross pathological findings were limited to lungs where pale or dark areas, pleural adhesions and depressed areas were seen in a few dogs from all groups. Minor histological findings included minor pulmonary changes (considered to be associated with lungworm parasitosis), occasional slight dilatation of brain ventricle (not dose related though outside the historical control range) and occasional slight to mild thymic atrophy. The thymic atrophy, which occurred in both control and treated dogs, is not uncommon to beagle dogs; the atrophy was within historical control range and, hence, considered to be of no toxicological significance (Table 15 and 16).

Table 15. Incidence of dilatation of brain ventricle and thymic atrophy in dogs treated with isoprothiolane for one year

Dose (mg/kg bw per day)	Number of animals with the finding			
	Males [b]		Females [a]	
	Dilatation of brain ventricle	Thymic atrophy	Dilatation of brain ventricle	Thymic atrophy
0	0	3	0	1
2	2	3	1	2
10	0	3	0	2
50	0	3	1	3
Historical control range	Max: 0% Min: 0%	Max: 100% Min: 0%	Max: 0% Min: 0%	Max: 83.33% Min: 0%

bw: body weight; max: maximum; min: minimum;

[a] Four dogs/sex per dose group.

Source: Osborne & Kalichman (1989)

Table 16. Historical control data for dilatation of brain ventricle and thymic atrophy in beagle dogs treated with isoprothiolane [a,b,c]

	Number of animals with the finding/study year					Range	
	2001	2005	2004	2007	2001	Minimum	Maximum
Males							
No. of animals examined	6	6	6	4	6	–	–
No. of animals with dilatation of brain ventricle	0	0	0	0	0	0%	0%
No. of animals with thymic atrophy	2	2	6	3	0	0%	100%
Females							
No. of animals examined	6	6	6	4	6	–	–
No. of animals with dilatation of brain ventricle	0	0	0	0	0	0%	0%
No. of animals with thymic atrophy	0	0	5	2	0	0%	83.33%

[a] Route of administration: All routes.

[b] Age of animals at necropsy: ~17–26 months.

[c] Treatment duration: 52–53 weeks.

Source: Harris (2017)

The NOAEL was 10 mg/kg bw per day based on reduced body weight gain in females, increased ALP activity in both sexes (3.3-fold in males and 1.6-fold in females), increased absolute (37%) and relative thyroid/parathyroid (72%) weights in females, and increased relative liver weight (17%) in males at 50 mg/kg bw per day (Osborne & Kalichman, 1989).

Two other submitted dog studies, one four-week oral toxicity study (Kennedy, 1974) and a two-year dietary toxicity study (Mastalski, 1977), were conducted by Industrial Bio-Test Laboratories in the 1970s. These studies were excluded from evaluation as no evidence of their validation by a government agency was provided.

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a carcinogenicity study performed in SPF mice (Crj:CD-1; 60/sex per group; five weeks at study start; males 18–22 g, females 17–21 g), isoprothiolane (lot no. DBBCFG; purity 97.1%) was administered in the diet at a dietary concentration of 0, 200, 1000 or 5000 ppm (equal to 0, 20, 103.8 and 501.4 mg/kg bw per day for males and 0, 18.2, 95.6 and 557.6 mg/kg bw per day for females, respectively) for 18 months (78 weeks).

Swelling of the skin was observed in five males at 5000 ppm, but this clinical finding disappeared before the terminal kill in all but one mouse. No other changes related to the dose levels were observed. No dose-related increase in mortality was observed.

At 5000 ppm, males and females had significantly lower body weight and decrease in feed consumption and feed efficiency throughout the treatment period compared to the control group. The changes in these parameters were considered to be adverse effects of treatment, and the highest dose was considered to be the maximum tolerated dose. The consistent lower body weight in males at 200 and 1000 ppm, from about eight weeks after treatment initiation but not earlier, might also be related to treatment since feed consumption in these groups was comparable to that of the control group. However, the changes were relatively minor: −3.4% at 200 ppm and −6.6% at 1000 ppm after 78 weeks.

In the differential count of leukocytes, males showed an increase in monocytes in all treated groups, an increase in unclassified cells at 5000 ppm and a decrease in stab type of neutrophil at 1000 and 5000 ppm after 52 weeks of treatment. However, at the 78-week examination, these parameters were

comparable to those in the control group. In females, there was significant decrease in monocytes in all treated groups after 78 weeks. Histopathological examination of these animals revealed no increased incidence and/or exacerbation of the lesions corresponding to the changes in leukocytes. Therefore, the changes in differential leukocytes count were considered to be incidental.

At autopsy, there was a significant increase in the incidence of small body size in high-dose females, probably due to significantly lower body weight. In both sexes at 5000 ppm, there was increase in the weight of the liver, which was enlarged and was dark in colour. These changes correspond to the microscopic findings diagnosed as periportal hepatocellular swelling and were considered to be a hepatotoxic change induced by the test compound. Other macroscopic changes observed were not dose related and were considered to be incidental.

Significant increases in relative weights of adrenals were noted in high-dose males and females in addition to the liver changes mentioned earlier. Males also showed an increased tendency to increased absolute liver weight, although the increased value was not statistically significant. The increased adrenal weight in high-dose males was attributable to amyloid deposition. No histopathological lesions were noted in the adrenals in high-dose females. In addition, absolute organ weight was decreased in females. Therefore, increased relative weight of adrenals in the female of the highest group is also result of lower body weight. It is likely that the decreased absolute weight and increased relative weight of brain and kidneys in high-dose males and females were also the result of the lower body weight rather than a toxic effect.

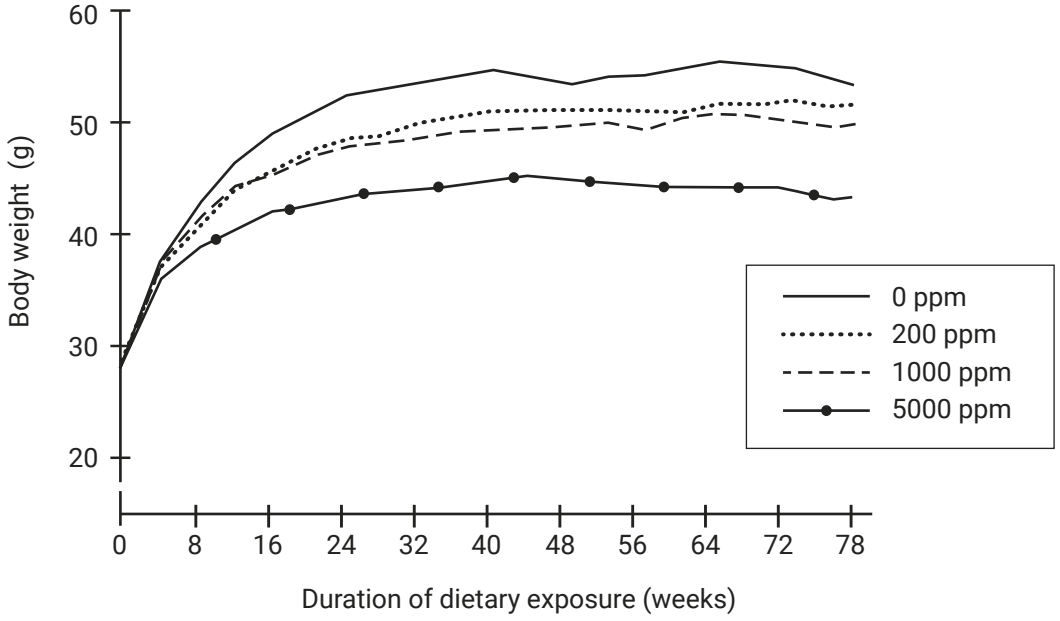
Males at 1000 ppm had body weights lower than the controls and a significantly increased incidence of periportal hepatocellular swelling. There were no marked changes related to treatment in females. At 200 ppm, males had significantly lower body weights, but no treatment-related effects were seen in females. The body weight changes in males at 200 and 1000 ppm were very similar (Fig. 3).

The NOAEL was 1000 ppm (equal to 95.6 mg/kg bw per day) based on reduced body weight in males at 5000 ppm (equal to 501 mg/kg bw per day).

There were no treatment-related differences between treated and control groups in the type, age of onset or incidence of neoplastic lesions.

Based on the results of the present study, it was concluded that isoprothiolane was not carcinogenic in ICR mice at dietary levels up to 5000 ppm for 78 weeks. The highest dose was considered to be the maximum tolerated dose (Inui, 1989).

Figure 3. Body weight changes in male mice over 78 weeks of dietary exposure with isoprothiolane in carcinogenicity study



ppm: parts per million

Source: Redrawn from Inui (1989)

Rat

In a combined chronic toxicity and carcinogenicity study, isoprothiolane (lot no. DBBCFG; purity 97.1%) was administered to Sprague Dawley rats (Crj:CD; 80/sex per group; five weeks old at study start; males 70–90 g, females 65–95 g) via feed at dietary concentrations of 0, 50, 300 or 3000 ppm (equal to 0, 1.82, 10.85 and 114.6 mg/kg bw per day for males and 0, 2.06, 12.62 and 138.9 mg/kg bw per day for females, respectively) for 24 months (104 weeks). After 26, 52, 78 and 104 weeks of treatment, 10 animals/sex per dose group were killed following urine analysis. The animals underwent haematology and blood biochemistry analyses and organ weight measurements. At 104 weeks of treatment, 10 animals/sex from the 0 and 3000 ppm groups underwent ophthalmological examination. All animals used in this study were necropsied and examined histopathologically.

At 3000 ppm, retardation of body weight gain and a reduction in feed consumption were observed in both sexes through the treatment period. Feed efficiency was also lowered in both sexes. Haematological examination showed a trend towards anaemia after 26 weeks of treatment in females. In blood biochemistry, there were increases in total cholesterol in both sexes and a decrease in total bilirubin and an increase in BUN in females. At necropsy, females were conspicuous for small body size. Macroscopic examinations of the liver found the incidence of darkening to be increased in both sexes and incidence of liver enlargement to be increased in males. There were increases in absolute and relative liver and kidney weights in males and in relative liver and kidney weights in females. In histopathological examination, an increase in the incidence of neoplastic lesions, dermal keratoacanthoma (all benign) was observed in males. The incidence in the high-dose group of 16.3% (13/80) compared with control incidence of 3.8% (3/79) was outside the range of historical controls (0.0–6.8%). With regard to microscopic non-neoplastic lesions, eosinophilic cytoplasmic inclusions, which was specific to treatment with isoprothiolane, were detected in hepatic cells in males. In addition, the incidences of increased brown pigment deposition (spleen), foam cell aggregation (lung), periportal hepatocellular fatty change (liver), eosinophilic cell foci of cellular alteration (liver) and spongiosis hepatis (liver) were elevated in males. On the other hand, in females, there were increases in incidence of epithelial reticulum cell hyperplasia (thymus), increased brown pigment deposition (spleen), centrilobular hepatocellular swelling (liver), eosinophilic cell foci of cellular alteration (liver) and ultimibranchial remnant (thyroid).

At 300 ppm, blood biochemistry examination found an increase in total cholesterol after 26 weeks of treatment in males but this was not significantly affected by treatment at later time points. There were no clear treatment-related changes in females at 300 ppm or in either sex at 50 ppm.

The NOAEL was 300 ppm (equal to 10.9 mg/kg bw per day) based on reduced body weight gain (up to 12% in males and 36% in females), increased BUN in females (16–25%, significant at weeks 26 and 52) and an increase in relative weight of liver (30–40%) and kidneys (10–50%) in both sexes at 3000 ppm (equal to 115 mg/kg bw per day) at the interim and final kills.

An increased incidence of benign dermal keratoacanthoma was observed in males in the highest dose group (13/80 compared to 3/79 in controls). No pre-neoplastic changes were observed in the skin. There were no compound-related increases in any other tumour incidence

The NOAEL for carcinogenicity was 3000 ppm (equal to 114.6 mg/kg bw per day), the highest dose tested (Inui, 1991).

2.4. Genotoxicity

Isoprothiolane was tested for genotoxicity in several studies, one of which was in vivo (Table 17). Isoprothiolane showed no evidence of genotoxicity in the in vivo micronucleus study and four in vitro studies. In one study of chromosomal aberrations (Chinese hamster lung fibroblast) in vitro, although isoprothiolane showed no evidence of genotoxicity without metabolic activation, there was a slight increase in the frequency of chromosome aberrations with metabolic activation. In the other study with Chinese hamster lung fibroblasts, isoprothiolane induced chromosomal aberration both with and without metabolic activation. On the basis of these studies, the Meeting concluded that isoprothiolane is unlikely to be genotoxic in vivo.

Table 17. Summary of genotoxicity studies with isoprothiolane

Test	Strain/species	Concentration	Purity (%)	Result	Reference
In vitro					
Bacterial reverse mutation (Ames test)	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, TA100; *Escherichia coli* WP2uvrAp	+S9: 5000, 1000, 200, 40 and 8 µg/plate	97.09	Negative	Asquith (1988a)
		−S9: 1000, 200, 40, 8 and 1.6 µg/plate		Negative	
DNA repair test (Rec assay)	Recombinant-wild (H17) and recombinant-deficient (M45) of *Bacillus subtilis*	20, 100, 200, 500, 1000, 2000 µg/disk (± S9)	99.0	Negative	Moriya (1977)
Reverse mutation	*E. coli* WP2hcr; *S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	± S9: 1, 10, 50, 100, 500, 1000, 5000 µg/plate	99.0	Negative	Moriya (1977)
Chromosomal aberrations	Chinese hamster lung fibroblast	−S9: 24–0 h: 28, 56, 112 µg/mL 48-0 h: 16.25, 32.5, 65 µg/mL	99.9	Negative	Asoh (1986a)
		+S9: 6–18 h: 33.5, 67, 134 µg/mL		Slight increase in chromosomal aberration	
Chromosomal aberrations	Chinese hamster lung fibroblast	−S9: 24–0 h: 28, 57, 114 µg/mL 48–0 h: 16.25, 32.5, 65 µg/mL +S9: 6–18 h: 55, 110, 220 µg/mL	97.5	Positive[a]	Kajiwara (1986a)
Human lymphocyte metaphase analysis	Human lymphocyte	± S9: 10, 20, 40 µg/mL	97.09	Negative	Asquith (1988b)
In vivo					
Micronucleus test	Male mice	600, 300 or 150 mg/kg bw	97.5	Negative	Kajiwara (1986b)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] The result was equivocal in 48-hour continuous treatment by the direct method. The results were positive in 24-hour continuous treatment by the direct method and in the metabolic activation method. The frequency of cells with chromosomal aberration was over 10% at high dose (5–10% is equal; > 10% is positive). Especially, the high percentage (32%) was recorded at 220 µg/mL in the metabolic activation method. The positive control, 4-NQO and DMN, induced chromosomal aberration. Based on these results, it was considered that isoprothiolane technical has the potential to induce chromosomal aberration in vitro.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rat

In a non-GLP-compliant reproductive toxicity study, isoprothiolane (lot no. not given; purity 99.9%) was administered to rats (25/sex per group) at a dietary concentration of 0, 30, 300 or 3000 ppm (equivalent to 0, 2, 20, 200 mg/kg bw per day) for three generations.

There were no differences in the female fertility index (gestation index) between F_1, F_2 and F_3 treated and control groups.

In F_1 animals, increase in body weight was suppressed in both sexes from the time of treatment initiation in the high-dose group. The decrease in body weight was significantly different from the control after 90 days. The female fertility index (gestation index) was the same in control and treated groups in both F_{1a} and F_{1b} animals. No differences were noted in the numbers of corpora lutea, implantations, dead fetuses and live fetuses and fetal weight in the prenatal test. No differences were noted in the number of delivered pups, number of dead pups, number of live pups, female fertility index and day 21 survival index (lactation index) during postpartum test. Although there were no significant differences between the high-dose group and the controls in body weight of the neonatal animals during the lactation period, body weight at weaning was significantly decreased compared to the control.

Skeletal examination revealed anomalies such as branching and splitting of the vertebral arch and insufficient ossification of the thoracic skeleton and lumbar rib, but the frequency was the same in all groups. The number of basal segment bones and tail vertebrae indicating the degree in which ossification was progressing also showed no change.

Postpartum examination of F_{1b} animals noted no differences between the control and treated groups in terms of the number of implantations, number of delivered pups, number of dead pups, number of live pups and in day 4 survival. The delivery index and day 21 survival index (lactation index) tended to be higher in the treated groups than the control group. The body weight of neonates was the same in the control group and treated groups; at weaning, neonate body weight in the high-dose group was slightly, but not statistically significantly lower, than the control. Examination of the skeleton revealed no remarkable anomalies except shortened 13th rib in one control animal.

The F_2 female fertility index was the same in the control and treated groups. The indices were similar to F_1 indices in the prenatal and postpartum tests. A decrease in the body weight of neonates was noted in the group treated with high dose compared to the control. This represented the only change.

In F_3 animals, suppression of body weight was noted in high-dose parental males and females shortly after treatment initiation. Body weight after 90 days were significantly lower than the control. No difference in the female fertility index (gestation index) between the control and treated F_{3a} and F_{3b} animals was noted.

Comparison of F_{3a} and F_{3b} animals found no difference between the control and treated groups in the number of delivered pups, number of dead pups, number of live pups, day 4 survival index and day 21 survival index (lactation index). The body weights of F_{3a} neonates were the same at delivery in all groups. A tendency towards suppression of any increase in the body weight appeared after one week in the treated groups. The body weight of the neonates in the high-dose group was significantly lower than the control at weaning. The weight of F_{3b} neonates tended to be suppressed in the high-dose group, but no difference was seen between control and treated groups as to time of opening of the earlobe, appearance of teeth, opening of eyelids or hair growth on the lower abdomen, indicating a normal development rate.

In a three-month test for subacute toxicity in F_{2b} animals, no difference was noted in pH, glucose, protein, ketone and occult blood in urine of control and treated groups. In haematology, the RBC count and haemoglobin concentration were significantly decreased in treated males compared to the control, although the observed changes lacked any relationship to dose. The white blood cell count was decreased in high-dose and mid-dose females, with no dose dependency. Total protein was decreased in high-dose males and females and low-dose males. The albumin/globulin ratio was dose-dependently increased in high-dose males and females and mid-dose males.

The ratios of wet liver to body weight and kidney to body weight were decreased in males. In females, the liver to body weight ratio was decreased in the high-dose group and the kidney to body weight ratio was decreased in the high- and mid-dose groups. The ratios of the weights of lungs and ovaries to body weight were decreased in treated groups.

A few histopathological findings were noted in the liver, lungs and kidneys of each group. However, all of these findings were slight, and no significant differences between treated groups and sex could be attributed to the administration of the compound. Similar findings were also noted in the control group.

In conclusion, suppression of body-weight gain in the high-dose group was the only characteristic treatment-related change observed. No changes were seen in the indices of filial generation during the reproduction tests. No influence of isoprothiolane was noted on the fetuses or delivered pups.

The NOAEL for parental and embryo/fetal toxicity was 300 ppm (equivalent to 20 mg/kg bw per day) based on decreased body weight at 3000 ppm (equivalent to 20 mg/kg bw per day).

The NOAEL for reproductive toxicity was 3000 ppm (equivalent to 200 mg/kg bw per day), the highest dose tested (Ito, 1976).

In a two-generation reproductive toxicity study, isoprothiolane (lot no. 002120; purity 98.2%) was mixed with basal diet to achieve concentrations of 0, 30, 300 or 3000 ppm (equal to 0, 1.9, 19.7 and 196 mg/kg bw per day in F_0 males; 0, 2.5, 25.0 and 242 mg/kg bw per day in F_0 females; 0, 2.3, 22.3, and 235 mg/kg bw per day in F_1 males; 0, 2.7, 27.6, and 276 mg/kg bw per day in F_1 females). The diet was administered to Crl:CD(SD) rats (24/sex per group; five weeks at study start; males 152–170 g, females 126–141 g, within ±20% of the mean body weight [160.6 g in males and 133.9 g in females]), over two successive generations to investigate its potential to cause effects on reproductive performance of parental animals and development and growth of pups.

In parental animals at 30 ppm, no toxic effects of the test substance administration were noted in general appearance, body weight, body weight gain, feed consumption, necropsy findings, organ weight or histopathological findings.

At 300 ppm, liver weight was significantly higher in F_0 and F_1 females; this was likely not toxicologically significant because it was not accompanied by histopathological changes. No effects of the test substance administration were noted in the other indices in males or females.

At 3000 ppm, body weights, body weight gain and feed consumption were significantly lower in both F_0 and F_1 males and females. Atrophy of the thymus was noted with significantly higher frequency in both F_0 and F_1 females, and hypertrophy of the spleen was noted in four F_1 females. Liver and spleen weights were significantly higher in F_0 and F_1 males and females, and the weights of the thymus, ovaries and uterus were significantly lower or tended to be lower in F_0 and F_1 females. Histopathological examination of these organs found centrilobular hypertrophy of hepatocytes, increase in haemosiderin deposits in the spleen, atrophy of the thymus cortex and atrophy of the endometrium and myometrium in the uterus, with significantly higher frequencies in both F_1 and F_2 females. The frequency of atrophy in the ovaries was significantly increased in F_0 females and tended to be increased in F_1 females. In F_1 females, the frequency of the increase in extramedullary haematopoiesis in the spleen was significantly increased. No changes were noted in the numbers of primordial follicles in F_1 females.

No effects were noted in the reproductive performance of parental animals in any treated groups of F_0 or F_1 generation. Completion of preputial separation in males and vaginal opening in females was significantly delayed at 3000 ppm; however, no significant differences were noted in the body weights on the day of completion compared to the control (Table 18). The delay was considered attributable to the inhibition of body weight gain in treatment weeks 0–3 (the time period of sexual maturation in this group). No changes were noted in the anogenital distance of F_2 pups in any treated groups.

Table 18. Sexual development in F_1 rats[a] in a two-generation reproductive toxicity study with isoprothiolane

		Sexual development parameter measures per dose level			
		Males		Females	
Dietary concentration	Measure[a]	Age at preputial separation (days)	Body weight on day of preputial separation (g)	Age at vaginal opening(days)	Body weight on day of vaginal opening (g)
Control	Mean	41.9	223.3	30.0	104.4
	SD	1.2	21.7	2.5	14.2
30 ppm	Mean	42.3	226.3	28.5	95.6*
	SD	1.8	20.4	1.8	9.0
300 ppm	Mean	42.1	227.8	29.3	100.4
	SD	1.8	23.1	2.2	11.0
3000 ppm	Mean	43.7**	209.5	32.0**	102.6
	SD	1.7	18.5	2.5	12.6

ppm: parts per million; SD: standard deviation *: $P \leq 0.05$ (Dunnett test); **: $P \leq 0.01$ (Dunnett test)

[a] Results expressed as means of 24 rats/sex per dose. *Source*: Katano (2007)

No effects were noted in general appearance, body weight, number of pups delivered, sex ratio, viability index, lactation index, physical development, reflex response tests, autopsy findings, organ weights or histopathological findings in pups in the 30 or 300 ppm groups.

At 3000 ppm, body weights during the lactation period were significantly lower in both F_1 and F_2 animals from postnatal days 7 to 21. In physical development, the completion ratio of eye opening was significantly lower in F_2 males and females. In organ weights of weanlings, uterus weight was significantly lower in F_1 and F_2 females. No changes related to the test substance administration were noted in other indices.

The NOAEL for parental toxicity was 300 ppm (equal to 19.7 mg/kg bw per day) based on lower body weights, body weight gains, feed consumption and other effects at 3000 ppm (equal to 196 mg/kg bw per day).

The NOAEL for offspring toxicity was also 300 ppm (equal to 22.3 mg/kg bw per day in F_1 animals) based on delayed sexual maturation (vaginal opening and preputial separation), lower completion ratio of eye opening and other secondary to general toxicity at 3000 ppm.

The NOAEL for reproductive toxicity was 3000 ppm (equal to 196 mg/kg bw per day), the highest dose tested (Katano, 2007).

(b) Developmental toxicity

Rat

A teratogenicity study was performed in order to investigate the potential embryo/fetal developmental toxicity of isoprothiolane in rats. Isoprothiolane (batch no. not given; purity 98.2%) was administered by oral gavage, via gastric tube, to female Crl:CD(SD) rats (24 mated females/group; 10 weeks; 204–252 g; $n = 24$), at doses of 0 (control), 12, 50 and 200 mg/kg bw per day from day 6 through day 19 of gestation. At the time of autopsy of maternal rats on gestation day 20, fetuses were removed from the uteri and externally, viscerally and skeletally examinations.

At 200 mg/kg bw per day, treatment-related effects in maternal rats included salivation and soiled perigenital fur in three animals in the latter half of the administration period. Body weight, body weight gain and feed consumption were significantly lower almost throughout the treatment period, and adjusted body weights were also significantly lower. Autopsy of maternal rats on gestation day 20 found atrophy of spleen and thymus in four animals and hypertrophy of the adrenals in nine animals. Examination of ovaries and uterine contents at the time of autopsy found no significant differences in the number of corpora lutea and implantations, implantation index, number of live fetuses, number of dead or resorbed

embryos and fetuses, viability index of fetuses, mortality of embryos and fetuses, and sex ratio of fetuses compared to the control group. However, gravid uterine weights of maternal rats tended to be low, and fetal body weights of males and females and placental weights were significantly lower.

External examination of live fetuses found many dwarfs but without accompanying morphological anomalies. In the visceral examination, no changes related to the test substance administration were observed. There were no increases in skeletal anomalies related to test substance administration. Skeletal variations included significantly higher fetal and litter incidences of unossified thoracic vertebral body. In fetuses, the number of ossified cervical, thoracic, sacrocaudal and total vertebral bodies, sternebra, and metacarpus were significantly lower, indicative of growth retardation.

At 50 mg/kg bw per day, fetal skeletal examination revealed significantly higher incidence of unossified thoracic vertebral body and significantly lower number of ossified cervical and total vertebral bodies. In the other test parameters, no treatment-related changes in maternal and fetal rats were observed.

At 12 mg/kg bw per day, no treatment-related changes were observed in any test parameters.

The NOAEL for maternal toxicity of isoprothiolane in this study was 50 mg/kg bw per day based on suppressed body weight gains and feed consumption and suppressed growth at 200 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 12 mg/kg bw per day based on the significantly higher incidence of incomplete ossifiction of the thoracic vertebral body and significantly lower number of ossified cervical and total vertebral bodies in fetuses at 50 mg/kg bw per day, in the absence of maternal toxicity. Isoprothiolane was not teratogenic (Fujii, 2007).

Rabbit

A teratogenicity study was performed in order to investigate the potential embryo/fetal developmental toxicity of isoprothiolane in rabbits. Isoprothiolane (lot no. DBADME; purity 97.5%) was administered by oral gavage via a gastric rubber catheter, to New Zealand White female rabbits (18 mated females/group; 5–6 months old at the time of mating; 2.96–3.60 kg on gestation day 0), at doses of 0 (control), 15, 80 or 400 mg/kg bw per day from gestation day 6 through 18.

Clinical signs were observed daily from pregnancy day 0 to 29. Body weight and feed consumption were measured daily from day 0 and day 1, respectively, until day 29. On pregnancy day 29, the animals were killed under anaesthesia and examined for number of corpora lutea, implantations, embryo/fetal deaths (early and late resorption and dead fetuses) and live fetuses. The fetuses were removed from dams by caesarean section and examined for sex, body weight, placental weight and external appearance. Live fetuses were examined for gross visceral abnormalities. Organs were also examined microscopically for any abnormalities. After evisceration, transparent skeletal specimens were prepared and examined for abnormalities, variations and ossification.

Number of pregnancies were 17, 14, 15 and 14, respectively, for control, 15, 80 and 400 mg/kg bw per day groups. There was one abortion and one premature delivery in the control group. Two control animals and one high-dose animal died because of dosing errors. As a result, the number of dams used for terminal autopsy were 13, 14, 15 and 13, respectively, for control, 15, 80 and 400 mg/kg bw per day groups.

There were no treatment-attributable clinical signs (Table 19).

Table 19. Clinical signs in rabbits after start of dosing in teratogenicity study with isoprothiolane

Signs	Number of rabbits with the finding per dose level[a]			
	Control	15 mg/kg bw per day	80 mg/kg bw per day	400 mg/kg bw per day
Aggressiveness	–	1	–	1
Lachrymation	–	–	2	–
Sneezing	1	–	–	–
Rhinorrhoea	3	2	9	5
Epistaxis	1	1	–	–
Staining of lower abdomen with urine	2	–	–	1

[a] Eighteen mated females/group. bw: body weight;

At 15 mg/kg bw per day, body weight gain was similar to that of the control. At 80 mg/kg bw per day, body weight gain tended to be retarded relative to that three days prior to start of dosing. At 400 mg/kg bw per day, body weight gain tended to be retarded from gestation day 7 to 22, with a concomitant decrease in feed consumption, though it did not differ significantly from that of the control.

Feed consumption at 15 mg/kg bw per day was similar to that of control, whereas at 80 mg/kg per day, feed consumption tended to decrease from about gestation day 14 and was decreased on gestation days 19 and 20. At 400 mg/kg bw, feed consumption decreased from gestation day 7 to 19.

Fetal examination did not show any abnormalities in reproductive parameters. External examination and visceral examination did not show any malformations in controls or at 80 mg/kg bw per day. At 15 mg/kg bw per day, one fetus had flexural contractures of the right and left forelimbs with shortened tail. At 400 mg/kg bw per day, one fetus had a diverticulum in the intestine. These changes were considered to be spontaneous.

Skeletal examination found no anomalies in the control group; variations included lumbar ribs, separation of lumbar vertebral arch, extra presacral vertebra and a cervical rib observed in 34, 12, 2 and 1 fetuses, respectively. At 15 mg/kg bw per day, fused sternebrae and fused caudal vertebral centra were found in two and one fetuses, respectively; variations included lumbar ribs, separation of lumbar vertebral arch and a bilobulated cervical vertebral centrum in 19, 6 and 1 fetuses, respectively. In addition, the ossification of 5th sternebrae tended to be retarded. At 80 mg/kg bw per day, separation of sternebrae was observed in two fetuses; variations included lumbar ribs, separation of lumbar vertebral arch and cervical rib in 40, 11 and 1 fetuses, respectively. At 400 mg/kg bw per day, there were no anomalies; variations included lumbar ribs and separation of lumbar vertebral arch in 33 and 4 fetuses, respectively. In addition, ossification of the caudal vertebral arch was retarded. These change were not considered treatment related in view of the absence of dose dependency and based on historical background data from the performing laboratory (mean number of caudal vertebral arch ± SD [n = 231]: right, 8.9 ± 0.67; left, 8.6 ± 0.66) (Table 20).

No treatment-attributable clinical signs were observed.

Table 20. External, visceral and skeletal findings of fetuses from dams dosed orally with isoprothiolane in a teratogenicity study

	Number of rabbits with the finding per dose level [a]			
	0 mg/kg bw per day	**15 mg/kg bw per day**	**80 mg/kg bw per day**	**400 mg/kg bw per day**
Number of dams	13	14	15	13
Number of fetuses examined	100	90	105	114
External findings				
No. of fetuses with anomalies	0	1 (1.1)	0	0
Flexion contracture of wrist joint	0	1 (1.1)	0	0
Short tail	0	1 (1.1)	0	0
Visceral findings				
Number of fetuses with anomalies	0	0	0	1 (0.9)
Cystic duplication of intestine	0	0	0	1 (0.9)
Skeletal findings				
Number of fetuses with anomalies	0	3 (3.3)	2 (1.9)	0
Separation of sternebrae	0	0	2 (1.9)	0
Fused sternebrae	0	2 (2.2)	0	0
Fused caudal vertebral bodies	0	1 (1.1)	0	0
Number of fetuses with variations	43 (45.0)	23 (25.6)	49 (46.7)	35 (30.7)

(*Continued on next page*)

	Number of rabbits with the finding per dose level [a]			
	0 mg/kg bw per day	15 mg/kg bw per day	80 mg/kg bw per day	400 mg/kg bw per day
Bilobulated cervical vertebral body	0	1(1.1)	0	0
Cervical rib	1 (1.0)	0	1 (1.0)	0
Separation of lumbar vertebral arch	12 (12.0)	6 (6.7)	11 (10.5)	4 (3.5)
Extra presacral vertebra	2 (2.0)	0	0	0
Lumbar rib	34 (34.0)	19 (21.1)	40 (38.1)	33 (28.9)

bw: body weight; *Source*: Asoh, (1986b)

[a] Expressed as number of fetuses with the finding and, in parentheses, as a percentage of the number of fetuses examined.

The NOAEL for maternal toxicity was 80 mg/kg bw per day based on decreased body weight gains and feed consumption at 400 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity in rabbits was 400 mg/kg bw per day, the highest dose tested. Isoprothiolane was not teratogenic in rabbits (Asoh, 1986b).

2.6 Special studies

(a) Studies on metabolites

The metabolites of importance in plant and animal commodities are:

- 4-hydroxy isoprothiolane (M-3),
- 1-hydroxypropan-2-yl propan-2-yl 1,3-dithiolan-2-ylidenemalonate (M-5,isoprothiolane- dediisopropoxy-carbonyl derivative)
- and the monoester (M-2) glucuronide conjugate.

4-hydroxy isoprothiolane (M-3; diisopropyl 4-hydroxy-1,3-dithiolan-2-ylidenemalonate)

A non GLP-compliant acute oral toxicity study of M-3, a major isoprothiolane metabolite found in animals and plants, was undertaken in male and female albino mice (dd strain; ~20 g). Signs of reduced locomotion were observed in males and females 10 minutes after administration. Hypertension was observed 30–60 minutes after administration, followed by convulsions at 2–3 hours after administration in both males and females.

Estimated oral LD_{50} values for males and females were greater than 6590 and 4510 mg/kg bw (range: 3290–5950 mg/kg bw), respectively (Nokata, 1991).

1,3-dithiol-2-ylidenmalonate

LD_{50} values of 1,3-dithiol-2-ylidenmalonate are shown in Table 21.

Table 21. Summary of acute toxicity effects of 1,3-dithiol-2-ylidenmalonate

Species	Route of administration	Solvent	Sex	LD_{50} (mg/kg bw)
Mouse	Oral	Gum arabic	Male	11 400
			Female	11 700
		Olive oil	Male	3120
			Female	3466
	Subcutaneous	Gum arabic	Male	> 10 000
			Female	12 000
		Olive oil	Both	> 5000
	Intramuscular	Gum arabic	Both	> 5000

(*Continued on next page*)

	Intraperitoneal	Gum arabic	Male	1220

Species	Route of administration	Solvent	Sex	LD_{50} (mg/kg bw)
			Female	1460
		Olive oil	Male	1265
			Female	1277
Rat	Oral	Gum arabic	Both	> 6000
		Olive oil	Male	2065
			Female	2340
	Subcutaneous	Gum arabic	Both	> 5000
		Olive oil	Both	> 5000
	Intramuscular	Gum arabic	Both	> 2000
	Intraperitoneal	Gum arabic	Male	1070
			Female	1160
		Olive oil	Male	750
			Female	803
Hamster	Oral	Olive oil	Male	2707
	Intraperitoneal	Olive oil	Female	1533
Guinea pig	Oral	Olive oil	Male	2101
	Intraperitoneal	Olive oil	Female	1011
Rabbit	Oral	Gum arabic	Male	706
			Female	731
		Olive oil	Male	594
	Subcutaneous	Olive oil	Male	> 5000
	Intraperitoneal	Olive oil	Male	656

bw: body weight; *Source*: Ito (1978)

(b) Genotoxicity of metabolites

Genotoxicity of malothilate (diisopropyl 1,3-dithiol-2-ylidenmalonate) was examined using microbial test systems, specifically rec-assay employing *Bacillus subtilis* strain H17 recombination-wild (rec+), and its mutant recombination-deficient (rec−) strain M45. The growth inhibitory effect of isoprothiolane (malothilate) toward both strains was identical, which suggests that malothilate induced no genetic injuries.

In Ames tests employing *Salmonella typhimurium* TA1595, TA1537, TA1538, TA100, TA98 and *Escherichia coli* WP2uvr A, in the presence and absence of 9000 × g supernatant fraction from rat liver (S9), malothilate showed negative results (Sugimoto, Kubo & Funayama, 1978).

(c) Receptor-mediated effects identified from the literature

A search of literature databases for published toxicological data on isoprothiolane identified only five relevant studies.

Oh et al. (2007) investigated the estrogenic activities of pesticides, including isoprothiolane, that had been detected in the Pal-dang reservoir. An in vitro E-screen assay with MCF-7BUS estrogen receptor (ER)-positive human breast cancer cells was used with ER-negative MDA MB 231 cell lines for comparison. Isoprothiolane was confirmed to have estrogenic activity as shown by the increasing MCF-7 BUS cell growth on its addition. Moreover, estrogen receptor alpha (ERα) protein, estrogen receptor-regulated progesterone receptor (PR) and pS2 messenger ribonucleic acid (mRNA) levels were measured with MCF-7 BUS cells. On the addition of isoprothiolane at concentration of 10^{-4} M, the levels of ERα protein decreased (slightly) and levels of PR and pS2 mRNA increased in the same manner as with the addition of 17β-estradiol.

Based on these results, it was confirmed that isoprothiolane exhibits weak estrogenic activity (Oh et al., 2007).

In a study of the estrogenic activities of 517 chemicals with a simple and rapid screening method using a yeast two-hybrid system based on the ligand-dependent interaction of nuclear hormone receptor with coactivators, isoprothiolane (and all of other pesticides) tested negative to 10^{-4} mol/L was negative in this study (Nishihara et al., 2000).

Screening of 200 pesticides, including isoprothiolane. for estrogen and androgen receptor activities by in vitro reporter gene assays using Chinese hamster ovary cells was undertaken by Kojima et al. (2004). Isoprothiolane did not exhibit estrogenic (ERα or ERβ) or androgen receptor agonism or antagonism (up to 10^{-5} mol/L) in this study.

An in vitro study demonstrated that isoprothiolane is able to activate the pregnane X receptor (PXR) receptor, a ligand-dependent transcription factor that regulates genes involved in xenobiotic metabolism. Kojima et al. (2011) characterized the human pregnane X receptor (hPXR) and mouse pregnane X receptorv(mPXR) agonistic activity of 200 pesticides and found that a large number, including isoprothiolane, possess these activities with greater or lesser interspecies differences. The REC_{20} values (the concentration of the test substance showing 20% of the agonistic activity of 1×10^{-5} mol/L rifampicin via hPXR, or 1×10^{-5} mol/L PCN via mPXR) of isoprothiolane were 4.2×10^{-6} and 7.3×10^{-6} mol/L, respectively. At 1×10^{-5} mol/L, isoprothiolane induced 49% of the response induced by rifampicin via hPXR and 23% of the response induced by PCN via mPXR. Studies have shown that PXR activation may affect energy metabolism as well as the endocrine and immune systems.

A study conducted by Ishizuka (1998) indicated that some agrochemicals, including isoprothiolane, are capable of inducing phase I and II xenobiotic-metabolizing enzyme activities in an isozyme selective manner. The induction of these activities may disrupt normal physiological functions related to these enzymes in exposed animals. Butachlor, pretilachlor and isoprothiolane were administered to male rats at 50 mg/kg bw daily for five days, and their effects examined on cytochrome P450 (P450), glutathione *S*-transferase (GST), UDP-glucuronosyltransferase (UDPGT) and NAD(P)H-quinone oxidoreductase 1 (NQO1)-related metabolism in the liver. Isoprothiolane had no effect on total microsomal P450 content on the liver. Activities of aminopyrine *N*-demethylase, aniline 4-hydroxylase and testosterone 6β-hydroxylase were unaffected. Ethoxyresorufin O-deethylase activity was marginally (114%) increased. In contrast, the CYP2B-dependent activities pentoxyresorufin O-depentylase (376% of control) and testosterone 16β-hydroxylase (305% of control) were markedly increased. Isoprothiolane had no noticeable effect on the expression of CYP1A1, CYP2C11, CYP2E1 or CYP3A, as determined by Western blotting, whereas expression of both CYP2B1 and CYP2B2 was appreciably elevated. Activities of GST toward 1-chloro-2,4-nitrobenzene and 3,4-dichloronitrobenzene were slightly induced (126–133% of control) in the liver of the rats treated with isoprothiolane. On the other hand, marked elevations of UDPGT activities toward *p*-nitrophenol (281% of control) and in NQO1-related metabolism (menadione reductase activity) (176% of control) were observed (Ishizuka,1998).

3. Observations in humans

3.1 Field exposure study in human volunteers

A field exposure study with human volunteers was conducted to assess the possible effects of spraying and exposure to the spray drift of an isoprothiolane formulation (FUJI ONE 40EC).

Ten apparently healthy human volunteers evaluated clinicopathologically two days before start of spray operations. The isoprothiolane formulation was sprayed on sugarcane crop (12–24 inches high) for three consecutive days (6 hours/day: 2 × 3 h) using three knapsack sprayers. Isoprothiolane formulation was sprayed at the maximum recommended concentration of 1 mL/L of water. The commonly suggested protective clothing (full sleeved apron, pyjamas, cap, mask, goggles and gloves) provided to volunteers was worn during their involvement in actual spraying operations (mixing, loading and spraying). Other routine precautions were observed during spraying. The volunteers remained in the spraying area when not spraying, at which time they wore their usual clothing (shorts, lungi and half-sleeved banian) and would have been exposed to the spray drift. The study was conducted in conjunction with a livestock exposure study.

Daily clinical examination and urine analysis of the volunteers and laboratory investigations on blood and plasma was carried out as per approved protocol at the predetermined schedules of pre-exposure, at the end of exposure and two days after spraying. Comparison of data on daily clinical evaluation, urine analysis (specific gravity, pH, nitrites, glucose, albumin, bilirubin, urobilinogen, ketone bodies, erythrocytes and leucocytes) and statistical analysis (by paired *t*-test) of data on haematology (white blood cell, RBC, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration values and percentage of neutrophils, lymphocytes, eosinophils and monocytes) and plasma clinical chemistry (glucose, BUN, GPT, GOT, ALP, albumin and total protein) data from the pre-exposure, exposure and post-exposure periods showed that there were:

- no exposure-related variations in clinical observations involving various systems of the body;
- no exposure-related changes in haematological parameters of biological significance; and
- no exposure-related changes in plasma clinical chemistry parameters of biological significance.

Isoprothiolane formulation (FUJI ONE 40EC) at 1 mL/L water applied using the recommended methods and concentration of application has no discernible effect on human health parameters based on assessments in human volunteers under the testing conditions adopted in the study (Prakash, 1995)

3.2 Health effects on manufacturing plant personnel

The sponsor intimated that searches of their internal records found that no health issues had been reported for isoprothiolane workers over the previous 10 years.

3.3 Information on accidental or intentional poisoning

No information on accidental or intentional poisoning in humans was available.

Comments

Biochemical aspects

In rats orally administered radiolabelled isoprothiolane, the test compound was almost completely absorbed. C_{max} in blood was achieved within six hours of administration of a low dose (5 mg) and within nine hours of administration of a high dose (500 mg), irrespective of sex. The radiolabel was widely distributed among tissues, with most found in the liver, kidney and gastrointestinal tract, irrespective of sex and dose administered. In the later phase, that is, 24 or 168 hours after the low and high dose, respectively, radioactivity in almost all organs and tissues was gradually eliminated, with elimination slowest from fur and skin. Radioactivity in these tissues appears to comprise amino acids incorporated into keratin. The radiolabel of orally-dosed isoprothiolane was excreted mainly via urine (24–34% of low dose and 46–53% of high dose) and expired air (~30%). In both low- and high dose groups, excretion of the radiolabel was rapid until 24 and 48 hours post dose, respectively, and became slower thereafter. After 168 hours post dose, the carcass still retained about 10% of the administered radiolabel (Yoshizane, 2007).

Orally dosed isoprothiolane was metabolized in rats by hydroxylation and hydrolysis, and by cleavage of the dithiolane ring, resulting in carbon dioxide and other low molecular weight metabolites. The glucuronic acid conjugate of the monoester was the most prominent metabolite in excreta (urine), accounting for 6% and 15% of dosed radioactivity in low and high-dose groups, respectively, irrespective of sex (Yoshizane, 2007).

Toxicological data

In one study in rats, the oral LD_{50} was estimated to be between 300 and 2000 mg/kg bw (Oda, 2010a). In another study, there were no mortalities below the dose level of 900 mg/kg bw (Miyazaki, 1970). The dermal LD_{50} in rats was greater than 2000 mg/kg bw (Miyazaki, 1970; Oda, 2010b). Following inhalation, the LC_{50} in rats was greater than 2.32 mg/L (Pouline, 2010). Isoprothiolane was not an irritant to rabbit skin (Suzuki, 1984a), but a mild irritant to the rabbit eye (Suzuki, 1984b, 2010a). Isoprothiolane was a dermal sensitizer in a Magnusson and Kligman maximization test in guinea pigs (Suzuki, 2010b).

Although findings in short- and long-term toxicity studies in mice, rats and dogs varied, the most consistently targeted organs were liver, kidney and the haematopoietic system.

In a 112–115 day oral toxicity study in mice using isoprothiolane at dietary concentrations of 0, 20, 100, 300, 900 or 2700 ppm (equal to 0, 3.32, 14.8, 48.0, 132 and 472 mg/kg bw per day for males and 0, 2.81, 14.3, 47.2, 140 and 444 mg/kg bw per day for females, respectively), the NOAEL was 900 ppm (equal to 140 mg/kg bw per day) based on decreases (by about 40%) in ovarian weight, the toxicological significance of which was equivocal because of the absence of specific histopathological findings, at 2700 ppm (equal to 444 mg/kg bw per day) (Miyazaki, 1972).

In a 90-day dietary study in rats, with isoprothiolane at dose levels of 0, 50, 300 or 3000 ppm (equal to 0, 3.4, 20.9 and 201 mg/kg bw per day for males and 0, 4.0, 23.4 and 223 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 3.4 mg/kg bw per day) based on increased relative weights of liver (10%) and kidneys (7%) and increased GGT activity (1.5-fold compared to controls) in males at 300 ppm (equal to 20.9 mg/kg bw per day) (Sunaga, 2006).

In a 52-week toxicity study in dogs, isoprothiolane was administered orally via gelatine capsule at dose levels of 0, 2.0, 10.0 or 50.0 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day based on reduced body weight gain in females (35%), increased ALP activity in both sexes (3.3-fold in males and 1.6-fold in females), increased absolute (37%) and relative (72%) thyroid/parathyroid weight in females and increased relative liver weight in males (17%) at 50 mg/kg bw per day (Osborne & Kalichman, 1989).

In a 78-week carcinogenicity study in mice using isoprothiolane at dietary concentrations of 0, 200, 1000 or 5000 ppm (equal to 0, 20, 104 and 501 mg/kg bw per day for males and 0, 18.2, 95.6 and 558 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 95.6 mg/kg bw per day) based on reduced body weight in males at 5000 ppm (equal to 501 mg/kg bw per day). There were no treatment-related increases in tumour incidence (Inui, 1989).

In an 104-week combined chronic toxicity and carcinogenicity study in rats using isoprothiolane at dietary concentrations of 0, 50, 300 and 3000 ppm (equal to 0, 1.82, 10.9 and 115 mg/kg bw per day for males and 0, 2.06, 12.6 and 139 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 10.9 mg/kg bw per day) based on reduced body weight gain (12% in males and 36% in females), increased BUN in females (16–25%, significant at weeks 26 and 52) and increased relative weights of liver (30–40%) and kidneys (10–50%) in both sexes at the interim and final kills, at 3000 ppm (equal to 115 mg/kg bw per day). An increased incidence of benign dermal keratoacanthoma was observed in males in the highest dose group (13/80 compared to 3/79 in controls). No pre-neoplastic changes were observed in the skin. There were no compound-related increases in any other tumour incidence (Inui, 1991).

The Meeting concluded that isoprothiolane is not carcinogenic in mice but caused benign skin tumours in male rats at the highest dose.

Isoprothiolane was tested for genotoxicity in an adequate range of in vitro and in vivo assays (Moriya, 1977; Asoh, 1986a; Kajiwara, 1986a,b; Asquith, 1988a,b). There was little evidence of genotoxicity in vitro and no evidence of genotoxicity in vivo.

The Meeting concluded that isoprothiolane is unlikely to be genotoxic in vivo.

In view of the lack of genotoxic potential in vivo, the absence of carcinogenicity in mice and the increase in benign skin tumours occurring only in male rats at the highest dose, the Meeting concluded that isoprothiolane is unlikely to pose a carcinogenic risk to humans at the levels occurring in the diet.

In a three-generation reproductive toxicity study in rats using isoprothiolane at dietary concentrations of 0, 30, 300 or 3000 ppm (equivalent to 0, 2, 20 and 200 mg/kg bw per day, respectively) for three generations, the NOAEL for parental and embryo/fetal toxicity was 300 ppm (equivalent to 20 mg/kg bw per day) based on decreased body weight gain at 3000 ppm (equivalent to 200 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equivalent to 200 mg/kg bw per day), the highest dose tested (Ito, 1976).

In a two-generation reproductive toxicity study in rats using isoprothiolane at dietary concentrations of 0, 30, 300 or 3000 ppm (equal to 0, 1.9, 19.7 and 196 mg/kg bw per day in F_0 males; 0, 2.5, 25.0 and 242 mg/kg bw per day in F_0 females; 0, 2.3, 22.3 and 235 mg/kg bw per day in F_1 males; 0, 2.7, 27.6 and 276 mg/kg bw per day in F_1 females), the NOAEL for parental toxicity was 300 ppm (equal to 19.7 mg/kg bw per day) based on decreased body weights, body weight gains and feed consumption and other effects at 3000 ppm (equal to 196 mg/kg bw per day). The NOAEL for offspring toxicity was 300 ppm (equal to 22.3 mg/kg bw per day) based on delayed sexual maturation, delayed eye opening and other effects secondary to general toxicity at 3000 ppm (equal to 235 mg/kg bw per day) and secondary to effects on body weights in the dams. The NOAEL for reproductive toxicity was 3000 ppm (equal to 196 mg/kg bw per day), the highest dose tested (Katano, 2007).

In a developmental toxicity study in rats, isoprothiolane was administered by oral gavage at doses of 0, 12, 50 or 200 mg/kg bw per day from gestation day 6 to 19. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on decreased body weight, body weight gain and feed consumption at 200 mg/kg bw per day. Isoprothiolane did not cause any fetal anomalies up to 200 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity in rats was 12 mg/kg bw per day, based on the significantly high incidence of incomplete ossification of the thoracic vertebral body and significantly low number of ossified cervical and total vertebral bodies at 50 mg/kg bw per day (Fujii, 2007).

In a developmental toxicity study in rabbits, isoprothiolane was administered by oral gavage at doses of 0, 15, 80 or 400 mg/kg bw per day from gestation day 6 through 18. The NOAEL for maternal toxicity was 80 mg/kg bw per day based on decreased body weight gains and feed consumption at 400 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Asoh, 1986b).

The Meeting concluded that isoprothiolane is not teratogenic.

Receptor-mediated effects identified from the literature

In in vitro studies of effects on estrogen or androgen receptors, isoprothiolane had little or no effect up to concentrations of 10^{-4} mol/L (Nishihara et al., 2000; Kojima et al., 2004; Oh et al., 2007).

An in vitro study demonstrated that isoprothiolane is able to activate the pregnane X receptor (PXR) receptor (Kojima et al., 2011). In vivo studies in rats showed that isoprothiolane can induce cytochrome CYP2B and UDPGT, characteristics of constitutive androstane receptor (CAR) activation (Ishizuka, 1998).

Toxicological data on metabolites and/or degradates

The metabolites of importance in plant and animal commodities are:

- 4-hydroxy isoprothiolane (M-3),
- 1-hydroxypropan-2-yl propan-2-yl 1,3-dithiolan-2-ylidenemalonate (M-5), and
- the monoester (M-2) glucuronide conjugate.

A structural comparison of these metabolites with isoprothiolane using Toxtree (version 2.6.13) identified no unique structural alerts that would not be covered by the toxicity tests on the parent. The Meeting therefore concluded that these metabolites are unlikely to be genotoxic.

In an acute toxicity study in mice, M-3 was less toxic than the parent, showing an LD_{50} equal or greater than 3290 mg/kg bw (Nokata, 1991). In a metabolism study, M-3 was found in small

amounts (<1%) in rat faeces. Based on these observations and the close structural similarity between M-3 and isoprothiolane, the Meeting concluded that it was unlikely that the metabolite M-3 or its conjugates would be of greater toxicity than the parent, isoprothiolane.

M-5 was not found in rat metabolism studies. However, based on its structure the Meeting concluded that it was unlikely that M-5 or its conjugates would be of greater toxicity than the parent, isoprothiolane.

There were no specific data on the toxicity of the monoester (M-2) glucuronide conjugate. However, given its low lipid solubility and structure, the intact glucuronide is unlikely to be toxic by the oral route. Although intestinal hydrolysis may lead to formation of the monoester (M-2), the structural similarity of M-2 to isoprothiolane suggests that it would not be of greater toxicity than the parent, isoprothiolane.

The Meeting concluded that these metabolites are not of greater toxicological concern than the parent and considered that they would be covered by the acceptable daily intake (ADI) established for isoprothiolane.

Human data

A report on a field exposure study in human volunteers spraying an isoprothiolane formulation (FUJI ONE 40EC) was provided. Human volunteers wearing the recommended protective clothing were exposed to spray drift during spraying. Subsequent monitoring for three consecutive days identified no effects on the health parameters assessed in this study (clinical examination, urine analysis and laboratory investigations on blood and plasma) (Prakash, 1995).

No adverse health effects were noted in reports on manufacturing plant personnel. No reports on accidental or intentional poisoning in humans were available.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.1 mg/kg bw on the basis of a NOAEL of 10.9 mg/kg bw per day from a 2-year study of toxicity and carcinogenicity in rats, based on an increase in BUN in females and an increase in the relative weight of liver and kidneys in both sexes at 115 mg/kg bw per day. Although the NOAEL of 3.4 mg/kg bw per day in the 90-day oral rat study was lower, the LOAEL in this study was based on marginal effects. The Meeting therefore concluded that the NOAEL of the 2-year combined toxicity/carcinogenicity study was the more appropriate on which to establish the ADI.

This ADI was supported by a NOAEL of 10 mg/kg bw per day from a 52-week toxicity study in dogs. A safety factor of 100 was applied.

The Meeting concluded that it was unnecessary to establish an acute reference dose (ARfD) for isoprothiolane in view of its low acute oral toxicity and absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of isoprothiolane

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity [a]	Toxicity	1000 ppm, equal to 95.6 mg/kg bw per day	5000 ppm, equal to 501 mg/kg bw per day
		Carcinogenicity	5000 ppm, equal to 501 mg/kg bw per day [b]	–
Rat	Ninety-day toxicity [a]	Toxicity	50 ppm, equal to 3.4 mg/kg bw per day	300 ppm, equal to 20.9 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	300 ppm, equal to 10.9 mg/kg bw per day	3 000 ppm, equal to 115 mg/kg bw per day
		Carcinogenicity	300 ppm, equal to 10.9 mg/kg bw per day (benign tumours)	3000 ppm, equal to 115 mg/kg bw per day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	3000 ppm, equal to 196 mg/kg bw per day [b]	–
		Parental toxicity	300 ppm, equal to 19.7 mg/kg bw per day	3000 ppm, equal to 196 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 22.3 mg/kg bw per day	3 000 ppm, equal to 235 mg/kg bw per day
	Three-generation study of reproductive toxicity [a]	Reproductive toxicity	3000 ppm, equivalent to 200 mg/kg bw per day [b]	–
		Parental toxicity	300 ppm, equivalent to 20 mg/kg bw per day	3000 ppm, equivalent to 200 mg/kg bw per day
		Offspring toxicity	300 ppm, equivalent to 20 mg/kg bw per day	3000 ppm, equivalent to 200 mg/kg bw per day
	Developmental toxicity study [c]	Maternal toxicity	50 mg/kg bw per day	200 mg/kg bw per day
		Embryo/fetal toxicity	12 mg/kg bw per day	50 mg/kg bw per day
Rabbit	Developmental toxicity study [c]	Maternal toxicity	80 mg/kg bw per day	400 mg/kg bw per day
		Embryo/fetal toxicity	400 mg/kg bw per day [b]	–
Dog	Fifty-two week study of toxicity [d]	Toxicity	10 mg/kg bw per day	50 mg/kg bw per day [b]

[a] Dietary administration.

[b] Highest dose tested.

[c] Gavage administration.

[d] Capsule administration.

Estimate of acceptable daily intake (ADI; applies to isoprothiolane and the metabolites M-2, M-3 and M-5)

0–0.1 mg/kg bw

Estimate of acute reference dose (ARfD)

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 isoprothiolane

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Almost completely absorbed; T_{max} at 6 h (low dose) or 9 h (high dose)
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	10% of dosed radioactivity in residual carcass at 168 h
Rate and extent of excretion	Excreted mainly into urine and expired air. Excretion is rapid until 24–48 h post dose and becomes slow afterwards
Metabolism in animals	Extensively metabolized through hydroxylation and hydrolysis
Toxicologically significant compounds in animals and plants	Isoprothiolane, M-2 glucuronide, M-3 and M-5
Acute toxicity	
Rat, LD_{50}, oral	≥ 300 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 2.32 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Mildly irritating
Guinea pig, dermal sensitization	Dermal sensitizer (maximization test)
Short-term studies of toxicity	
Target/critical effect	Liver and kidneys
Lowest relevant oral NOAEL	3.4 mg/kg bw (rat)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver, kidney
Lowest relevant NOAEL	10.9 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice; benign dermal keratoacanthoma only in male rats at highest dose tested [a]
Genotoxicity	Unlikely to be genotoxic in vivo [a]
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	19.7 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	20 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	196 mg/kg bw per day (highest dose tested; rat)
Developmental toxicity	
Target/critical effect	Delayed ossification
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	12 mg/kg bw per day (rat)

Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
M-3	Acute oral toxicity data in mice: LD_{50}: ≥3290 mg/kg bw
M-5	No data
Monoester (M-2) glucuronide conjugate	No data
Human data	No adverse health effects reported

[a] Unlikely to pose a carcinogenic risk to humans at the levels occurring as residues in the diet.

Summary

	Value	Study	Safety factor
ADI [a]	0–0.1 mg/kg bw	Two-year chronic toxicity/carcinogenicity (rat)	100
ARfD	Unnecessary	–	–

[a] Applies to isoprothiolane and to M-2, M-3 and M-5.

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NATAMYCIN

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Explanation

Natamycin (synonym pimaricin) is the World Health Organization-approved, non-proprietary name (according to IUPAC, the International Union of Pure and Applied Chemistry) for:

(8*E*,14*E*,16*E*,18*E*,20*E*)-(1*R*,3*S*,5*R*,7*R*,12*R*,22*R*,24*S*,25*R*,26*S*)-22-(3-amino-3,6-dideoxy-β-*D*-mannopyranosyloxy)-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.05,7] octacosa-8,14,16,18,20-pentaene-25-carboxylic acid,

with the Chemical Abstracts Service number 7681-93-8.

No International Organization for Standardization (ISO)-approved name is available.

Natamycin is a fungicide of the polyene macrolide class of antifungal antimicrobials used in the protection of foods and for treatment of human disease. In food production, natamycin can be used as surface treatment of cheeses and dried sausages. It is also used as for the topical treatment of fungal infections in humans and animals, such as mycotic keratitis. Natamycin was originally produced by *Streptomyces natalensis* in submerged aerobic batch culture fermentation but is also now known to be produced by several other *Streptomyces* species. Natamycin acts as fungicide by preventing the germination of fungal spores via binding to ergosterol located in fungal cellular membranes.

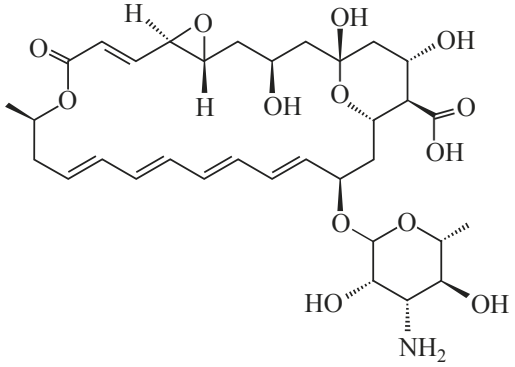
Natamycin (Fig. 1) has not been evaluated previously by Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

Natamycin was previously evaluated by the 12th, 20th and 57th Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1968, 1976 and 2001 at the request of the Codex Committee on Food Additives and Contaminants (FAO/WHO, 1969, 1976, 2002a). The 20th JECFA established an acceptable daily intake (ADI) of 0–0.3 mg/kg body weight (bw) based on gastrointestinal effects in humans and application of a safety factor of 10 (FAO/WHO, 1976). The 57th JECFA reaffirmed its previous conclusion on the ADI (FAO/WHO, 2002a).

This evaluation is based mainly on the study reports made available to the Meeting. A comprehensive literature search was also conducted (see Appendix 1). The articles relevant for the human health risk assessment were included in the evaluation and are described in the appropriate sections. Only a few submitted studies contained statements of compliance with good laboratory practice (GLP) or were conducted according to internationally recognized guidelines (such as the United States Environmental Protection Agency/Federal Insecticide, Fungicide, and Rodenticide Act or Organisation for Economic Co-operation and Development [OECD]). Most submitted studies pre-dated the GLP requirements or similar test guidelines. However, GLP status was not specifically checked. In general, studies were conducted with technical material; however, neither impurity profiles of the tested materials nor current specifications were provided. Hence, it could not be assessed whether the tested material was representative of the currently available commercial technical material.

In line with the JMPR mandate, only the impact of natamycin residues on human health was assessed in this evaluation. This included the assessment of the potential impact on the intestinal microbiota and possible induction of resistant microorganisms due to the presence of residues in the gastrointestinal tract. The possible impacts on workers involved in natamycin manufacture and operators using natamycin-containing products was not assessed.

Figure 1. Structural formula of natamycin



Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

The stability/recovery of natamycin was investigated in faeces of mice and humans, and was found to be of low stability/recovery (~50% recovery within 24 h; under certain conditions ~20%). Further investigations on the amount of natamycin in blood, urine and faeces in mice, rats, guinea pigs, dogs, pigs and humans were described (Anonymous, 1968). However, both the experimental study design (e.g., groups size, dose levels, time to analysis [due to instability of the test material]) and the results are reported in too little detail and in a too confusing way to allow an independent evaluation of the findings. Natamycin amounts were determined via antimycotic activity. The study report stated that low concentrations of natamycin were found in dog blood but not in rat, guinea pig and human blood; no natamycin was found in rat, guinea pig and human urine, but it was detected in dog urine; natamycin was detected in mouse, pig and human faeces.

An evaluation by the United States Environmental Protection Agency (USEPA, 2012) mentioned a study by Shirk (1963)[1] on faecal excretion of natamycin in animals. This study was not submitted to JMPR, despite a request from the sponsor.

Rat

The distribution of natamycin was studied by autoradiographic and bioautographic techniques. In the autoradiographic study, five female Wistar rats (TNO, specific pathogen-free) were each given a single dose of 50 mg/kg bw of [^{14}C]natamycin (50 mg in 5 mL of 1% amylum) orally. In the bioautographic study, four female rats were each given a single dose of 50 mg/kg bw (70 mg in 7 mL of 1% amylum) orally. No information on the purity of the compound was provided. Before treatment, the animals were fasted for 24 hours but were given a 5% glucose drinking-water solution. One animal in each group was killed by immersion in a solid CO_2 and acetone mixture under mild ether anaesthesia 1, 2 (autoradiographic study only), 4, 8 and 24 hours after treatment. Whole-body sections of the animals were cut in a cryostat at −20 °C. In the autoradiographic study, sections were freeze-dried (48 h) and exposed on photographic plates at −20 °C for 93 days (a few for 150 days). In the bioautographic study, the antibiotic activity of the sections was evaluated by exposure on Whiffen agar plates inoculated with *Saccharomyces cerevisiae* strain ATCC 9763 for 5, 10, 15 or 20 minutes (20, 40, 60 and 120 minutes for sections from the animals killed 24 hours after treatment). After exposure, the agar plates were incubated at 30 °C for 20 hours and photographed.

In the autoradiographic study, the radioactivity was confined to the gastrointestinal tract after 93 days' exposure (1 h: oesophagus, stomach, small intestine; 2 h: oesophagus, stomach, small intestine, caecum; 4 h: stomach, small intestine, caecum, colon; 8 h: stomach, intestine; 24 h: caecum, colon). After exposure for 150 days, the radioactivity was visible only faintly, after magnification of the pictures, in the liver, kidneys and fatty tissue in addition to the gastrointestinal tract.

In the bioautographic study, the antibiotic activity of natamycin was restricted to the gastrointestinal tract (1 h: stomach, small intestine; 4 h: stomach, small intestine, caecum; 8 h: stomach, small intestine, caecum) and lasted less than 24 hours. No antibiotic activity was observed in the colon. The results of the autoradiographic study indicate that natamycin is minimally absorbed into the bloodstream and excreted almost entirely in faeces. The lack of antibiotic activity and the presence of radioactivity in the caecum and colon 24 hours after dosing are consistent with the breakdown of natamycin into microbiologically inactive compounds by bacterial flora in the caecum and colon (Blankwater & Hespe, 1979).

A series of experiments was conducted to study the excretion and resorption of[^{14}C]natamycin and its degradation products in normal and cholestatic Wistar rats (induced by tying the bile ducts with

[1] Shirk RJ (1963). The fecal excretion and non-absorption of pimaricin in animals: Report FS 3. Progress rept.

a ligature). GLP status was not mentioned in the report. The report was available only in Dutch; only summary tables were reported and not individual results for all animals.

In the first series, the excretion pattern of radiolabelled compound was investigated in groups of three young male Wistar rats given [^{14}C]natamycin at a dose of 0.1, 1 or 10 mg/kg bw by quantifying the amount of radioactivity in the urine and faeces at 24-hour intervals for 72 hours and in expired air hourly for up to seven hours. Another group received a single dose of 10 mg/kg bw by intraperitoneal injection. A similar experiment was performed in which 10 mg/kg bw of the degradation products of [^{14}C]natamycin, obtained by acid hydrolysis to simulate that in the stomach, were administered orally or via intraperitoneal injection to three Wistar rats; their urine, faeces and expired breath were analysed as described above.

Separate experiments were conducted to determine the elimination of [^{14}C]natamycin in bile by giving 10 mg/kg bw to two rats orally and to four rats by intraperitoneal injection. Bile was obtained via a cannula in the bile duct at one-hour intervals for 7 h and analysed for radioactivity. In a similar experiment, the elimination of [^{14}C]natamycin via bile was determined after oral administration of 10 mg/kg bw of the test substance; in this experiment, 0.1 mL of bile obtained from rats not treated with natamycin was placed in the duodenum of treated animals. Bile was collected hourly for 7 h and analysed for radioactivity.

A series of analyses was also carried out to quantify the radioactivity in the stomach, small intestine, caecum and large intestine of groups of two rats at 1, 2, 4, 8 and 24 hours after administration of 10 mg/kg bw of [^{14}C]natamycin. Sections of the stomach, small intestine, caecum and large intestine were extracted in methanol, and the extracts were analysed for radioactivity by thin-layer chromatography. One rat was given the non-radioactive form of the test material and killed after four hours. The concentration of natamycin was analysed in each section of the gastrointestinal tract by high-performance liquid chromatography. The results were compared with those obtained with the radioactive form. In each experiment, all animals were fasted for 20 hours before treatment. Water was available during fasting.

The results are summarized in Table 1. When 10 mg/kg bw of [^{14}C]natamycin was administered orally to normal or cholestatic rats, most of the radioactivity (93–103%) was found in faeces. Cholestatic rats had about 5% more radioactivity in their urine than normal rats at this dose. The results were similar in rats treated with 0.1 or 1 mg/kg bw. When natamycin was delivered by intraperitoneal injection at a dose of 10 mg/kg bw, about 16% of the radioactivity was found in urine and about 76% in faeces by 72 hours, indicating significant elimination in the bile. Most of the elimination (63%) occurred within 24 hours after administration of natamycin.

Intraperitoneal administration of acid-hydrolysed [^{14}C]natamycin resulted in approximately twice as much radioactivity in the urine (61%) as in faeces (30%). In contrast, after oral administration of acid-hydrolysed [^{14}C]natamycin, most of the radioactivity was recovered in faeces (94% as compared with 6.7% in urine); hydrolysis resulted in higher urinary excretion than seen with intact natamycin. Little radioactivity associated with either intact or acid-hydrolysed natamycin was eliminated as $^{14}CO_2$ in expired breath (< 1%) after either oral or intraperitoneal administration.

In experiments to determine the amount of radioactivity in bile after an oral or intraperitoneal dose of 10 mg/kg bw [^{14}C]natamycin, 40% of the total radioactivity was recovered over seven hours from the bile of rats treated via intraperitoneal injection and only 1% from the bile in rats treated orally. However, results were presented graphically but not tabulated. When "blank" bile was administered via the duodenum each hour for seven hours to animals treated orally, the amount of radioactivity recovered in the bile was similar to that recovered in animals not given bile.

In the stomach and small intestine, natamycin was mostly untransformed, as indicated by thin-layer chromatography. Most degradation took place in large intestine. The degradation products were more hydrophobic than natamycin and were found from about four hours after treatment. Most of the dose of 10 mg/kg bw was degraded about eight hours after treatment.

Overall, no more than 5–7% of the total radioactive dose was excreted via urine after oral administration of [^{14}C]natamycin. Approximately 90% of the administered compound was eliminated in the faeces (Anonymous, 1979).

The English language version[2] of this report was not submitted to JMPR.

[2] Hespe W, Meier AM (1979). The metabolism of pimaricin in rats. II. Investigation, with the help of ^{14}C-pimaricin, of its resorption, its decomposition in the gastrointestinal tract, and its elimination. Unpublished report no. 20.504 by Gist-Brocades NV, Haarlem, the Netherlands.

Rabbit

Rabbits were induced with uveitis by intravitreous injection with solutions of human albumin. The rabbits were assigned to one of two groups, each with comparable degrees of severity of effects, consisting out of 21 animals. Animals received a single dose of 5 or 10 mg/kg bw of natamycin (suspended in 5% glucose) by intravenous injection. Subgroups of three animals were killed 0.5, 1, 3, 6, 12 and 24 hours after administration. The remaining three animals were treated daily with natamycin for one week and were killed 24 hours after the last dose. Natamycin levels were determined in aqueous, vitreous and blood samples by ultraviolet–visible (UV–VIS) photometry after sample preparation.

The half-life of natamycin in serum was approximately three hours. No natamycin was detectable 12 hours after a single dose (Ellison, 1979).

Table 1. Excretion of radioactivity administered to normal and cholestatic rats

Administered dose / Excreta	Time (h)	% of administered dose of [^{14}C]natamycin [a]		
		Oral administration		i.p. administration
		Normal rat	Cholestatic rat	Normal rat
10 mg/kg bw				
Urine	0–24	1.87 ± 0.44	5.92 ± 1.95	13.95 ± 1.25
	24–48	0.08 ± 0.01	0.56 ± 0.25	1.01 ± 0.25
	48–72	0.03 ± 0.01	0.12 ± 0.03	0.65 ± 0.12
	Sum (urine)	1.98 ± 0.51	6.60 ± 1.82	15.61 ± 1.00
Faeces	0–24	91.60 ± 7.94	50.36 ± 4.78	62.80 ± 8.72
	24–48	10.69 ± 7.77	35.82 ± 0.20	9.10 ± 3.62
	48–72	0.36 ± 0.37	6.77 ± 4.83	4.48 ± 3.23
	Sum (faeces)	102.65 ± 1.08	92.95 ± 3.41	76.38 ± 6.83
$^{14}CO_2$	0–7	0.27 ± 0.03	0.22 ± 0.13	0.14 ± 0.01
	Total	104.90 ± 0.92	99.77 ± 1.80	91.99 ± 7.29
1 mg/kg bw				
Urine	0–24	1.82 ± 0.46	3.28 ± 0.61	–
	24–48	0.50 ± 0.10	0.65 ± 0.09	–
	48–72	0.17 ± 0.02	0.40 ± 0.18	–
Faeces	0–24	78.34 ± 13.10	68.45 ± 12.20	–
	24–48	21.46 ± 15.00	34.16 ± 10.10	–
	48–72	4.67 ± 2.78	5.73 ± 2.49	–
0.1 mg/kg bw				
Urine	0–24	1.58 ± 0.33	3.79 ± 0.74	–
	24–48	0.31 ± 0.09	0.81 ± 0.55	–
	48–72	0.27 ± 0.10	0.93 ± 0.23	–
Faeces	0–24	85.12 ± 6.53	66.15 ± 12.30	–
	24–48	7.06 ± 5.40	17.91 ± 0.55	–
	48–72	4.12 ± 2.60	6.10 ± 4.13	–
10 mg equivalents/kg bw (acid hydrolysed)				
Urine	0–24	5.75 ± 1.28	4.99 ± 0.94	58.63 ± 10.22
	24–48	0.66 ± 0.42	0.45 ± 0.33	1.77 ± 0.63
	48–72	0.33 ± 0.48	0.23 ± 0.04	0.98 ± 0.36
	Sum (urine)	6.74 ± 1.99	5.67 ± 0.76	61.38 ± 10.12
Faeces	0–24	55.99 ± 8.04	67.74 ± 8.44	27.29 ± 3.79
	24–48	32.25 ± 9.75	10.46 ± 4.92	1.59 ± 1.15
	48–72	1.85 ± 2.40	1.64 ± 1.77	0.91 ± 0.70
	Sum (faeces)	94.09 ± 4.11	79.84 ± 14.67	29.79 ± 4.55

(*Continued on next page*)

Administered dose / Excreta	Time (h)	% of administered dose of [^{14}C]natamycin [a]		
		Oral administration		i.p. administration
		Normal rat	Cholestatic rat	Normal rat
$^{14}CO_2$	0–7	0.87 ± 0.26	0.93 ± 0.12	0.58 ± 0.03
	Total	101.70 ± 2.91	86.44 ± 14.41	91.76 ± 7.53

bw: body weight; i.p.: intraperitoneal; *Source*: Anonymous (1979)

[a] Mean ± standard deviation.

Dog

The excretion of natamycin was studied in dogs (n = 2/group) following administration of gelatine capsules containing [^{14}C]natamycin at 1.00–1.03 mg/kg bw or in a 1% starch suspension at 0.95–1.0 mg/kg bw. In another experiment, [^{14}C]natamycin in 5 mL of propylene glycol at a concentration of 1 mg/mL was administered intravenously to one dog. The study pre-dates GLP or OECD test guideline (TG) requirements. In other experiments, natamycin was administered via natamycin-coated cheese, but the results from these experiments were not assessed in the present evaluation.

A single batch of natamycin was labelled with ^{14}C by incorporating labelled sodium acetate as the substrate in the usual fermentation process. Three female beagle dogs, weighing 10–12.5 kg , were used in these experiments. Two of the three dogs were used in multiple tests, but at least two weeks were allowed to elapse between experiments to ensure complete elimination of radiolabelled material from earlier experiments. Before dosing, the animals were fasted for about 16 hours but given drinking-water. The animals were housed individually in metabolism cages after dosing. Faeces and urine were collected daily for 2–4 days. The samples were processed appropriately, and radioactivity was measured with a liquid scintillation counter.

After oral administration of natamycin, most of the radioactivity was eliminated in faeces within 24 hours, with less than 4% of the total dose in urine (Table 2). Approximately equal amounts of radioactivity were measured in faeces and urine after intravenous administration of natamycin, suggesting that resorption occurred via biliary elimination. The amount of radioactivity recovered was less than 100% after administration in an oral capsule or a suspension. According to the study director, this low recovery was possibly due to the short observation periods.

Based on the results of this study, and taking into account the excretion via bile, the oral bioavailability of natamycin is higher in dogs than indicated by the amounts detected in urine (Hespe & Meier, 1980).

Human

No specific information was submitted on the absorption, distribution, excretion or metabolism of natamycin in humans. No antimycotic activity (equivalent to natamycin at <1 µg/mL) could be detected in blood after ingestion of 500 mg natamycin by human study participants (Anonymous, 1968). Raab (1972) interpreted this lack of antimycotic activity as an indication that natamycin is not absorbed after oral administration.

Table 2. Excretion of radioactivity via urine and faeces after administration of ^{14}C-labelled natamycin to female dogs

Animal ID	Dose level (mg/kg bw)	Method of administration	Experiment duration (days)	Recovery of radioactivity (% of dose)		
				Urine	Faeces	Total recovery
1	1.00	Capsule	2	2.23	85.79	88.02
2	1.03	Capsule	2	3.22	81.51	87.73
2	1.00	Suspension	3	0.74	81.96	82.70
3	0.95	Suspension	3	0.75	75.87	76.62
1	0.47	Intravenous	4	39.20	43.77	82.97

bw: body weight; *Source*: Hespe & Meier, 1980

(b) Dermal route

No reports from absorption, distribution, metabolism and excretion studies with dermal administration were submitted by the sponsor.

1.2 Biotransformation

No reports from studies on biotransformation were submitted by the sponsor. The Meeting noted that natamycin-related radioactivity in rat colon did not have antimycotic activity (Blankwater & Hespe, 1979), which would indicate degradation to antimycotically inactive metabolites. However, chemical structures of metabolites or degradation products were not elucidated.

1.3 Effects on enzymes and other biochemical parameters

No reports from studies on enzymes and other biochemical parameters were submitted by the sponsor. A number of articles investigated pharmacokinetic parameters (1) in tear samples after administration of the natamycin-containing drug Natamet into the conjunctival sac of rabbits (Bhatta et al., 2011, 2012; Chandasana et al., 2014) or (2) in artificial tear fluid (Bhatta et al., 2011). At 1, 6, 12, 24 and 48 hour after administration of an unknown amount of natamycin, concentrations in aqueous humour and cornea were below the limit of detection of 0.5 µg/mL (Ellison & Newmark, 1973).

Uptake of natamycin was investigated in rabbit cornea (O'Day et al., 1986, Zhou et al., 2014) and in rabbit cornea and humour (Tang et al., 2016). Due to lack of relevance for dietary natamycin exposure, the Meeting did not evaluate these studies.

Groups of six male Wistar rats were treated with oral doses of natamycin in glycerol formal at 0.3, 1, 3 or 10 mg/kg bw per day for six days. Another group received the vehicle only. Twenty-four hours after the last dose was administered, animals were killed and livers were isolated. Microsomes were prepared and used to determine the activities of several cytochrome P450 enzymes (CYP).

None of the treated animals showed signs of toxicity. Over the six-day dosing period, treatments with natamycin had no significant effect on body weight gain, liver weight, liver/body weight ratio or microsomal protein content when compared to the control group. The lowest dose of natamycin (0.3 mg/kg bw per day for six days) had no significant effects on the activities of the hepatic CYP enzymes studied. For the 1, 3 and 10 mg/kg bw doses of natamycin, significant dose-dependent decreases in total hepatic CYP content and in the activities of aniline hydroxylase (associated with CYP2E1), aminopyrine *N*-demethylase, 7-methoxyresorufin *O*-demethylase (MROD; associated with CYP1A2), 7-ethoxyresorufin *O*-deethylase (EROD; associated with CYP1A1) and pentoxyresorufin-*O*-dealkylase (PROD; associated with CYP2B1/2) enzymes were observed. Natamycin also produced a significant decrease in the 12- and 11-hydroxylation of lauric acid (associated with CYP4A subfamily) (Martinez et al., 2013).

The sponsor considered the published results of the Martinez et al. (2013) study to be unreliable (Wilms, 2015). However, as specific reasons to support this claim were not given, this could not be followed up.

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with natamycin administered orally, dermally or by inhalation as well as those of the studies investigating skin and eye irritation/corrosion and skin sensitizing potential are summarized in Table 3.

Table 3. Summary of acute toxicity studies with natamycin

Species	Strain	Sex	Route/end-point	Purity (%)	Result	Reference
Rat	Sprague Dawley	F	Oral	~98	LD_{50} > 2000 mg/kg bw	Kuhn (2008a)
Rat	Sprague Dawley	F	Oral	50.49–51.59	1750 < LD_{50} < 5000 mg/kg bw (Natamycin Instant)	Kuhn (2009)
Rat	Carworth Farms Nelson	M + F	Oral	NR	LD_{50} = 2.73/4.67 g/kg bw (male/female) within 7 days	Levinskas (1959); Levinskas, Ribelin & Shaffer (1966)
Rabbit	NR	M	Oral	NR	LD_{50} = 1.42 g/kg bw	Levinskas, Ribelin & Shaffer (1966)
Rat	Sprague Dawley	M + F	Dermal	~98	LD_{50} > 5050 mg/kg bw	Kuhn (2008b)
Rabbit	NR	M	Dermal	NR	LD_{50} > 1.25 g/kg bw	Levinskas, Ribelin & Shaffer (1966)
Rat	Sprague Dawley	M + F	Inhalation (4 hour)	~98	LC_{50} > 2.39 mg/L	Crutchfield (2008)
Rabbit	New Zealand White	M	Dermal irritation	~98	Slightly irritating	Kuhn (2008c)
Rabbit	New Zealand White	M	Eye irritation	~98	Irritating after 1 h, resolving within 24 hours	Kuhn (2008d)
Mouse	CBA/J	F	Skin sensitization	98.17	Non-sensitizing (LLNA)	Kuhn (2008e)

bw: body weight, M: male; F: female; LC_{50}: median lethal concentration, LD_{50}: median lethal dose; NR: not reported

(a) Lethal doses

Natamycin (purity: ~98%) was administered by gavage (40% weight per volume in deionized water) to five female Sprague Dawley rats at a dosage of 2000 mg/kg bw each (according to USEPA OPPTS TG 870-1100, 2002). It is unclear why the top dose of 2000 mg/kg bw was selected instead of the recommended top dose of 5000 mg/kg bw. The test animals were fasted overnight prior to dosing. The test animals were observed for signs of toxicity on the day of dosing and at least once daily thereafter for the remainder of the 14-day observation period. Body weights were recorded on the day of dosing (day 0), day 7 and at study termination (day 14). Gross necropsies were performed on all the test animals. Besides one dead animal (day 7), which was observed with slight polyuria, no overt signs of toxicity were observed in any of the other test animals during the 14-day observation period. The animal that died prematurely had following gross necropsy findings: yellow spots on anogenital area, pale lungs and intestines full of light red fluid. In the surviving animals, no significant gross pathological findings could be attributed to ingestion of natamycin.

The acute oral median lethal dose (LD_{50}) for natamycin in female rats was greater than 2000 mg/kg bw under the conditions of the study (Kuhn, 2008a).

Natamycin instant (purity: ~50.49–51.59%) was administered by gavage (40% weight per volume in deionized water) to eight female Sprague Dawley rats at dosages of 175, 550, 1750 or 5000 mg/kg bw each (according to USEPA OPPTS TG 870-1100, 2002) using a total of eight animals. The test animals were fasted overnight prior to dosing. The test animals were observed for signs of toxicity on the day of dosing and at least once daily thereafter for the remainder of the 14-day observation period. Body weights were recorded on the day of dosing (day 0), day 7 and at study termination (day 14). Gross necropsies were performed on all the test animals. Initially five animals received consecutively 5000 mg/kg bw, of which three animals died between days 4 to 6. Subsequently doses of 175, 550 or 1750 mg/kg bw were administered to one animal/dose, and all animals survived. In the animals treated at 5000 mg/kg bw, the following clinical signs were observed: polyuria, piloerection, red stain on muzzle or tail and decreased activity. No overt signs of toxicity were observed in any of the test animals treated with lower doses during the 14-day observation period. The animals that died prematurely had the following gross necropsy findings: stained/wet/crusted facial/leg/abdominal areas; discoloured liver and contents in the gastrointestinal tract; and empty stomach/intestines. In the surviving animals, there were no significant gross pathological findings that could be attributed to ingestion of natamycin.

The acute oral LD_{50} for natamycin instant in female rats was between 1750 and 5000 mg/kg bw under the conditions of the study (Kuhn, 2009).

The acute dermal toxicity of natamycin (purity: approx. 98%) was investigated in five male and five female Sprague Dawley rats by topical application of a dose level of 5050 mg/kg bw (moistened with deionized water) to the clipped skin of the dorsal trunk. The test material was covered for 24 hours, after which test material and the wrap were removed. The test animals were observed for signs of toxicity at least once daily for the 14-day observation period. Observations for evidence of dermal irritation were made at approximately 60 minutes after removal of wrappings and on days 4, 7, 11 and 14. Body weights were recorded on the day of dosing (day 0), day 7 and at study termination (day 14). Gross necropsies were performed on all the test animals at the end of the 14-day observation period.

No mortality or signs of toxicity were observed in any of the test animals during the 14-day observation period. No visible gross lesions were observed in any of the test animals. The acute dermal LD_{50} for natamycin in male and female rats was greater than 5050 mg/kg bw under the conditions of the study (Kuhn, 2008b).

Groups of five male and five female Sprague Dawley rats underwent a 4-hour nose-only exposure to dust of natamycin (purity: ~98%) at an analytically determined concentration of 2.39 mg/L. It is unclear whether the maximum attainable concentration was reached. The mass median aerodynamic diameter (MMAD) of the dust was 3.3 µm. Mortality and clinical signs were recorded on the day of exposure and at least daily for 14 days after dosing. Individual body weights were measured before dosing on day 0 and on days 7 and 14.

No mortalities occurred at the only dose level tested. A decrease in activity and piloerection was observed until day 3 in males and females. Normal body weight development was observed. There were no treatment-attributable gross necropsy findings.

The acute inhalation median lethal concentration (LC_{50}) of natamycin in male and female rats was greater than 2.39 mg/L air under the conditions of the study (Crutchfield, 2008).

Groups of 10 male and five female Carworth Farms Nelson rats and groups of three male albino rabbits received natamycin (dispersed in 0.2% aqueous agar solution plus 0.1% Tween 80) to determine the acute oral toxicity. Similarly, acute dermal toxicity was determined in groups of five or 10 rabbits. The rats were observed for one week and the rabbits for two weeks. Reported oral and dermal LD_{50} values are listed in Table 3 (Levinskas, 1959; Levinskas, Ribelin & Shaffer, 1966). However, insufficient details were reported to allow for an independent evaluation of the effects.

An article summarizing the results of acute toxicity studies reported that the oral LD_{50} values in rats, mice and guinea pigs were 1500, 1500 and 450 mg/kg bw, respectively. The LD_{50} values after intraperitoneal, intramuscular and subcutaneous administration of natamycin to rats were 250, 2000 or 5000 mg/kg bw, respectively. However, insufficient details were reported to allow for an independent evaluation of the effects (Struyk et al., 1958).

(b) Dermal irritation

Natamycin (purity: ~98%) was applied at a dose of 0.5 g to the clipped dorsal regions of three male New Zealand White rabbits under a gauze patch moistened with water and kept in contact with the skin for 4 hours under an occlusive dressing. Cutaneous observations for erythema and oedema were performed, using the Draize scheme, at 1, 24, 48 and 72 hours after application. Very slight erythema was observed at one hour. No further cutaneous reactions were observed at any of the test sites during the study (Kuhn, 2008c).

(c) Ocular irritation

Natamycin (purity: ~98%) was instilled at a dose of 0.1 mL (43.7 mg) into the conjunctival sac of one eye of each of three male New Zealand White rabbits. Observations for ocular lesions were conducted at 1, 24, 48 and 72 hours after instillation with the aid of fluorescein/ultraviolet light at the 24-hour time-point and later if lesions were positively identified. Moderate effects were seen in cornea, iris and conjunctivae in all animals one hour after instillation of the test substance. Twenty-four hours after dosing, all three treated eyes exhibited minimal conjunctival redness. All animals were free of ocular irritation by 48 hours.

The test material caused transient slight irritation to the eyes of rabbits (Kuhn, 2008d).

The results of an eye irritation study in rabbits as conducted by American Cyanamid Company are described in an article by Levinskas, Ribelin & Shaffer (1966). However, insufficient details are reported to allow for an independent evaluation of the effects.

Rabbits received natamycin by intravitreal (Ellison, 1976) or intraocular (Ellison & Newmark, 1976) injection. Effects on eyes and antimycotic efficacy were investigated; however, due to lack of relevance for dietary natamycin exposure, these articles were not further evaluated.

(d) Dermal sensitization

In a dermal local lymph node sensitization study, natamycin (purity ~98%) was applied dermally to the dorsum of each ear of groups of five female mice on three consecutive days. The test material was applied undiluted or as 25% or 50% dilution in propylene glycol. Concurrent control groups consisting of five females each received the vehicle or 90% volume per volume (v/v) alpha-hexylcinnamaldehyde (in acetone:olive oil; 80:20). After two days, tritiated methylthymidine was injected and five hours later, the mice were killed, the draining auricular lymph nodes were excised and further processed, and incorporated radioactivity was measured. No adverse clinical signs were reported in test and control groups during the treatment and observation periods. The positive control produced satisfactory results (stimulation index of 8.8).

Under the conditions of the study, natamycin did not lead to meaningful increases in stimulation index (Kuhn, 2008e).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

No reports from oral short-term studies in mice were submitted by the sponsor.

Rat

In a 13-week dietary toxicity study, groups of 10 male and 10 female Wistar rats received diets containing natamycin (purity: 88.5%) at a concentration of 0, 125, 500 or 2000 ppm (equal to 0, 11, 42 and 204 mg/kg bw per day for males and 0, 12, 48 and 238 mg/kg bw per day for females, respectively; when considering feed scatter, the substance intakes were 10–30% lower). Rats were observed daily for signs of overt toxicity, morbidity and mortality. Ophthalmological examinations were conducted

pretest and in week 13. Detailed clinical observations, individual body weights and individual feed consumptions were recorded at least weekly. A set of neurobehavioural observations were recorded in weeks 12 or 13. Haematological and clinical chemistry parameters were determined at study termination. At scheduled kill, all surviving animals underwent a gross necropsy and a range of organs were weighed. Samples of a range of tissues from all test rats underwent histopathological evaluation. Urine analysis was not conducted.

No mortalities were observed during the 13-week study period. No treatment-attributable clinical signs of toxicity were reported. Body weights of high-dose but not mid- or low-dose animals were reduced. Body weight gains were reduced from treatment week 3/4 onwards, amounting to an approximate 20% weight gain deficit relative to controls at study termination. Feed consumption in both sexes was comparable to that of control animals throughout the study. No biologically relevant statistically significant changes in haematological parameters were reported for either sex at any treatment level. In high-dose groups, alanine aminotransferase activity increased in individual cases in male rats; urea and inorganic phosphate levels increased in male and female rats; potassium levels increased in male rats; and cholesterol and total protein levels were reduced in female rats (Table 4). No biologically relevant statistically significant changes in clinical chemistry parameters were reported for either sex at low and mid dose. No neurobehavioural changes were reported. Apparent changes in some absolute or relative organ weights in individual dose groups were not considered to be substance related or adverse due to the lack of a dose–response relationship and corroborating findings. No gross pathological findings were attributed to treatment with natamycin. Higher incidences of discolouration of mandibular lymph nodes in high-dose males were not corroborated by microscopic findings. No toxicologically relevant histopathological changes were reported in this study.

Table 4. Summary of clinical biochemistry and body weights in a 13-week dietary study with natamycin in rats

	Measures per dose level of natamycin							
	0 ppm		125 ppm		500 ppm		2000 ppm	
	M	F	M	F	M	F	M	F
Clinical biochemistry								
Alanine aminotransferase (U/L)	35.2	37.4	36.6	35.7	35.8	37.6	55.1**	39.1
Urea (mmol/L)	5.3	6.0	5.3	5.8	5.7	6.5	7.3**	8.3**
Inorganic phosphorus	1.87	1.75	1.87	1.52**	1.88	1.53**	2.49**	1.98**
Potassium (mmol/L)	3.80	3.36	3.86	3.20	3.67	3.29	4.13**	3.52
Total cholesterol (mmol/L)	1.96	1.97	1.91	2.02	1.81	1.74	2.03	1.58**
Total protein (g/L)	62.0	68.0	61.7	65.7	62.3	65.8	60.3	64.9**
Body weights (g)								
Week 1	192	162	192	158	189	157	192	157
Week 13	539	308	517	305	523	296	463**	278*

F; females; M: males; ppm: parts per million; U: Units; *Source*: Otterdijk (2003)
*: $P < 0.05$, **: $P < 0.01$ (Dunnett test based on pooled variance significant at 5% or 1% level)

The no-observed-adverse-effect level (NOAEL) was 500 ppm (equal to 42 and 48 mg/kg bw per day for males and females, respectively) based on reduced body weights and clinical chemistry findings such as changes in alanine aminotransferase, urea, inorganic phosphorous, cholesterol and total protein at 2000 ppm (equal to 204 and 238 mg/kg bw per day) (Otterdijk, 2003).

In another 13-week dietary toxicity study, groups of 20 male and 20 female Carworth Farms Nelson rats received diets containing natamycin (purity: 95%) at a concentration of 0, 125, 500, 2000 or 8000 ppm (equal to 0, 9.6, 39, 170 and 710 mg/kg bw per day for males and 0, 13, 48, 210 and 780 mg/kg bw per day for females). The study pre-dates GLP or OECD TG requirements. Rats were observed daily for signs of overt toxicity, morbidity and mortality. No ophthalmological examinations were

conducted. Individual body weights and individual feed consumptions were recorded weekly. No neurobehavioural observations were recorded. Haematological and clinical chemistry parameters were determined in 5 animals/sex per group after 35 days and at study termination. At scheduled kill, all surviving animals underwent a gross necropsy; selected a range of organs from 5 rats/sex per group were weighed. Samples of kidneys and livers from five rats/sex per group underwent histopathological evaluation. Urine analysis was not conducted.

No treatment-related mortalities were observed during the 13-week study period. No treatment-attributable clinical signs of toxicity were reported. Body weights and feed intake were reduced at 2000 and 8000 ppm but not at 125 and 500 ppm. At scheduled kill, body weights of males and females at 2000 ppm amounted to approximately 86% and 88% of weights of control males and females; at 8000 ppm, body weights were 63% and 78% of the weights of control males and females. No biologically relevant statistically significant changes in haematological parameters were reported for either sex at any treatment level. No changes in absolute or relative organ weights were reported. No gross pathological or histopathological findings were attributed to treatment with natamycin due to the lack of dose response.

The tentative NOAEL was 500 ppm (equal to 39 and 48 mg/kg bw per day for males and females, respectively) based on reduced body weights and reduced feed intake at 2000 ppm (equal to 170 and 210 mg/kg bw per day for males and females, respectively) (Levinskas, 1959; Levinskas, Ribelin & Shaffer, 1966).

Dog

In a seven-week dietary toxicity study, beagle dogs received diets containing natamycin (purity: 95%) at a concentration of 312, 625, 1250 or 5000 ppm (equivalent to 7.8, 15.6, 31 and 125 mg/kg bw per day). No control group was employed. The study pre-dates GLP or OECD TG requirements. Group size was small (n = 2 vs 4 according to OECD TG requirements). The age of the animals was not indicated in the report. The animals had previously been used in other studies with diets containing organic phosphates. Findings were described only as narrative and not in tabulated form.

Clinical changes, body weight and feed consumption were monitored. Haematological, clinical chemical, urinary, electrocardiography and ophthalmological examinations were not conducted. Animals were not killed after the treatment period and therefore necropsy with gross examination, determination of organ weights and histopathological evaluations could not be conducted.

Treatment at 5000 ppm was discontinued after four days because of severe vomiting, diarrhoea, which started a few hours after the first meal, and the animals' refusal to eat the treated feed. No dose- or treatment-related effects were seen in males or females with respect to mortality rate. Mean body weight gain was reduced at 312 and 1250 ppm. Feed intakes were reduced at 312 and 1250 ppm. Diarrhoea was observed at 625 and 1250 ppm.

No firm NOAEL/LOAEL could be determined due to the low number of investigated parameters and the lack of necropsy. Body weights gains and feed intakes were already low at the lowest dose of 312 ppm (equivalent to 7.8 mg/kg bw per day for males and females) (Levinskas, 1959; Levinskas, Ribelin & Shaffer, 1966).

In a 90-day dietary toxicity study, beagle dogs received diets containing natamycin (purity: 90.5%) at a concentration of 0, 375 or 750 ppm (equivalent to 0, 12 and 25 mg/kg bw per day). According to the sponsor this study was not conducted according to GLP principles. Group size was small (n = 2 vs 4 according to OECD TG 409 requirements). The age of the animals was not indicated in the report.

Clinical changes, body weight and feed consumption were monitored. Haematological, clinical chemical, urine analysis, electrocardiography (wave intervals and heart rate at weeks 0, 4, 8, and 12) and ophthalmology assessments were made and pupillary reactions observed. After scheduled kill by intravenous overdose of pentobarbital, all animals were necropsied, and thymus, heart, liver, kidneys, adrenals, spleen and testes were weighed and gross lesions noted. The following tissues were preserved in buffered formaldehyde saline and examined microscopically: brain, thyroid, thymus, lung, heart, liver, kidneys, adrenals, spleen, pancreas, lymph nodes, urinary bladder, ovaries, testes, stomach, ileum, colon, jejunum, caecum and oesophagus. The statistical evaluations included analysis of variance (ANOVA) and the Student t-test.

No dose- or treatment-related effects were seen in males or females with respect to mortality rate, haematological, clinical chemical or urinary end-points; electrocardiography; ophthalmology; absolute and relative organ weights; gross pathology; and histopathology. Mean body weight gain was lower in high-dose groups than in control and low-dose groups. Feed intake tended to be lower in high-dose groups. The number of days animals had diarrhoea was highest in males and females at the high dose; diarrhoea was also observed on a few days in controls and animals at the low dose. Heart frequency was lower in high-dose females than in controls and low-dose animals throughout the study period; this finding may be related to uneven distribution of the animals.

The NOAEL was 375 ppm (equivalent to 12 mg/kg bw per day) based on lower body weight gains and increased number of days with diarrhoea at 750 ppm (equivalent to 25 mg/kg bw per day) (Van Eeken et al., 1984).

In a two-year dietary toxicity study, groups of three male and three female beagle dogs received diets containing natamycin (purity: 97.5%) at a concentration of 0, 125, 250 or 500 ppm (equivalent to 0, 3.1, 6.3 and 12.5 mg/kg bw per day). The study pre-dates GLP or OECD TG requirements. Group size was small (n = 3 vs 4 according to OECD TG requirements). Ophthalmological and urinary parameters were not determined. Haematology and clinical chemistry results, organ and body weights, and gross and histopathological findings were presented individually and not as summary tables.

The animals were monitored for changes in clinical signs, body weight, feed consumption and haematological and clinical chemistry parameters. After scheduled kill by intravenous injection with magnesium sulfate solution, all the animals were necropsied, organs weighed and gross lesions noted. The tissues preserved in formaldehyde for subsequent microscopic examination were bone and marrow, lymph nodes, gall bladder, stomach, small and large intestine, caecum, urinary bladder, gonads, prostate, uterus and aorta. No statistical evaluations were presented, although some statistical results of organ weight changes were discussed in the report.

No dose- or treatment-related effects were seen in males or females with respect to mortality rate, clinical signs, feed intake, haematological and clinical chemistry end-points, gross pathology or histopathology. Mean body weight gain was lower in high-dose males than in control and low-dose groups. Relative liver weights in low- and mid-dose males were significantly lower than in control animals. However, due to lack of a dose response in the high-dose groups, these findings were considered less relevant. Other organ weights were comparable to the range seen in control animals.

The NOAEL was 250 ppm (equivalent to 6.3 mg/kg bw per day) based on lower body weight gains in males at 500 ppm (equivalent to 12.5 mg/kg bw per day) (Fogleman, 1963). (It seems that the results of this study were also published in the open literature by Levinskas, Ribelin & Shaffer, 1966).

(b) Dermal application

No reports from short-term studies with dermal exposure were submitted by the sponsor.

(c) Exposure by inhalation

No reports from short-term studies with exposure by inhalation were submitted by the sponsor. An evaluation by USEPA (2012) mentioned a 90-day inhalation study in rats; this was not submitted to JMPR. According to the sponsor, this reference in the USEPA report is a typographical error.

(d) Exposure by other routes

No reports from short-term studies with exposure by other routes were submitted by the sponsor.

Rabbits were induced with uveitis by intravitreous injection with solutions of human albumin. Based on the severity of the effects, rabbits were assigned to one of two groups, each aiming for comparable degrees of severity and each consisting out of 21 animals. Three animals/group received either 5 mg/kg bw per day of natamycin (suspended in 5% glucose) by intravenous injection or 10 mg/kg bw per day for one week, and were killed 24 hours after the last dose. Blood samples were used to determine clinical chemistry parameters. The remaining animals were used for toxicokinetic

investigations (see above). In other rabbits, right eyes were infected by intraocular injection with viable yeast cells of *Candida albicans*; the left eyes served as uninfected controls. Based on the severity of the resulting effects after 36 hours, rabbits were assigned to one of three groups, each aiming for comparable degrees of severity, each consisting of five animals. Treatment groups received 5 or 10 mg/kg bw per day of natamycin (suspended in 5% glucose) by intravenous injection for three weeks; the control group received glucose solution only. Blood samples were taken from each animal one day before infection; 3 days, 10 days and 3 weeks after start of natamycin administration; and 1 and 3 weeks after cessation of natamycin administration. Blood samples were used to determine clinical chemistry parameters. Apparently, these animals were used for histopathological assessment.

Effects on eyes due to the infection were not considered in this assessment.

Reported findings are difficult to relate to individual treatment groups because they were presented in the study report mainly as a narrative.

Ninety-six hours after the administration of natamycin at 5 mg/kg bw per day, a decrease in haematocrit was noted; this subsequently further decreased over the rest of the treatment period. All rabbits at 10 mg/kg bw died within five days after start of treatment, usually following tonic and clonic convulsions and respiratory collapse. Following the single dose of 5 or 10 mg/kw bw, a transitory increase in blood urea nitrogen, uric acid and serum bilirubin was noted. Serum glutamic oxaloacetic transaminase (SGOT; aspartate transaminase) and lactate dehydrogenase (LDH) were elevated. These elevated levels lasted for approximately 12 hours and disappeared upon continuance of the drug. However, following continued administration of 10 mg/kg bw, irreversible renal, liver and myocardial damage was noted. Electrolyte changes over the 24-hour period following a single dose of natamycin included lower calcium levels after 24 hours and slightly elevated phosphate levels. Following continuous intravenous treatment at 5 mg/kg bw for three weeks, serum calcium levels were significantly depressed. Serum phosphate levels were decreased after one week. Calcium and phosphate levels normalized during a two-week recovery period after the three-week treatment period. Serum sodium, potassium and chloride remained basically unaltered throughout the study. SGOT and total bilirubin were increased throughout the three-week treatment period and the three-week recovery phase. Creatinine and uric acid remained basically unaltered throughout the study. Histopathological evaluation found no ocular or retinotoxicity in the normal eyes following intravenous administration at 5 mg/kg bw. However, toxicity was noted in the lymph nodes, spleen, kidney, liver, thyroid and adrenal glands. Endocrine glands were severely affected, with the thyroid gland showing marked hyperplasia and loss of colloid and the adrenals showing extensive lipidosis of the cytoplasm in the area of the stratum fasciculate. The pancreas was normal. The spleen and lymph nodes showed reticular hyperplasia of the germinal centres; the liver showed extensive cellular lipidosis and constriction of the hepatic sinuses. The kidneys showed mild swelling of the renal capillaries and nephrons with necrosis in 10% of the glomeruli (Ellison, 1979).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

No reports from long-term toxicity or carcinogenicity studies in mice were submitted by the sponsor.

Rat

In a long-term toxicity and carcinogenicity study, groups of 35 male and 35 female Carworth Farms Nelson rats received diets containing natamycin (purity: 97.5%) at a concentration of 0, 125, 250, 500 or 1000 ppm (equivalent to 0, 6, 12.5, 25 and 50 mg/kg bw per day) for up to two years. Additional groups of five male and five female rats were treated similarly with control diet or high-dose diet for one year. The study pre-dates GLP or OECD TG requirements. Five males and 10 females from the control and high-dose groups were temporally used for a multigeneration study (Levinskas et al., 1963b; section 2.5(b)). The rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed clinical observations were recorded weekly. No ophthalmological examinations were performed. Individual body weights and feed consumption data were recorded weekly during the first three months of the study, biweekly until the end of the first year and monthly for the remainder of the 24-month study period. Samples

for haematological assessments were collected from five rats per sex per dose after 3, 6, 12, 21 and 24 months. No clinical chemistry assessments or urine analysis were performed. At interim and final kill, all surviving animals underwent a gross necropsy, and a range of organs were weighed. Gross necropsies were also performed on animals found dead, euthanized moribund or that died accidentally during the study. Samples of selected tissues from five high-dose males and females at interim kill and 10 surviving test animals per sex at study termination underwent histopathological examination. At interim kill, no tissues from control animals underwent histopathology.

No treatment-related clinical signs or mortalities were reported. Of the 35 animals per sex in each group, 19, 22, 21, 24 and 25 males and 25, 20, 17, 18 and 24 females at, respectively, 0, 125, 250, 500 or 1000 ppm, survived until study termination. Body weights were significantly lower in high-dose males and females than in control animals; no significant effects on body weights were reported for other treatment groups. Feed intakes were 5–10% lower in high-dose males and females than in control animals (no statistical analysis conducted), but feed intakes in the other treatment groups were comparable to the respective controls. Haematological parameters and organ weights showed no dose-related alterations.

Animals (17/sex per group), particularly those in a debilitated or abnormal condition rather than randomly selected animals, were subjected to necropsy. Microscopic evaluation was conducted in 10 animals per sex and per treatment group only. No gross or microscopic findings were considered treatment related. No treatment-related increase in neoplasm was described.

Due to the low number of animals per dose group and sex when compared with OECD TG 453 or 451, and the gross and microscopic examination of some not randomly selected animals, the results of this study do not contribute this assessment (Levinskas et al., 1963a). (It seems that the results of this study were also published in the open literature by Levinskas, Ribelin & Shaffer, 1966).

An evaluation by USEPA (2012) mentioned a study by Shirk & Lovesky (1963)[3] on faecal flora of rats maintained on a diet containing natamycin diet for up to two years. This study was not submitted to JMPR, despite a request from the sponsor.

2.4 Genotoxicity

Natamycin was tested in selected in vitro and in vivo genotoxicity assays. Results are summarized in Table 5 (see next page). The Meeting noted that no report on in vitro mammalian cell gene mutation assay was submitted. The summary report on the assessment of the veterinary drug natamycin (EMEA, 1998) listed GLP-compliant studies on mutagenicity that included Ames test, mouse lymphoma assay and chromosomal aberration in Chinese hamster ovary cells, which gave negative results. When requested to submit these studies, the sponsor indicated an inability to access these studies.

The sponsor drew attention to the fact that the presence of an epoxide ring on the natamycin molecule is a structural alert for genotoxicity.

[3] Shirk RJ, Lovesky RL (1963). A gross examination of the fecal flora of rats sustained for two years on diets containing from 0 to 1000 ppm. Pimaricin: Report FS 3. Progress rept. 21 June 1962 to 1 August 1962.

Table 5. Results of genotoxicity studies performed with natamycin

End-point	Organism/cells	Dose range tested	Batch purity (%)	Result	Reference
In vitro					
Gene mutation	Ames: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA	± S9: 0, 33, 10 330 µg/plate	88.5 [a]	± S9: Negative	Verspeek (2002)
Chromosomal aberration	Human lymphocytes	3 h exposure, 24 h fixation: ± S9: 1, 3, 10 µg/mL 24/48 h exposure, 24/48 h fixation time: −S9: 1, 3, 10 µg/mL 3 h exposure, 48h fixation time: +S9: 1, 3, 10 µg/mL	88.5 [a]	± S9: Negative [b]	Meerts (2002)
Chromosomal aberration	Human lymphocytes	24/48 h exposure, −S9: 13, 18, 23, 28 µg/mL	Not reported	Positive	Rencuzogullari et al. (2009)
Micronucleus	Human lymphocytes	24/48 h exposure, −S9: 13, 18, 23,28 µg/mL	Not reported	Positive	Rencuzogullari et al. (2009)
In vivo					
Chromosomal aberration	Mouse bone marrow (i.p. administration)	0, 200, 400 and 800 mg/kg bw	Not reported	Negative	Rasgele & Kaymak (2013a,b)
Chromosomal aberration	Rat bone marrow (oral administration; animals derived from multigeneration study)	0, 5, 15, 50 and 100 mg/kg bw per day	Not reported	Negative [b]	Cox, Bailey & Morgareidge (1973)
Micronucleus	Mouse bone marrow (i.p. administration)	0, 200, 400 and 800 mg/kg bw	Not reported	Positive	Rasgele & Kaymak (2013a,b)
Dominant lethal	Rat male germ cells / implants	0, 5, 15, 50 and 100 mg/kg bw per day (7 days)	Not reported	Negative [b]	Cox, Bailey & Morgareidge (1973)

bw: body weight; i.p.: intraperitoneal; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] No correction for low purity.

[b] Study design was less powerful than required according to current test guidelines.

(a) In vitro studies

Natamycin (purity 88.5%) suspended in ethanol was tested for its mutagenic potential on *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA in a plate incorporation test conducted according to the Ames test procedure at concentrations up to 5000 µg/plate in range-finding experiments in the absence or presence of S9 mix. The experiments were independently replicated; concentrations were plated in triplicate in each experiment. Natamycin was not cytotoxic up to 5000 µg/plate, but precipitation was observed at 3330 and 5000 µg/plate. No consistent or dose-related increase of revertants was observed when compared with concurrent controls.

Natamycin did not show mutagenic potential under the conditions of this test. Satisfactory positive control results were obtained (Verspeek, 2002).

Abstracts summarized the results of a growth inhibition mutation assay in bacteria and an Ames test conducted with natamycin (possibly formulated as Delvocid-50%). However, insufficient details were reported to allow for an independent evaluation of the methods and the effects (Khoudokormoff, 1977, 1978). The authors concluded from the Ames test that natamycin did not induce reverse mutation when tested alone or with nitrite in any of the strains of *Salmonella typhimurium* or *Escherichia coli* tested. The study author reported that natamycin at a concentration of 1% and its degradation products aponatamycin, dinatamycinolidediol and mycosamine at 0.5% and at different pH (3.0–6.5) and nitrite (100–400 ppm) levels had no mutagenic activity in *Bacillus subtilis* under the conditions tested. The author commented on the slight positive response with nitrite at about 0.2 mol/L and concluded that natamycin did not enhance the mutagenic effect.

Natamycin (purity: 88.5%) suspended in ethanol was tested for its clastogenic potential in cultured human lymphocytes at concentrations up to 10 µg/mL in the presence and absence of S9 mix. The study was conducted according to OECD TG 473. Duplicate cultures were used for each treatment. Cultures were initiated with phytohaemagglutinin two days before treatment. In the absence of S9, cells were exposed to natamycin for 3 (plus 17–19 hours recovery), 24 or 48 hours; for the last 2.5 hours, cells were incubated with colchicine. The exposure time in the presence of S9 was three hours, after which the cells were washed and cultured in fresh media until 20–22 or 48 hours had been reached, the last 2.5 hours of which was in the presence of colchicine. Appropriate positive controls showed positive results. Usually, 100 metaphases from each culture were examined. The study design was less powerful than required according to the current test guideline (200 metaphases were scored instead of 300). While the study report mentions historical negative control ranges, it is unclear how they were generated. No cytotoxicity was observed up to the highest dose tested, which was based on the observation of precipitates; for non-precipitating, non-cytotoxic compounds, the TG-recommended top dose is 10 mol/L, 2 mg/mL or 2 µg/mL, whichever is the lowest. No evidence of clastogenicity was observed up to a maximum concentration of 10 µg/mL (Meerts, 2002).

Natamycin dissolved in methanol was tested for its chromosomal aberration and micronucleus-inducing potential in cultured human lymphocytes from four healthy volunteers. Natamycin was tested at concentrations of up to 28 µg/mL, in the absence of S9 mix. The study was conducted similarly to OECD TGs 473 and 487, but not according to GLP principles. The report also included results of an sister chromatid exchange study as an indicator end-point only; these results were not assessed. For the chromosomal aberration assay, cells were exposed to natamycin for 24 or 48 hours; for the final two hours of exposure, cells were incubated with colchicine. Appropriate positive controls were used and showed appropriate results. Usually 100 metaphases from each culture were examined, or a total of 400 metaphases per concentration. Mitotic index was determined by scoring 3000 cells per donor and concentration. For the micronucleus assay, cells were exposed to natamycin for 24 or 48 hours; cells were incubated with cytochalasin B to inhibit cell division. Appropriate positive controls were used and showed appropriate results. From each culture, 3000 binucleated cells were examined for micronuclei, or a total of 12 000 binucleated cells per concentration.

Results are summarized in Table 6. Natamycin induced chromosomal aberrations and micronuclei in cultured human lymphocytes (Rencuzogullari et al., 2009).

Table 6. Results of chromosomal aberration and micronucleus studies performed with natamycin

Test substance	Treatment period (h)	Concentration (µg/mL)	Structural CA ± SE (%) [a]	MI ± SE (%) [a]	MNBNC ± SE (%) [b]	NDI ± SE [b]
Control	–	–	2.00 ± 1.52	4.10 ± 0.41	0.86 ± 0.13	1.37 ± 0.07
Methanol	24	6	3.00 ± 0.40	4.19 ± 1.08	0.92 ± 0.10	1.29 ± 0.02
MMC	24	0.25	13.00 ± 2.00	3.35 ± 0.31	2.00 ± 0.25	1.21 ± 0.07
Natamycin	24	13	5.25 ± 1.31	4.76 ± 0.82	0.89 ± 0.09	1.25 ± 0.04
Natamycin	24	18	5.75* ± 0.75	4.39 ± 1.11	1.12 ± 0.08	1.19 ± 0.06
Natamycin	24	23	4.75* ± 0.47	3.94 ± 0.72	1.17 ± 0.12	1.17* ± 0.04
Natamycin	24	28	6.25** ± 0.50	2.94* ± 0.84	1.19* ± 0.08	1.11** ± 0.02
Methanol	48	6	4.25 ± 0.41	3.17 ± 0.98	0.66 ± 0.19	1.33 ± 0.09
MMC	48	0.25	12.25 ± 1.60	2.61 ± 0.82	7.41 ± 1.33	1.28 ± 0.03
Natamycin	48	13	4.25 ± 0.47	2.54 ± 0.37	1.01 ± 0.15	1.33 ± 0.05
Natamycin	48	18	6.00* ± 0.91	2.46 ± 0.46	1.12** ± 0.07	1.35 ± 0.07
Natamycin	48	23	7.25* ± 0.44	2.50 ± 0.63	1.19** ± 0.10	1.27 ± 0.06
Natamycin	48	28	7.75* ± 0.75	2.71 ± 0.12	1.24** ± 0.06	1.21 ± 0.05

CA: chromosomal aberration; MI: mitotic index; MNBNC: micronucleus binucleated cells; MMC: mitomycin C; NDI: nuclear division index; SE: standard error;
*: $P < 0.05$; **: $P < 0.01$, significant from solvent control (methanol, t-test)

[a] A total of 400 cells were scored for CAs and 12 000 cells for MI.

[b] A total 12 000 binucleated cells were scored for MNBNC, and 12 000 cells were scored to calculate the NDI.

Source: Rencuzogullari et al. (2009)

(b) In vivo studies

In in vivo assays in bone marrow, groups of five male and five female mice were treated with natamycin (Delvocid by DSM, dissolved in distilled water) by intraperitoneal injection at 0, 200, 400 and 800 mg/kg bw for 6, 12 and 24 hours in chromosomal aberration assays and for 24, 48 and 72 hours in micronucleus assays. The studies were conducted similar to OECD TGs 475 and 474 but not according to GLP principles. Mitomycin C served as positive control substance and gave appropriate results. For the chromosomal aberration assay, mice received an intraperitoneal dose of colchicine three hours prior to scheduled kill. After the scheduled kill, bone marrow cells were collected from the femur and slides were prepared. Coding of slides in either the chromosomal aberration or micronucleus assays was not mentioned. At least 3000 cells per animal were analysed to determine the mitotic index. One hundred metaphases per group were analysed from five animals per sex. The study design was less powerful than required according to the current test guideline (100 metaphases were scored instead of 200).

For the micronucleus assay, bone marrow cells were collected from the femur and slides were prepared. At least 1000 cells/animal were analysed to determine the number of micronucleated polychromatic erythrocytes (MNPCE) and the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE). For either assay, the individual counts for each of the animals were not reported.

Results are summarized in Table 7. Natamycin induced no chromosomal aberrations but micronuclei in mouse bone marrow. Cytotoxicity as measured by mitotic index or PCE/NCE ratio was not excessive (Rasgele & Kaymak, 2013a,b).

The Meeting noted the high frequency of micronuclei in bone marrow in control animals (up to 20%) and in treated animals (up to 56%). Such high frequencies were considered unusual when compared to other in vivo micronucleus assays in mice. It was speculated whether the unit "%" was reported wrongly in the report, but this issue could not be resolved.

Table 7. Results of chromosomal aberration (excluding gaps) and micronucleus studies with natamycin in mice

Test substance	Period (h)	Dose level (mg/kg bw)	Chromosomal aberration study				Micronucleus study			
			Females		Males		Females		Males	
			CA (%)	MI (%)	CA (%)	MI (%)	MNPCE% ± SE	PCE/NCE ± SE	MNPCE% ± SE	PCE/NCE ± SE
Distilled water	6	–	0	3.17	0	3.97				
MMC	6	2	12***	2.0**	13***	2.40***				
Natamycin	6	200	2	2.26*	1	2.73**				
Natamycin	6	400	2	2.07**	1	2.50**				
Natamycin	6	800	4	1.96**	2	2.26***				
Distilled water	12	–	1	3.63	0	4.53				
MMC	12	2	12*	2.30**	19***	2.73***				
Natamycin	12	200	1	2.66*	2	3.20**				
Natamycin	12	400	2	2.37**	3	2.53***				
Natamycin	12	800	3	2.17***	5	2.13***				
Distilled water	24	–	1	3.93	2	4.37	6.20 ± 2.20	1.67 ± 0.20	19.60 ± 0.75	1.48 ± 0.03
MMC	24	2	9*	2.67**	21***	2.53***	56.40 ± 6.65***	0.98 ± 0.12	37.20 ± 3.83***	0.91 ± 0.02***
Natamycin	24	200	1	3.00*	0	3.03**	21.20 ± 3.72*	1.10 ± 0.14	23.60 ± 3.54	1.26 ± 0.09*
Natamycin	24	400	1	2.37***	3	2.96**	28.80 ± 2.06***	1.05 ± 0.28	34.00 ± 2.28*	0.96 ± 0.05***
Natamycin	24	800	2	1.93***	7	1.87***	30.40 ± 0.98***	0.88 ± 0.14*	30.00 ± 1.79*	0.86 ± 0.05***
Distilled water	48	–					5.20 ± 1.02	1.90 ± 0.19	16.00 ± 2.28	1.70 ± 0.11
MMC	48	2					51.20 ± 2.58***	0.90 ± 0.08**	37.60 ± 2.71***	0.81 ± 0.03***
Natamycin	48	200					12.40 ± 1.72*	1.01 ± 0.29**	19.20 ± 2.58	1.00 ± 0.05***
Natamycin	48	400					16.00 ± 1.79**	0.84 ± 0.09***	35.20 ± 4.32***	0.97 ± 0.03***
Natamycin	48	800					26.80 ± 3.32***	0.99 ± 0.10**	34.40 ± 1.94***	0.81 ± 0.03***
Distilled water	72	–					9.20 ± 0.80	1.51 ± 0.18	15.20 ± 1.36	1.52 ± 0.05
MMC	72	2					33.60 ± 7.55***	1.06 ± 0.20	36.80 ± 2.87***	0.82 ± 0.08***
Natamycin	72	200					12.80 ± 2.15	1.39 ± 0.13	19.20 ± 1.96	1.35 ± 0.10
Natamycin	72	400					10.80 ± 2.42	1.31 ± 0.09	16.80 ± 0.80	1.23 ± 0.02
Natamycin	72	800					23.20 ± 3.32*	1.01 ± 0.10*	19.20 ± 1.96	1.26 ± 0.11

bw: body weight; CA: chromosomal aberration; MI: mitotic index; MNPCE: micronucleated polychromatic erythrocytes; MMC: mitomycin C; NCE: normochromatic erythrocytes; PCE: polychromatic erythrocytes; SE: standard error;
*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: Rasgele & Kaymak (2013a,b)

The results of the study by Rasgele & Kaymak (2013a,b) were published in another journal but subsequently retracted at the request of the publisher due to the authors' use of the trade name Delvocid rather than the compound name natamycin (Rasgele & Kaymak, 2010a,b). In addition, it was claimed that there were "some fundamental scientific errors in the paper that were not addressed by the authors during manuscript revision". However, as these errors were not specified, this claim cannot be followed up.

This initial article also described the induction of abnormal sperm after treatment of groups of three male mice with natamycin at 0, 200, 400 or 800 mg/kg bw for 6, 12 and 24 hours (Rasgele & Kaymak, 2010a,b).

The sponsor considered the published Rasgele & Kaymak (2010a,b, 2013a,b) study results to be unreliable (Wilms, 2015). However, as few specific reasons were given to support this claim, it could not be followed up.

For an in vivo chromosomal aberration assay in rat bone marrow, groups of five male and five female rats were selected from the F_0, F_{1b}, F_{2b} and F_{3a} litters of a multigeneration study (Cox, Bailey & Morgareidge, 1973; section 2.5a). The rats had continuously received feed containing natamycin (formulated as Delvocid-5%) at concentrations adjusted to maintain target dose levels of 0, 5, 15, 50 and 100 mg/kg bw per day (achieved dose levels were not reported). The study pre-dates GLP or OECD TG requirements. No positive control was used. Rats received an intraperitoneal dose of colchicine 3–4 hours prior to scheduled kill. After scheduled kill, bone marrow cells were collected from the femur and slides were prepared. Coding of slides was not mentioned in the report. Neither the ratio of polychromatic erythrocytes to normochromatic erythrocytes nor the mitotic index were reported. At least 500 cells per animal were analysed to determine the mitotic index. Two hundred fifty metaphases per group were analysed from five animals per sex; however the individual counts for each of the animals were not reported. The study design was less powerful than required according to the current test guideline (50 metaphases were scored instead of 200). No results were reported for animals selected from F_0 and F_{3a} litters.

Under the conditions of this study, there was no increase in the incidence of chromosomal aberrations in the bone marrow cells of animals selected from F_{1b}, F_{2b} litters (Cox, Bailey & Morgareidge, 1973).

For a dominant lethal test, groups of 10 male rats from the F_{1b} litter of the Cox, Bailey & Morgareidge (1973) multigeneration study (section 2.5a) were reared on basal diet until 10 weeks of age, when they were administered undiluted natamycin (formulated as Delvocid-5%) orally at doses of 0, 5, 15, 50 or 100 mg/kg bw per day for seven consecutive days (the same dose level as the F_0 generation received). The study pre-dates GLP or OECD TG requirements. Thereafter, each male was co-housed with two virgin females for 7 days for the first mating. According to current OECD TG, males should be mated preferably with one female and the number of females per mating interval should be sufficient to provide at least 400 total implants. The matings were repeated weekly for seven days with two virgin females per male for a total of nine weeks. The females were killed 13 days after vaginal plugs were observed, and the numbers of corpora lutea and live and dead implants were counted and recorded. It is unclear whether body weights were measured. No positive control group was used in this study.

Under the conditions of the study, natamycin did not produce dominant lethal effects in male rats at the doses administered as measured by preimplantation and postimplantation losses (Cox, Bailey & Morgareidge, 1973).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

To evaluate the effects of natamycin on the ability to reproduce, groups of five males and 10 females were selected from the two-year long-term toxicity study control group and 1000 ppm group (section 2.3) and mated in pairs of one male with two females to generate two groups of offspring. The study pre-dates GLP or OECD TG requirements. Due to low mating success in control and treated animals, fresh groups of five males and 10 females were placed into control group and 1000 ppm group. These animals were used for three mating periods (~48 days, 184 days or 260 days in test). Animals from second mating were raised to maturity and mated to produce a second generation. Again the mating

success was low (often less than 50%) in control and treated animals. The parental animals and offspring were discarded without autopsy or histopathological evaluation when no longer needed for the study.

At weaning, offspring weights were lower in the treatment groups than in control groups. In the treatment groups (first litter of second experimental part), two pups from two litters had unilateral or bilateral anophthalmia. Unilateral anophthalmia was also reported in one pup of the control animals (second mating of first experimental part).

Due to the small group sizes and the low number of litters produced, the experimental power of the study to assess effects on reproduction or fertility is limited (Levinskas et al., 1963b; results published in Levinskas, Ribelin & Shaffer, 1966).

In a three-generation study of reproductive toxicity, groups of 10 male and 20 female Wistar-derived rats were fed diets containing natamycin (formulated as Delvocid-5%) at concentrations adjusted to maintain target dose levels of 0, 5, 15, 50 and 100 mg/kg bw per day (achieved dose levels were not reported) for three generations. The study was conducted in two parts: a first control group was treated concurrently with the 100 mg/kg bw per day group and a second control group was treated concurrently with the 5, 15 and 50 mg/kg bw per day groups. The study pre-dates GLP or OECD TG requirements. A clear and complete description of the materials and methods used including the measured parameters is missing. Parental generations were treated during a premating period of 10 weeks, and treatment continued during both a two-week mating period and a post-mating period. Animals continued to be treated during the ensuing gestation, lactation and post-weaning periods until scheduled kill. Each generation was mated twice to produce two offspring groups. Pups were weaned on lactation day 21. Offspring from the first mating (F_{1a} and F_{2a}) were used to produce the next generation. F_{1b} males were used for a dominant lethal test (see section 2.4b), Cox, Bailey & Morgareidge, 1973), whereas the respective females were used for a developmental toxicity study (see section 2.5b, Cox, Bailey & Morgareidge, 1973). Parental (F_0), F_{1b}, F_{2b} and F_{3a} groups were used for analysis of possible chromosomal aberrations (see section 2.4a, Cox, Bailey & Morgareidge, 1973). Animals from the parental generation and F_{3b} groups were used for gross examination, organ weights (liver, spleen, kidney) and histopathological evaluation (half of the animals from control and 50 and 100 mg/kg bw per day groups: liver, spleen, kidneys, adrenals, gonads, thyroids, pituitary, lymph nodes, heart and sites of gross pathology). Body weights were recorded weekly during the premating treatment periods. Randomly selected pups from the F_{1a} litters (at least 1 pup/sex per litter) were chosen to become the F_1 parental generation.

At scheduled kill, parental (F_0) animals underwent gross necropsy, and selected F_{3b} animals were histopathologically evaluated.

When compared to current TG requirements, several parameters were not checked or reported: including clinical observations for adverse effects, measurement of body weight and feed intake during all study periods, vaginal smear to determine estrous cycling, histopathological evaluation of reproductive and other tissues, evaluation of offspring for external and internal irregularities, criteria for culling, timing of pup developmental landmarks (such as pinna detachment, eye opening, hair growth and incisor eruption, preputial separation and vaginal opening).

Results are summarized in Table 8. There were no treatment-related deaths. During the premating treatment period, total feed consumption for males and females of all parental generations at 50 and 100 mg/kg bw per day was slightly lower than that of controls. Mean body weights for males and females of all parental generations at 50 and 100 mg/kg bw per day tended to be lower than that of controls. This was corroborated by lower feed utilizations in males of the high-dose groups.

There was an increase in the number of stillborn fetuses in the litters of most matings of high-dose animals and a decrease in numbers of liveborn pups and of pup survival until postnatal day 21. Pup weights in all treatment groups were comparable with control groups on postnatal day 4. Pup weights were low on postnatal day 21 in high-dose groups of all generations and at 15 and 50 mg/kg bw per day in F_2 and F_3 generation litters.

No gross macroscopic findings were observed for either parental or pup generations. No microscopic compound-related changes were observed. In summary, the types and frequencies of observations/lesions seen in treated animals were similar to those seen commonly in the strain of rats used in the performing laboratory.

Table 8. Summary of selected findings in the multigeneration study with natamycin in rats

Parameter (g)	Measure per target dose level of natamycin [a]											
	0 mg/kg bw per day		5 mg/kg bw per day		15 mg/kg bw per day		50 mg/kg bw per day		0 mg/kg bw per day		100 mg/kg bw per day	
	M	F	M	F	M	F	M	F	M	F	M	F
Total feed intake (pre-mating period)												
P	1 512	1 220	1 562	1 222	1 524	1 272	1 460	1 197	1 489	1 166	1 407	1 149
F_1	1 453	1 132	1 549	1 144	1 455	1 149	1 310	1 098	1 572	1 204	1 425	1 050
F_2	1 637	1 300	1 756	1 234	1 632	1 266	1 582	1 270	1 664	1 245	1 523	1 204
Body weights												
P, week 10	346	217	354	218	340	219	327	204	329	202	303	199
F_1, week 10	335	206	326	206	309	190	279	187	352	216	284	182
F_2, week 10	359	207	357	206	333	200	329	207	365	210	300	200
	L_1	L_2	L_1	L_2	L_1	L_2	L_1	L_2	L_1	L_2	L_1	L_2
Pup weights												
F_1, PND 4	10.8	11.2	11.0	10.2	10.9	10.3	10.3	10.6	10.1	10.8	10.3	10.1
F_1, PND 21	47.5	49.1	48.8	47.6	48.2	48.9	45.0	42.8	49.4	51.1	43.0	37.5
F_2, PND 4	10.9	10.3	10.5	9.7	9.1	9.8	10.2	10.3	10.2	10.1	10.6	10.1
F_2, PND 21	45.2	44.2	42.7	42.1	33.9	45.1	37.9	43.8	45.3	45.9	42.8	34.2
F_3, PND 4	9.95	9.56	10.2	10.3	10.6	10.6	9.50	10.3	10.2	10.1	9.94	10.0
F_3, PND 21	48.6	47.5	47.6	50.9	35.3	43.9	35.9	43.0	46.5	51.5	36.2	37.4

bw: body weight; M: males; F: females; PND: postnatal day ; F_1: first filial generation; F_2: second filial generation; L_1, litter of first mating period; L_2, litter of second mating period; P: parental generation (F_0)

[a] No statistical analysis was mentioned in the report. *Source*: Cox, Bailey & Morgareidge (1973)

The NOAEL for parental toxicity was 15 mg/kg bw per day based on lower body weights at 50 mg/kg bw per day. The NOAEL for offspring toxicity was 5 mg/kg bw per day based on lower pup weights at 15 mg/kg bw per day. The NOAEL for reproductive toxicity was 50 mg/kg bw per day based on low pre- and postnatal survival at 100 mg/kg bw per day (Cox, Bailey & Morgareidge, 1973).

(b) Developmental toxicity

Rats

Groups of 21–27 Wistar-derived female rats from the second litters of the first filial generation (F_{1b}) of the three-generation study of reproductive toxicity (section 2.5a) were reared to maturity on a control diet and mated with untreated males. The females were given the same dose of natamycin (formulated as Delvocid-5%) as their parents (0, 5, 15, 50 or 100 mg/kg bw per day of natamycin base) by gastric intubation during gestation days 6–15, and were killed and examined on gestation day 20. The study pre-dates GLP or OECD TG requirements. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; as such, the study design may have been less sensitive than required according to current standards. A clear and complete description of the materials and methods used including the measured parameters is missing. Apparently, the offspring were examined only for skeletal and soft tissue alterations; it is unclear whether external findings were recorded.

Slight changes in body weight gain were seen in the treatment groups compared to control groups, but these were considered unrelated to treatment because of the lack of a clear dose response. No differences were found between control and test animals with respect to the numbers of pregnancies, live litters, implantation sites, resorption sites, live and dead fetuses, or skeletal and soft tissue abnormalities.

The NOAELs for maternal toxicity and for embryo/fetal toxicity were 100 mg/kg bw per day, based on the absence of adverse effects up to the highest dose level tested (Cox, Bailey & Morgareidge, 1973).

Rabbit

In a developmental toxicity study, groups of 10–12 presumed-pregnant Dutch Belted artificially inseminated rabbits were treated with natamycin (formulated as Delvocid-5%) at a dose of 0, 5, 15 or 50 mg/kg bw per day via gavage (the vehicle was not reported) on gestation days 6–18. The report was unclear as to whether the dose levels refer to Delvocid-5% or to natamycin. The study pre-dates GLP or OECD TG requirements. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; as such, the study design may have been less sensitive than required according to current standards. All the rabbits were observed daily for appearance and behaviour with attention to feed consumption and body weight. Body weights were recorded on days 0, 6, 12, 18 and 29 of presumed gestation. Feed consumption values were not recorded. The rabbits were killed on day 29 of presumed gestation and necropsied. The number of corpora lutea in each ovary was recorded. The uterus was excised, weighed and examined for pregnancy, number and distribution of implantations, resorptions, and live and dead fetuses. Each fetus was weighed, sexed, assessed for 24 hour survival and examined for soft tissue and skeletal alterations. Statistical analysis was not conducted. It is unclear whether external findings were recorded.

All animals were pregnant (10, 12, 10 and 11 from the 0, 5, 15 and 50 mg/kg bw per day dose groups, respectively) and survived until scheduled kill on gestation day 29. The Meeting noted that the low number of does reaching gestation day 29 hampered reaching a firm conclusion on maternal and offspring effects in individual dose groups, as indicated by current OECD TG 414 requirements ("groups with fewer than 16 animals with implantation sites may be inappropriate").

No adverse alterations in body weights were reported throughout the study period. Higher incidences of dead fetuses were reported for the high-dose group (11 vs 1 in the control group). Fetal weights were 96.9%, 92.5% or 90.1% of weights in control groups for low-, mid- and high-dose groups. Soft tissue and skeletal examinations of the fetuses found no treatment-related adverse effects. However, the Meeting noted that compared to current studies, only a few skeletal and no soft tissue findings were observed.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 15 mg/kg bw per day based on increased number of dead fetuses and decreases in pup body weight of equivocal toxicological relevance at 50 mg/kg bw per day (Cox, Bailey & Morgareidge, 1973; [Addendum III by Bailey & Morgareidge, 1974[4]]).

In a developmental toxicity study, groups of 20–26 mated Dutch Belted rabbits were treated with aqueous suspensions of natamycin (formulated as Delvocid, containing 5% natamycin) at a dose of 0, 5, 15 or 50 mg/kg bw per day via gavage on gestation days 6–18. The study pre-dates GLP or OECD TG requirements. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; as such, the study design may have been less sensitive than currently required. A positive control group received 2.5 mg/kg bw of 6-aminonicotinamide by gavage on gestation day 9, but the results were not considered in this evaluation. All the rabbits were observed daily for signs of toxicity. Body weights were recorded on days 0, 6, 9, 12, 15, 18 and 29 of presumed gestation. The rabbits were killed on day 29 of presumed gestation and necropsied. The number of corpora lutea in each ovary was recorded. The uterus was excised and examined for number of implantations, resorptions, and live and dead fetuses. Each surviving fetus was weighed, sexed, assessed for 24-hour survival and examined for gross external, soft tissue and skeletal alterations. Feed consumption was not recorded. No gross examination of does was conducted at necropsy.

Treated does showed no clinical signs of toxicity. Animals died or were euthanized when moribund at the low dose (1/18), mid dose (3/19) and high dose (2/18); the cause of these deaths was not indicated in the report. One doe at mid dose delivered the day before scheduled caesarean section. Of control, low-dose, mid-dose and high-dose groups, 15, 17, 16 and 16 animals, respectively, were pregnant and survived until scheduled kill on gestation day 29.

[4] Bailey DE, Morgareidge K (1974). Teratologic evaluation (delvocid 5%) in rabbits. Unpublished report No. 1-1052, Addendum III of Cox, Bailey & Morgareidge, 1973, dated 5 April 1974, by Food & Drug Research Laboratories Inc., Waverly, New York, USA.

No clear indications of adverse effects on body weights were reported in all dose groups. The Meeting noted that virtually no increase in body weights was observed during gestation day 6–18. Maternal body weight gain was not calculated.

The following parameters were comparable in treatment and vehicle control groups: pregnancy rate, number of implantation sites, number of resorption sites, numbers of live and dead fetuses, fetal sex ratio, per cent viability and incidence of soft tissue anomalies. In addition, although the number of corpora lutea in each doe and the occurrence of external anomalies were determined, these data were not summarized or analysed statistically. The average fetal weight in the mid-dose group was significantly lower than that of the vehicle control group but not that of the high-dose group. The incidence of extra sternebrae was significantly increased in the mid- and high-dose groups when compared to the vehicle control group; however, compared to current studies, only a few skeletal or soft tissue findings were observed.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 5 mg/kg bw per day based on the increased incidence of extra sternebrae and decreases in pup body weight of equivocal toxicological relevance at 15 mg/kg bw per day (Knickerbocker & Re, 1978, 1979).

2.6 Special studies

(a) Neurotoxicity

No reports from specific neurotoxicity studies were submitted by the sponsor. The 90-day rat study (Otterdijk, 2003) did not indicate neurotoxic potential based on the absence of clinical signs.

In a study investigating the relationship between gut microorganisms and neurological parameters, groups of 15–47 (depending on the investigated parameter) BALB/c mice received an aqueous mixture of neomycin, bacitracin and natamycin (5 mg/mL, 5 mg/mL and 1.25 µg/mL, respectively) via drinking-water for seven days. Another group received sterile water. In another experiment, mice received the compounds (1% of daily dose) or saline via intraperitoneal injection for seven days.

In mice treated with the mixture via drinking-water, administration induced a significant perturbation of microbiota composition in the absence of changes in total cultivable bacteria counts. Sequence analysis using excised DGGE bands and dominant cultivable bacteria showed that treatment increased the proportion of *Lactobacilli* (dominant species: *Lactobacillus intestinalis*, *L. johnsonii*/*gasseri* and *L. plantarum*) and Actinobacteria populations. At the same time, there was a decrease in the γ-proteobacteria (dominant genus: *Shigella*/*Klebsiella*) and Bacteroidetes populations (dominant genus: *Bacteroides*). Mouse behaviour was assessed on day 7 of treatment using standard techniques of step-down and light/dark preference tests. Treated mice showed more exploratory and less apprehensive behaviour than controls. Specifically, they stepped down faster from the elevated platform, spent more time in the illuminated compartment of the apparatus and displayed an increased number of zone entries between the dark and light compartments. However, their overall locomotor activity, assessed by total distance covered or average velocity, was not affected. After two weeks of recovery, both bacterial populations and behaviour were comparable to control animals. Brain-derived neurotropic factor levels in compound-treated mice were much higher in the hippocampus and lower in the amygdala than in control mice. In mice treated via intraperitoneal administration, no difference was observed in behaviour or microbiota profiles (Bercik et al., 2011).

In a study investigating the relationship between gut microorganisms and neurological parameters, untreated Sprague Dawley rats were mated and offspring was randomly assigned to treatment groups consisting of 9–10 animals/sex. From postnatal day 4 to 13, animals received either a mixture of antimicrobial agents (natamycin 5/2.5 mg/kg bw, bacitran 100/50 mg/kg bw, neomycin 100/50 mg/kg bw) or sterile water, which also served as the vehicle. Another group received vancomycin, but these results were not assessed in the present evaluation. During weeks 4 and 11, colorectal distension, a measure of visceral sensitivity, was conducted. During week 12, animals were killed and samples from lumbosacral spinal cord and colorectum were extracted and frozen. For each investigation (colorectal distension in weeks 4 and 11 and sample collection during section) different animals were used. Colorectal distension in females was conducted during diestrous 1 or 2.

At four weeks of age, there was no significant difference between the male treatment groups with regard to the threshold for visceral hypersensitivity. A one-way ANOVA showed that at 11 weeks of age, the males treated with antimicrobial agents in early life had a decreased threshold to colorectal distension. The number of pain behaviours did not change in a statistically significant way in weeks 4 and 11. Early-life treatment with the antimicrobial agents did not affect visceral hypersensitivity at four weeks of age or in adult female rats, as assessed by the threshold and the total number of pain behaviours. A one-way ANOVA followed by Dunnett post hoc test indicated that male animals treated with the cocktail of antimicrobial agents in the neonatal period express lower lumbosacral spinal cord levels of cholecystokinin B receptor mRNA (O'Mahony et al., 2014).

(b) Immunotoxicity

No reports from specific immunotoxicity studies were submitted by the sponsor.

Stimulation of DNA synthesis was measured in isolated and cultured spleen cells from C57BL or A.CA mice or from the respective congenitally athymic mice from Balb/c background. Irrespective of the mouse strain, natamycin induced no or only low increases of DNA synthesis when measured by ^{3}H-thymidine incorporation. Similarly, in T-cells isolated from mice pretreated with cortisone acetate or B-cells isolated from athymic mice, natamycin induced no or only low increases of DNA synthesis (Hammarstrom & Smith, 1977).

Interleukin (IL)-1β secretion was investigated in vitro in dendritic cells isolated from C57BL/6 mice. IL-1β release depended on toll-like receptor-mediated induction of pro-IL-1β as well as the NLRP3 inflammasome, its adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 for enzymatic cleavage of pro-IL-1β into its mature form. Potassium efflux was induced from the cells as a known mechanism for NLRP3 activation, but the P2X7 receptor was not required for this process. Natamycin-induced (50 µg/mL) IL-1β secretion also involved phagocytosis, as cathepsin activation as described for crystal-induced IL-1β release. Natamycin triggered IL-1β secretion by causing potassium efflux which activates the NLRP3-ASC-caspase-1 (Darisipudi et al., 2011).

The effect of incubating human polymorphonuclear neutrophils with natamycin on chemotaxis to cytoplasmic extracts of *Trichophyton rubrum* was assessed. The viability of leukocytes was not altered at concentrations of up to 10 mg/L. Lysis of sensitized red blood cells occurred in the presence of natamycin and complement, but no lysis occurred in the control wells from which complement was excluded, indicating that the effects on polymorphonuclear neutrophil migration were not secondary due to an effect on the complement system. In the presence of 1 mg/L of natamycin, the percentage reduction of migrating polymorphonuclear neutrophils in comparison to natamycin-free controls was significantly lower (37–61%; Davies & Zaini, 1985).

(c) Other end-points

An article summarizing the influence of intravenous injection of natamycin (dissolved in propylene glycol) on blood pressure and heart frequency in cats and dogs reported insufficient details to allow for an independent evaluation of the effects, namely that injections of natamycin at 5 and 10 mg/kg bw led to drops in blood pressure for less than one minute. Sometimes brief halving of heart frequency was observed (Struyk et al., 1958).

Isolated hearts of guinea pigs were perfused with freshly prepared solutions of 35 µg/mL of natamycin (dissolved in propylene glycol at 85 °C) in Ringer-Locke solution. Experiments with ouabain and pentamycin were not considered for the present evaluation. Systolic arrest was observed within 5–15 minutes. Five experiments with natamycin and six control experiments were conducted. The heart rate was not significantly altered but auriculoventricular block occurred in two experiments shortly before cessation of ventricular activity. The coronary flow increased by 50–100% above the control values. The auricles continued to beat for 5–10 minutes after the ventricles had stopped. Mean water content of hearts was unaltered but mean sodium content per concentration was significantly increased compared to controls. Potassium content per concentration was significantly decreased compared to controls. Concentration of sodium plus potassium was not statistically significantly different from controls (Arora, 1965).

Isolated hearts of rabbits were perfused with freshly prepared solutions of 35 µg/mL of natamycin (dissolved in propylene glycol at 85 °C) in Ringer-Locke solution. Experiments with amphotericin A and F-17 C were not considered for the present evaluation. Systolic arrest was observed within 10–15 minutes. Five experiments with natamycin and six control experiments were conducted. Natamycin induced an initial decrease in the amplitude of contraction, lasting for 0.5–1 minute, followed by a decrease terminating in systolic arrest in one experiment; diastolic arrest in one experiment; and mid-diastolic arrest in three experiments. A progressive decrease in ventricular rate was observed in one experiment, and an initial decrease followed by recovery and then a decrease terminating in ventricular arrest was observed in the remaining four experiments. An intermittent, irregular, partial auriculoventricular block developed in three experiments. Natamycin increased the coronary flow by 28.2 ± 9.5%; this was followed by a return to 96.0 ± 2.9% of control values in 10– 15 minutes, that is, at the time of ventricular arrest. A decrease in potassium concentration and an increase in sodium concentration were reported. Water content of the heart and concentration of sodium plus potassium did not differ from controls (Arora, 1966).

Yeast cells were transformed to express the mouse *ABCA1* gene for the ABCA1 transporter. Similarly, a non-functional gene was integrated into yeasts. The GFP-ABCA1 transcript was localized on the plasma membrane. Growth of ABCA1-expressing yeasts was reduced to 49% of the growth rate of control yeast, while the yeasts expressing the non-functional ABCA1 transporter showed 90% of control yeasts (Bocer et al., 2012).

Antitumorigenic activity of bleomycin against Ehrlich ascites carcinoma in mice (intraperitoneal injection of 2.5×10^6 cells per mouse) was examined in the presence and absence of natamycin. Survival was checked daily for up to 60 days. Natamycin at a dose of 25 mg/kg bw was given by intraperitoneal injection once on the next day, followed by bleomycin at a dose of 50 mg/kg bw by intraperitoneal injection one or six hours later. This treatment produced a synergistic life-prolongation effect compared with that of each agent alone (mean survival of ~40 days as compared to ~20–30 days). Accumulation of abdominal ascites was almost completely blocked by bleomycin in combination with natamycin. Frequent intraperitoneal injections of small doses of bleomycin and natamycin on day 1 resulted in a much greater increase in lifespan (mean survival was up to 47 days) than the single intraperitoneal injection (Komiyama et al., 1983).

To assess the influence of natamycin on the kidney, groups of four male Jcl:ICR mice were treated via intravenous injection with vehicle or natamycin at 25 or 50 mg/kg bw formulated in physiological saline at a dose volume of 10 mL/kg. Animals were killed 24 hours after administration. Other groups received doses of amphotericin B deoxycholate, liposomal amphotericin B, amphotericin B methyl ester, nystatin, candicidin, cephalothin, cefepime, ceftazidime, piperacillin; results of these groups were not considered for the present evaluation.

Tubular necrosis of Henle's loop and infiltration with neutrophils in renal medulla were observed. In addition, dilatation of renal tubules in cortex was reported. In plasma samples, creatinine and urea nitrogen levels were increased but did not reach statistical significance. In kidney samples, gene expression of *Kim1*, *Timp1*, *Lcn2*, *Clu*, *Spp1*, *Krt19*, *Fgg*, *Fga*, *Cp* were significantly increased, while *Vim*, *Mki67*, *Krt8*, *Krt18* and *A2m* were not statistically significantly different from the control groups (Kondo et al., 2012).

Isolated adipocytes from Sprague Dawley rats were incubated with natamycin and assessed for changes in glucose uptake, glucose oxidation and lipogenesis. Treatment of cells with approximately 100–200 µmol/L of natamycin stimulated glucose uptake, oxidation and lipogenesis. Higher concentrations were less effective or led to inhibition. Treatment of cells with natamycin inhibited lipid oxidation. Natamycin induced the release of cyclic adenosine monophosphate (cAMP) into the medium of cells treated with or without norepinephrine or corticotropin. Intracellular amounts were decreased by treatment with norepinephrine or corticotropin (Kuo, 1968; Kuo & De Renzo, 1969).

(d) Toxicity of metabolites

A few reports on specific toxicological studies with individual metabolites were submitted by the sponsor. Some reports on non-identified degradation products were also submitted.

Acute toxicities of natamycin, aponatamycin, mycosamine HCl and dinatamycinolidediole were assessed in mice. After intraperitoneal administration, survival of the animals was observed for seven days (current TGs require an observation period of 14 days). The aponatamycin and mycosamine HCl treatment group sizes were small. Overall, the report contained only a few details on the identity of the test materials and the results. Experimental details and results are summarized in Table 9 (Van Eeken & Wubs, 1976).

Table 9. Acute toxicity of natamycin and related compounds in mice

Test material	Group size	Dose levels (mg/kg bw)	Intraperitoneal LD_{50} (mg/kg bw)
Natamycin	5 M, 5 F	500, 583, 680, 793, 925, 1080, 1260, 1470, 1710, 2000	Male: 1636; Female: 421 950 (M & F combined)
Aponatamycin	2 M, 2 F	2000, 2710, 3690, 5000	3189 (M & F combined)
Mycosamine HCl	2 M, 2 F	2000, 2710, 3690, 5000	3722 (M & F combined)
Dinatamycinolidediole	NR	NR	>4000 (M & F combined)

bw: body weight; F: females; M: males; LD_{50}: median lethal dose; NR: not reported

Source: Van Eeken & Wubs (1976)

Breakdown of natamycin was determined in acidic aqueous solution (pH of 1.5 with diluted hydrochloric acid); this solution was allowed to stand at 40 °C for two weeks. An ochre yellow suspension was formed, which was isolated by precipitation followed by further clean-up. The light-yellow amorphous powder darkens at approximately 250 °C, but appears to be unaltered at temperatures up to 350 °C. Equivalent weight determined by alkalimetric titration was about 595. The molecular weight determined by osmosis was 1250 ± 100 g/mol. While the authors speculated about possible structure of the reaction product, no elucidation of the structure (in modern terms) was performed (Brik, undated, 1976a,b).

The 57th JECFA meeting considered a study by Brik (1975)[5] on decomposition products of natamycin with intact lactone ring; this study was not submitted to JMPR. According to the sponsor, this reference was a preliminary version of the article by Brik (1976a).

According to a summary of preliminary results (GLP status not mentioned), approximately 50% natamycin was broken down within one hour in simulated gastric juice. The losses from the stomachs of fasted and non-fasted rats were 33–43% and 0–31%, respectively. No further details on the in vitro experiment were available. Six fasted and three non-fasted rats were operated on to introduce a rubber balloon into duodenum (just after the pylorus) to collect all material leaving the stomach. Three of the fasted animals were killed after the operation to serve as controls. The remaining animals received an oral dose of 30 mg of natamycin in 1% amylum and were killed three hours post administration. Stomachs and rubber balloons were removed and frozen. Natamycin amounts in frozen specimen of the in vivo study were determined by antimicrobiological activity. The Meeting noted that details on in vitro and in vivo experimental procedures or results were not sufficient enough to allow an independent evaluation of the findings. There was large interindividual variability between replicates of the in vivo experiments. Stability of natamycin in the specimen under frozen condition and under experimental procedures was not determined (Morgenstern & Muskens, 1976[6]).

The results of studies of the acute toxicity of the decomposition products of natamycin kept under various conditions were summarized in a statement (Ottens, 1965). Results after intraperitoneal administration in mice are presented in Table 10. The Meeting noted that details on experimental procedures or results were not sufficient enough to allow an independent evaluation of the findings.

[5] Brik H (1975). Natamycin (pimaricin). New high-molecular decomposition products with intact lactone-ring.

[6] Neither enclosures mentioned in the summary nor the final study reports were submitted by the sponsor.

Table 10. Acute toxicity of natamycin decomposition products in mice

Treatment of suspension	Decomposition (%)	I.p. LD_{50} (mg/kg bw)
pH 2.2 with citric acid	74	200
pH 6.3 in the dark	13	200–400
pH 6.3 in the light	80	400–600
pH 8.5 (NaOH)	0	150–250
pH 8.5 (NaOH)	5	450
pH 10.4 with "soda"	100	> 800
pH 6.3 with 0.1% H_2O_2	9	200–400
pH 5.0 in ultraviolet light	0	170

bw: body weight; i.p.: intraperitoneal; LD_{50}: median lethal dose

Source: Ottens (1965)

In a dietary toxicity study, groups of 15 male and 15 female rats were given diets containing 5% water, 5% of 0.5% citric acid, natamycin at 500 ppm or 5% of a solution of acid-degraded natamycin (suspended in 0.5% citric acid until only 14% of the activity remained) for 98 days. The study pre-dates GLP or OECD TG requirements.

Rats were observed daily for signs of overt toxicity, morbidity and mortality. No ophthalmological examinations were conducted. Individual body weights were recorded weekly and individual feed consumptions were recorded biweekly. No neurobehavioural observations were recorded. Haematological parameters were determined pretest and towards the end of the study, in five animals/sex per group. At scheduled kill, 10 animals/sex per group underwent gross necropsy; kidneys and livers were weighed. Samples of the following tissues from five rats per sex from the group treated with acid-degraded natamycin underwent histopathological evaluation: skeletal muscle, femur with marrow, spleen, mesenteric lymph node, pancreas, liver, stomach, ilium, colon, urinary bladder, gonads, uterus, prostate and seminal vesicles, adrenals, kidneys, thyroids and parathyroids, trachea, oesophagus, lungs, heart, pituitary and brain. Urine or clinical chemistry analysis were not conducted.

No animals died. Weight gain was unaffected by treatment; no adverse effects were seen in haematological tests or in absolute or relative weights of liver and kidneys. Minor differences in relative organ weights were considered to be coincidental and not due to treatment. No gross pathological or histopathological findings were attributed to treatment (Hutchison, Levinskas & Ribelin, 1966).

Groups of 15 male and 30 female rats received, for seven weeks, diets containing fresh cheese dressed with 0%, 0.05% or 5.0% natamycin or cheese dressed with 0%, 0.05% or 5% suspensions of natamycin and stored for three weeks (Wieriks, 1966). This report was not further evaluated due to the lack of relevance for the present assessment.

In a feeding study, groups of 10 male and 10 female rats (strain not reported) were fed, for three months, diets containing apple peel that was either untreated, freshly treated with natamycin or treated with natamycin and stored for 2–8 weeks to allow degradation to take place. The study pre-dates GLP or OECD TG requirements. In a similar experiment, sausage skins that were either untreated, freshly treated or stored with natamycin were fed to rats; results of these experiments were not evaluated due to the lack of relevance to the present assessment. The doses of natamycin and its degradation products cannot be calculated (one part apple peel was mixed with three parts feed). The homogeneity of the feed mixture, the concentration in feed and the identity of the degradation products were not confirmed analytically. Some isolated changes were noted, but due to the low magnitude of change or the lack of dose response or consistency, none were considered adverse. Due to the missing information on achieved dose levels, this study provides only little information (Wieriks, 1971).

3. Observations in humans

The sponsor stated that no natamycin-related health incidents, whether through production or use, had been observed (JS Moore, Technology Sciences Group, personal communication, 2017). The Meeting noted that the submitted statement contained no information on the health checks used or their frequency. Nor was there a confirmation by the responsible occupational physician. No statement from the medical officer was submitted. No reports on clinical cases / poisoning incidences were available for review.

In a clinical trial, 145 patients were treated dermally with a natamycin-containing ointment (2% natamycin) against yeast or fungal infections. The report provides no information on whether the patients gave informed consent or whether the study was approved by an ethics committee/review board. Skin areas were treated two to three times per day for 4 to 30 days (average: 15 days). Of the patients initially included in the study, only 111 were available for follow-up. According to the report, no signs of irritation or sensitization were observed. However, it is unclear how such symptoms were determined and how they were distinguished from the symptoms of mycosis (Grupper, undated). The Meeting noted that the report was undated and unsigned.

A published summary about patients treated with natamycin-containing ointment reported that neither skin irritation nor skin sensitization was induced in 66 cases (Grupper, 1967). However, it is unclear how such symptoms were determined or how they were distinguished from the symptoms of mycosis.

The 57th JECFA meeting discussed a study by Grupper (1964)[7] on the use of natamycin in the treatment of mucocutaneous monoliasis. This report was not submitted to JMPR, despite a request by the sponsor. According to the sponsor, this reference was "not available on short notice".

Healthy, occupationally exposed and non-exposed persons were recruited from a natamycin-producing factory. In the report there is no information whether the study was approved by an ethics committee/review board. Seventy-one previously exposed and 37 previously non-exposed participants were treated with solutions of natamycin: right forearms were scarified three times; one served as control, one was treated with a drop of a 1% natamycin solution and one with a drop of a 1% ultraviolet-irradiated natamycin solution. The left upper arms received patches of the same solutions for 24 hours. Finally, the participants received intracutaneous injections of 0.1 mL of a 0.0025% natamycin solution and of a 0.0025% ultraviolet-irradiated natamycin solution in the right upper arm. The exposure points were examined after 20 minutes, 24 and 48 hours and 5–7 days. The exposure was repeated after two weeks in 66 exposed, and 37 non-exposed participants. The author indicated that there were no skin reactions indicative of sensitization due to the application (Malten, 1967).

Repeated patch tests were conducted on 102 patients with various forms of eczema. In the report there is no information whether the patients gave informed consent or whether the study was approved by an ethics committee/review board. Patients received dermal patches for a duration of 48 hours; the procedure was repeated after 14 days until the patients had received patches 2 (1/3 of the participants) to 3 times (2/3 of the participants). The author indicated that there were no skin reactions indicative of sensitization due to the applications (Malten, 1968).

The 57th JECFA meeting considered a 1966 study by an anonymous writer on the effects of natamycin in humans. This study was not submitted to JMPR, despite a request to do so. The sponsor indicated that this reference was not known to their organization and hypothesized that

[7] Grupper C (1964). Pimaricin in the treatment of superficial mucocutaneous monoliasis. In: *Proceedings of the International Congress on Tropical Dermatoses*, Naples, June 1964.

this reference actually refers to another report (Anonymous, 1965 [8]), which was also not submitted to JMPR. The monograph of the 57th JECFA meeting concluded:

> "Nausea, vomiting, and diarrhoea have been observed occasionally after an oral dose of 300–400 mg of natamycin daily; no changes in peripheral blood cells were observed (Anonymous, 1966)"
>
> (FAO/WHO, 2002b).

A report summarized clinical observations in 10 patients with systemic mycoses who were treated with oral doses of 50–1000 mg/day for 13–180 days. Nausea, vomiting and diarrhoea occurred in those receiving 400–1000 mg/day (Newcomer et al., 1960).

In the European database on adverse drug reactions (www.adrreports.eu), 75 cases related to natamycin were reported (search conducted on April 10, 2017). Most cases related to reaction groups "eye disorders" (16 cases), "general disorders and administration conditions" (24 cases), "infections and infestations" (13 cases), "injury, poisoning and procedural complications" (seven cases). Other affected reaction groups had three or fewer cases. No details on the cases were available to the Meeting.

In the German database on adverse drug reactions (nebenwirkung.bfarm.de), four cases related to natamycin were reported (search conducted on April 10, 2017). One case had pruritus, ocular oedema and erythema and burning eyes, and received chloramphenicol/natamycin. One case had fungal keratitis, and eyes were treated with natamycin and other drugs. One case received natamycin orally and other drugs to treat bacterial sepsis, cardiac insufficiency and cerebral thrombosis. The fourth case received about 40 different drugs due to several multimorbid indications, including several antibiotics and chemotherapy for leukaemia.

The teratogenicity of vaginal natamycin treatment during pregnancy was investigated using the population-based dataset of the Hungarian Case-Control Surveillance of Congenital Abnormalities. Data were collected between 1980 and 1996 and included a total of 38 151 pregnant women whose newborn infants had no congenital abnormalities (control group) and 22 843 pregnant women who had fetuses or newborns with congenital abnormalities. Pair analysis of cases with congenital abnormalities and matched healthy controls was carried out; 62 (0.27%) and 98 (0.26%) pregnant women were treated with the natamycin in the case and control groups, respectively (crude odds ratio [OR] = 1.1; 95% confidence interval [CI]: 0.8–1.5). A teratogenic potential of vaginal natamycin treatment during the second and third months of pregnancy, the critical period of most major congenital abnormalities, was not indicated in the case-control pair analysis (adjusted OR = 0.9; 95% CI: 0.4–1.8). A somewhat higher mean birth weight (72 g) was found in newborn infants born to mothers with natamycin treatment compared with newborn infants without this treatment (adjusted $P = 0.01$), though mean gestational age was shorter (Czeizel, Kazy & Vargha, 2003).

4. Microbial aspects

4.1 Mechanism and type of antimicrobial action

The antifungal activities of natamycin are due to irreversible binding to sterols, primarily ergosterol, an important component of the plasma cell membrane found in the growing tips of germinating fungal spores and vegetative hyphae (Te Welscher et al., 2008; Van Leeuwen et al., 2008, 2010, 2013; Van Leeuwen, Golovina & Dijksterhuis, 2009). Gram-positive and Gram-negative bacteria are not susceptible to natamycin because their cell walls and cell membranes lack ergosterol and other sterols, and therefore they are naturally resistant to natamycin. Natamycin binding to ergosterol is a critical reaction required for antifungal action; the lactone ring of polyenes inserts into the lipid bilayer of fungi and interferes with cellular processes. In addition to ergosterol binding, natamycin impairs membrane fusion via perturbation of ergosterol-dependent priming reactions that precede membrane fusion (Te Welscher et al., 2010, 2012). The effect is cessation of active fungal growth. While some polyenes, for example, amphotericin B and nystatin, form complexes that lead to leakage of the

[8] Anonymous (1965). Data on the safety of the use of pimaricin as preservative against mold growth on cheese. Summary of the results of acute and chronic toxicity tests. Unpublished report submitted to WHO by the Royal Netherlands Fermentation Industries Ltd, Delft, the Netherlands.

plasma membrane, natamycin does not permeabilize cells (Te Welscher et al., 2008). In addition, natamycin interferes with ergosterol-dependent cellular processes, such as membrane trafficking and fusion (Te Welscher et al., 2012). Natamycin inhibits endocytosis in germinating conidia of *Penicillium discolor* (Van Leeuwen, Golovina & Dijksterhuis, 2009) and the fusion of prevacuolar compartments in *S. cerevisiae* (Te Welscher et al., 2010). Natamycin also inhibits transport of amino acids and sugars into cells in a reversible manner (Te Welscher et al., 2012).

4.2 Microbiome of the human gastrointestinal tract

When ingested, natamycin used as an antifungal agent in food may interact with the indigenous microbiota in the gastrointestinal tract. The human gastrointestinal tract ecosystem consists of complex and diverse microbial communities (bacteria, fungi, viruses) termed the intestinal microbiome (Zoetendal, Rajilić-Stojanović & de Vos, 2008). The total number of microorganisms inhabiting the gastrointestinal tract is estimated to be 10^{14} cells, representing more than 1000 bacterial species. While the literature on the bacterial intestinal microbiome is extensive, less is known about the fungal microbiome of the intestinal tract (Seed, 2014; Suhr & Hallen-Adams, 2015). The development of advanced culturing methodologies and polymerase chain reaction (PCR) and next generation sequencing has enabled a more in-depth survey of both culturable and unculturable intestinal fungi (Huffnagle & Noverr, 2013; Suhr, Banjara & Hallen-Adams, 2016). Fungi are detectable in all sections of the gastrointestinal tract of about 70% of healthy adults, normally at up to 10^3 fungal cells per mL or g of intestinal contents (Schulze & Sonnenborn, 2009). In other words, yeasts and filamentous fungi are minor constituents in the gastrointestinal tract compared to the high amounts of bacteria.

Culture-independent analyses show that fungal genes constitute less than 0.1% of the human gut microbiome (Qin et al., 2010). At least 267 distinct fungal taxa have been reported in the gut, a number considerably lower than the estimated 500–1000 bacterial taxa therein (Qin et al., 2010). Approximately 40% of the fungal taxa reported from the gut are yeasts. Yeasts represent 63.4% of the taxa reported from multiple studies, and 10 of the 12 most commonly detected fungi are yeasts. Culture-dependent studies identified *Candida* spp. as the most common fungi in the gastrointestinal tract. The most commonly detected fungi in the gastrointestinal tract of humans are *C. albicans*, *S. cerevisiae*, *C.vtropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *Cladosporium cladosporioides, Penicillium allii*, *Malassezia globosa*, *M. restricta*, *Debaryomyces hansenii* and *Galactomyces geotrichum*.

The use of natamycin as an antifungal agent in food could result in residues that may interact with commensal microbiota; however, no data are available on the effect of natamycin on the indigenous human intestinal microbiota. Because bacteria do not contain ergosterol in their cells, they are not affected by polyenes, and it seems likely that natamycin residues would not have any potentially harmful effects on bacteria in the gastrointestinal tract. Furthermore, as fungi are found in small quantities in the human gastrointestinal tract compared to the high levels of intestinal bacteria, the risk of disruption of the colonization barrier due to dietary natamycin residues would be minimal.

4.3 Antimicrobial spectrum of activity

Natamycin inhibits a wide range of fungal species at low concentrations (in the micromolar range). However, there are differences between different fungal species and within strains of the same species in sensitivity to natamycin (Table 11). Natamycin shows in vitro activity against yeasts and filamentous fungi, such as *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., *Candida* spp., *Cephalosporium* spp., *Cladosporium* spp., *Cunninghamella* spp., *Curvularia* spp., *Exserohilum* spp., *Fusarium* spp., *Lasiodiplodia* spp., *Penicillium* spp., *Pseudallescheria* spp., *Rhizopus* spp., *Rhodotorula* spp., *Saccharomyces* spp., *Scedosporium* spp., *Scopulariopsis* spp. and *Trichosporon* spp., but is inactive against aerobic and anaerobic Gram-positive and Gram-negative bacteria. In general, yeasts are more sensitive to natamycin than filamentous fungi.

Table 11. Minimum inhibitory concentrations (MICs) of natamycin and amphotericin B tested in vitro against fungal and yeast isolates

		Natamycin (mg/mL)				Amphotericin B (mg/mL)				
Genus	**Species**	**MIC avg**	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC avg**	**MIC_{50}**	**MIC_{90}**	**MIC range**	**Reference**
Filamentous fungi										
Acremonium	*implicatum*	–	–	–	2–4	–	–	–	–	Zhang et al., 2012
Acremonium	*sp.*	6.3	–	–	4–16	–	–	–	–	Pradhan et al., 2011
Acremonium	*sp.*	2.12	2	4	1–4	5.7	32	>32	<0.13–>32	Rotowa et al., 1990
Acremonium	*sp.*	22	2	2	1–2	4.9	4	32	<0.13–>32	Rotowa et al., 1990
Acremonium	*sp.*	–	–	–	4	–	–	–	–	Shapiro et al., 2010
Alternaria	*alternata*	–	4	4	–	–	–	–	–	Homa et al., 2013
Alternaria	*alternata*	–	4	4	2–8	–	0.125	4	0.0625–2	Xu et al., 2013
Alternaria	*sp.*	–	2	–	2–2	–	–	–	–	Lalitha et al., 2014
Alternaria	*sp.*	–	2	–	2–2	–	–	–	–	Sun et al., 2014
Aspergillus	*flavus*	–	32	64	8–64	–	–	–	–	Lalitha et al., 2008
Aspergillus	*flavus*	–	32	32	–	–	–	–	–	Lalitha et al., 2012
Aspergillus	*flavus*	–	32	64	2–64	–	–	–	–	Lalitha et al., 2014
Aspergillus	*flavus*	19	–	–	8–32	–	–	–	–	Pradhan et al., 2011
Aspergillus	*flavus*	–	32	64	16-64	–	–	–	–	Shapiro et al., 2010
Aspergillus	*flavus*	–	32	64	8–64	–	–	–	–	Sun et al., 2014
Aspergillus	*flavus*	–	32	32	8–32	–	2	2	1–32	Xu et al., 2009, 2010
Aspergillus	*fumigatus*	–	4	4	1–4	–	–	–	–	Lalitha et al., 2008
Aspergillus	*fumigatus*	–	4	8	–	–	–	–	–	Lalitha et al., 2012
Aspergillus	*fumigatus*	–	4	64	2–64	–	–	–	–	Lalitha et al., 2014
Aspergillus	*fumigatus*	3.9	–	–	2–8	–	–	–	–	Pradhan et al., 2011
Aspergillus	*fumigatus*	–	–	–	8–8	–	–	–	–	Shapiro et al., 2010
Aspergillus	*fumigatus*	3.12	–	–	3.12	3.12	–	–	3.12	Stern, 1978
Aspergillus	*fumigatus*	–	4	–	2–64	–	–	–	–	Sun et al., 2014
Aspergillus	*fumigatus*	–	4	4	4–32	–	1	2	0.5–4	Xu et al., 2009, 2010
Aspergillus	*niger*	–	–	–	1–4	–	–	–	–	Lalitha et al., 2008
Aspergillus	*niger*	–	5	–	2–8	–	–	–	–	Lalitha et al., 2014
Aspergillus	*niger*	–	2	–	–	–	–	–	–	Pradhan et al., 2011
Aspergillus	*niger*	–	–	–	8–32	–	–	–	–	Shapiro et al., 2010
Aspergillus	*niger*	2.34	–	–	1.56-3.12	4.3	–	–	2.34–6.25	Stern, 1978
Aspergillus	*niger*	–	5	–	2–8	–	–	–	–	Sun et al., 2014
Aspergillus	*niger*	–	–	–	0.25-4	–	–	–	–	Xu et al., 2009
Aspergillus	*oryzae*	–	32	32	4–32	–	1	2	1–2	Xu et al., 2009, 2010
Aspergillus	*sp.*	–	32	>32	–	–	2	4	–	Lalitha et al., 2007
Aspergillus	*sp.*	–	16	16	–	–	–	–	–	Lalitha et al., 2011
Aspergillus	*sp.*	–	32	32	–	–	–	–	–	Lalitha et al., 2012

		Natamycin (mg/mL)				Amphotericin B (mg/mL)				
Genus	Species	MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	Reference
Aspergillus	*sp.*	–	32	64	1–64	–	–	–	–	Lalitha et al., 2014
Aspergillus	*sp.*	–	32	64	2–64	–	–	–	–	Sun et al., 2014
Aspergillus	*sp.*	–	4	32	0.25-32	–	1	2	0.125–2	Xu et al., 2009, 2010
Aspergillus	*terreus*	–	–	–	4–16	–	–	–	–	Lalitha et al., 2008
Aspergillus	*terreus*	–	16	–	8–16	–	–	–	–	Lalitha et al., 2014
Aspergillus	*terreus*	–	16	–	–	–	–	–	–	Pradhan et al., 2011
Aspergillus	*terreus*	–	–	–	8–32	–	–	–	–	Shapiro et al., 2010
Aspergillus	*terreus*	–	16	–	8–16	–	–	–	–	Sun et al., 2014
Aspergillus	*versicolor*	–	8	32	4–32	–	1	2	0.5–2	Xu et al., 2009, 2010
Bipolaris	*sp.*	–	4	4	–	–	–	–	–	Lalitha et al., 2012
Bipolaris	*sp.*	2	–	–	2–2	–	–	–	–	Lalitha et al., 2014
Bipolaris	*sp.*	2	–	–	2	–	–	–	–	Pradhan et al., 2011
Bipolaris	*sp.*	–	–	–	32	–	–	–	–	Shapiro et al., 2010
Bipolaris	*sp.*	2	–	–	2–2	–	–	–	–	Sun et al., 2014
Cladosporium sphaerospermum		–	–	–	8–32	–	–	–	–	Zhang et al., 2012
Cunninghamella	*sp.*	2.83	2	4	2–4	5.04	8	16	0.25–16	Rotowa et al., 1990
Cunninghamella	*sp.*	2.83	2	4	2–4	8	16	32	0.25–>32	Rotowa et al., 1990
Curvularia	*sp.*	–	4	256	–	–	–	–	–	Lalitha et al., 2012
Curvularia	*sp.*	–	2	2	1–16	–	–	–	–	Lalitha et al., 2014
Curvularia	*sp.*	1.7	–	–	1–2	–	–	–	–	Pradhan et al., 2011
Curvularia	*sp.*	–	–	–	4	–	–	–	–	Shapiro et al., 2010
Curvularia	*sp.*	–	2	2	1–2	–	–	–	–	Sun et al., 2014
Exserohilum	*sp.*	–	4	4	–	–	–	–	–	Lalitha et al., 2012
Exserohilum	*sp.*	2	–	–	1–2	–	–	–	–	Lalitha et al., 2014
Exserohilum	*sp.*	4	–	–	4	–	–	–	–	Pradhan et al., 2011
Exserohilum	*sp.*	1.5	–	–	1–2	–	–	–	–	Sun et al., 2014
Fusarium	*avenaceum*	–	8	8	4–32	–	2	4	0.5–8	Xu et al., 2009, 2010
Fusarium	*dimerum*	–	–	–	2–8	–	–	–	4–64	Homa et al., 2013
Fusarium	*fujikori*	–	–	–	4–>64	–	–	–	16–>64	Homa et al., 2013
Fusarium incarnatum-equiseti		–	–	–	4–>64	–	–	–	32–>64	Homa et al., 2013
Fusarium moniliforme		–	4	8	4–8	–	2	2	1–8	Xu et al., 2009, 2010
Fusarium	*oxysporum*	–	–	–	8–>64	–	–	–	4–>64	Homa et al., 2013
Fusarium	*oxysporum*	–	–	–	4	–	–	–	1	Mukherjee et al., 2012
Fusarium	*oxysporum*	–	–	–	4–8	–	–	–	–	Xu et al., 2009
Fusarium	*poae*	–	–	–	4–8	–	–	–	–	Xu et al., 2009
Fusarium	*solani*	–	–	4.8	–	–	–	–	–	Alfonso, 2008
Fusarium	*solani*	–	–	–	2–>64	–	–	–	0.125–>64	Homa et al., 2013
Fusarium	*solani*	–	–	–	2–4	–	–	–	1	Mukherjee et al., 2012

Genus	Species	Natamycin (mg/mL)				Amphotericin B (mg/mL)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Fusarium	*solani*	4.21	–	–	3.12–6.25	20.2	–	–	3.12–50	Stern, 1978
Fusarium	*solani*	–	4	8	4–32	–	1	2	0.5–16	Xu et al., 2009, 2010
Fusarium	*solani*	–	–	–	4–16	–	–	–	–	Zhang et al., 2012
Fusarium	*sp.*	–	–	4.2	–	–	–	–	–	Alfonso, 2008
Fusarium; multiresistant isolate		–	16	–	–	–	–	–	4	Edelstein et al., 2012
Fusarium	*sp.*	–	8	16	–	–	4	4	–	Lalitha et al., 2007
Fusarium	*sp.*	–	4	4	2–8	–	–	–	–	Lalitha et al., 2008
Fusarium	*sp.*	–	16	16	–	–	–	–	–	Lalitha et al., 2011
Fusarium	*sp.*	–	4	8	–	–	–	–	–	Lalitha et al., 2012
Fusarium	*sp.*	–	4	8	2–32	–	–	–	–	Lalitha et al., 2014
Fusarium	*sp.*	6.7	–	–	4–8	–	–	–	–	Pradhan et al., 2011
Fusarium	*sp.*	–	2	4	2–4	–	1	2	1–2	Reuben et al., 1989
Fusarium	*sp.*	–	–	–	3.12–6.25	–	–	–	0.25–50	Reuben et al., 1989
Fusarium	*sp.*	3.56	4	4	0.5–>32	0.71	0.5	4	<0.13–>32	Rotowa et al., 1990
Fusarium	*sp.*	3.06	2	4	1–>32	1.79	2	8	<0.13–>32	Rotowa et al., 1990
Fusarium	*sp.*	–	8	16	4–16	–	–	–	–	Shapiro et al., 2010
Fusarium	*sp.*	–	4	8	2–32	–	–	–	–	Sun et al., 2014
Fusarium	*sp.*	–	4	8	4–8	–	1	2	0.5–2	Xu et al., 2009, 2010
Lasiodiplodia	*sp.*	–	4	32	–	–	–	–	–	Lalitha et al., 2012
Lasiodiplodia	*sp.*	–	2	–	2–2	–	–	–	–	Lalitha et al., 2014
Lasiodiplodia	*sp.*	–	2	–	2–2	–	–	–	–	Sun et al., 2014
Penicillium	*lilacinus*	9.37	–	–	9.37	4.68	–	–	4.68	Stern, 1978
Penicillium	*sp.*	2.34	–	–	2.34	4.68	–	–	4.67	Stern, 1978
Pseudallescheria boydii		2.1	2	4	1–4	4.64	2	>32	1–>32	Rotowa et al., 1990
Pseudallescheria boydii		1.64	2	4	1–4	4.42	2	>32	0.5–>32	Rotowa et al., 1990
Rhizopus	*sp.*	9.37	–	–	6.25–12.5	–	–	–	2.34–>50	Stern, 1978
Scedosporium apiospermum		–	–	–	2	–	–	–	–	Pradhan et al., 2011
Scedosporium sp.		–	8	8	–	–	–	–	–	Lalitha et al., 2012
Scopulariopsis brevicaulis		3.12	–	–	3.12	>50	–	–	>50	Stern, 1978
Yeasts										
Candida	*albicans*	–	–	–	–	0.48[1]	–	–	0.25–1[1]	Athar & Winner, 1971
Candida	*albicans*	2.7	–	–	–	0.23	–	–	–	Gray et al., 2012
Candida	*albicans*	3.27	–	–	1.56–4.68	0.36	–	–	0.2–0.6	Stern, 1978
Candida	*krusei*	1.56	–	–	1.56	1.56	–	–	1.56	Stern, 1978
Candida parapsilosis		5.2	–	–	4.68–6.25	2.35	–	–	0.8–3.12	Stern, 1978

Genus	Species	Natamycin (mg/mL)				Amphotericin B (mg/mL)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Candida	*sp.*	–	–	–	0.437 –43.5	–	–	–	–	Kuratowska & Horwatt, 1998
Rhodotorula	*sp.*	2.34	–	–	2.34	6.25	–	6.25	–	Stern, 1978
Saccheronyces	*cerevisiae*	1.3	–	–	–	0.46	–	–	–	Gray et al., 2012
Saccheronyces	*cerevisiae*	1.1	–	–	–	–	–	–	–	te Welscher et al., 2008
Trichosporon	*beigelii*	3.03	2	8	2–8	0.32	0.25	0.5	<0.13–0.5	Rotowa et al., 1990
Trichosporon	*beigelii*	2.64	2	16	1–16	0.22	0.25	0.5	<0.13–0.5	Rotowa et al., 1990
Algae										
Prototheca	*zopfii*	–	3.7	6.3	1–32	–	–	–	–	Buzzini et al., 2008

MIC avg: average minimum inhibitory concentration; *Source*: Streekstra (2015)
MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms;
MIC_{90}: minimum concentration required to inhibit the growth of 90% of organisms

[1] Numbers in Table II if the source are 1000-fold higher, which must be a mistake in view of its Table VI and common sense.

Reported MICs of natamycin for bacteria that include *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus haemolyticus*, *Bacillus cereus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *Proteus mirabilis* and *Pseudomonas aeruginosa* are higher than 250 µg/mL (EFSA, 2009). The high MIC values for bacteria are expected since the bacterial cell walls and cell membranes do not contain sterols. Streekstra et al. (2016) evaluated the adaptation of a selection of 20 fungal strains to higher concentrations of natamycin. The fungal species were selected for their association with food and crop spoilage or for their medical relevance, and comprised various taxonomic groups in the Ascomycota and Zygomycota. The MICs of natamycin ranged from 1.8 to 19.8 µmol/L in the fungal species (Table 12). These differences may be caused by various factors, including the composition of the cell wall (permeability), the plasma membrane (sterol type and multidrug-resistant proteins) (Stern, 1978; Rotowa, Shadomy & Shadomy, 1990; Chamilos & Kontoyiannis, 2005), or the presence of protective proteins (Blum et al. 2013). The range of MICs before (1.8–19.2 µmol/L) and after (1.8–19.8 µmol/L) training, that is, exposure to sublethal concentrations of natamycin for a prolonged time, did not change the MICs for most of the fungal species surveyed. The average MIC increased from 6.1 to 8.6 µmol/L, whereas the median MIC value of the untrained strains increased from 4.2 to 7.8 µmol/L by the training procedure.

Table 12. Influence of training on the MIC

Fungal species		MIC (µm) before and after training procedure		
		Agar surface	Microtiter assay	
		Trained	Control	Trained
1	*Saccharomyces cerevisiae*	1.8	1.8	1.8
2	*Candida parapsilosis*	5.4	3.0	4.8
3	*Candida albicans*	3.6	3.6	4.2
4	*Candida krusei*	2.2	1.8	2.4
5	*Rhodotorula mucilaginosa*	2.5	3.0	3.0
6	*Trichosporon asahii*	8.1	4.2	10.8
7	*Geotrichum candidum*	6.3	6.6	10.8
8	*Fusarium solani*	15.6	9.6	8.4
9	*Aspergillus terreus*	18	19.2	19.8
10	*Aspergillus terreus*	14.3	16.8	19.2

Fungal species		MIC (µm) before and after training procedure		
		Agar surface	Microtiter assay	
		Trained	Control	Trained
11	*Aspergillus fumigatus*	5.3	5.4	5.4
12	*Cladophialophora potulentorum*	2.1	3.0	3.0
13	*Neosartorya spinosa*	3.6	4.8	4.8
14	*Penicillium discolor*	2.2	3.6	6.0
15	*Mucor plumbeus*	6.0	6.0	8.4
16	*Aspergillus ochraceus*	8.1	4.2	12.0
17	*Verticillium fungicola* [a]	8.4	–	–
18	*Colletotrichum musae*	6.0	2.4	7.8
19	*Fusarium oxysporum*	14.9	7.2	16.2
20	*Trichoderma aggressivum*	7.5	9.6	13.8

MIC_{avg}: minimum inhibitory concentration

Source: Streekstra et al. (2016)

4.4 Antimicrobial resistance mechanisms and genetics

Resistance against polyenes used in food (e.g. natamycin) and clinical applications (e.g. amphotericin B) is rare in fungi because of the mode of action of these antimicrobial agents (Khoudokormoff, 1984; Sorensen, McNall & Sternberg, 1958–1959). Delvocid, a 50% natamycin preparation, has been used for more than 20 years for preserving cheese and sausages (Jay, 1996). Surveys in cheese warehouses and in dry sausage factories where Delvocid had been used for up to 10 years showed no change in the composition or the sensitivity of the contaminating fungal microbiota (de Boer et al., 1979; Hoekstra & Van der Horst, 1998).

De Boer & Stolk-Horsthuis (1977) reported on laboratory experiments intended to induce tolerance to natamycin in strains isolated from cheese warehouses. The results of the study indicated that, after approximately 30 transfers to media containing concentrations equal to or greater than the MIC of natamycin, eight out of 26 strains had become less sensitive to natamycin.

An updated literature search also supports the earlier findings that the daily use of natamycin in the cheese and sausage industries over many years does not alter the sensitivity of the contaminating fungi. Moreover, in contrast with the main polyenes used therapeutically in clinical applications, for example, amphotericin B, the fungistatic and fungicidal minimal concentrations of natamycin differ only slightly (Table 11), which further reduces the opportunity for establishment of resistance. Induction of polyene- and especially natamycin-resistant *Candida* species mutants is difficult (Athar & Winner, 1971). Such mutants invariably show reduced metabolic and growth rates in vitro, and in the absence of polyenes, they readily revert to normal metabolism, growth and sensitivity to natamycin. One way of obtaining such resistant isolates is by successive subculturing in vitro in media with the presence of gradually increasing concentrations of a polyene. Typically, such isolates are resistant up to the highest concentration to which they are exposed, and these laboratory-based in vitro culture conditions are not likely to result from the technical practical application of natamycin as a food preservative.

The antifungal action of polyene antimycotics is based on their binding to sterols in the cytoplasmic membrane of the fungal cell wall, which distends the wall (Gray et al., 2012). The sensitivity of fungal cells to polyenes depends on the characteristics of the sterols (Littman, Pisano & Lancaster, 1958; Molzahn & Woods, 1972; Subden et al., 1977). *Candida* strains resistant to nystatin contain more ergosterol than sensitive ones (Athar & Winner, 1971; Safe et al., 1977). Sensitivity to polyene antimycotics is a consistent feature of wild-type fungal strains. Prolonged therapy with an antimycotics results in increased resistance to it. Induced resistance to polyene antimycotics has been observed in *Candida, Torulopsis* and *Cryptococcus* strains (Macura, 1991).

Natamycin has been given orally for the treatment of intestinal candidosis at a daily dose of up to 400 mg. It is highly active against yeast-like fungi (MIC, 1.5 µg/mL) but less effective against

dermatophytes (MIC, 3.0–100 µg/mL). Strains resistant to natamycin are rare, but the effectiveness of this drug in the treatment of vaginal candidosis has decreased (Lovgren & Salmela, 1978). The MIC values were 2.9–31 µg/mL for strains isolated from untreated women but 9.8–64 µg/mL for strains from women who had been previously treated with natamycin.

There is concern that the use of natamycin as a food preservative could compromise the clinical efficacy of polyene antifungal drugs (Dalhoff & Levy, 2015). The effect of natamycin on resistance development in the fungal microbiota in the intestinal tract is not known. Still, the potential for cross-resistance is possible since binding to ergosterol is the primary and necessary event in the working mechanism of all polyene antifungals (Gray et al., 2012) and the primary mechanism for lowering the sensitivity towards polyene antimycotics is believed to be lowering the ergosterol content of the membrane (Morace, Perdoni & Borghi, 2014). In spite of the potential for cross-resistance, only one article clearly shows that laboratory-induced resistance towards polyenes (amphotericin B and nystatin in *C. albicans*) can lead to cross-resistance towards other polyenes such as natamycin (Athar & Winner, 1971).

Although there is a potential risk of development of resistance in fungal microbiota as a consequence of prolonged, repeated application of natamycin in the food industry, published studies on the mechanism of action of natamycin and its susceptibility to fungi, historical use of natamycin as a food preservative with a minimal or no increase in the selection of resistant fungi and small number of intestinal fungi compared to bacteria indicate that colonization barrier disruption and the level of resistance to intestinal fungal microbiota would be low and not likely in the gastrointestinal tract. Furthermore, bioautographic studies of rats orally dosed with radiolabelled natamycin indicate that natamycin is poorly absorbed, with most of the administered dose eliminated in the faeces, and that no antifungal activity was found in the colon (Blankwater & Hespe, 1979). Although no direct evidence was provided by the authors, they indicate that the degradation of natamycin to biologically inactive compounds was due to the bacterial microbiota in the colon and caecum.

Comments

Biochemical aspects

The absorption, excretion, tissue residues and metabolism of ^{14}C-labelled natamycin were investigated in rats and, to some extent, in dogs. The radioactivity in oral doses of 0.1 to 50 mg/kg bw administered to rats was readily excreted, with more than 95% of the dose eliminated in the faeces within 72 hours. Urinary elimination of administered radioactivity amounted to below 2%. Radioactivity eliminated as ^{14}C-labelled carbon dioxide was low. Sex differences were not investigated. Upon intraperitoneal administration, amounts excreted via faeces were seven-fold those excreted via urine. Based on comparisons of urinary amounts after oral and intraperitoneal administration, oral bioavailability was approximately 13% or less. In dogs, excretion via urine amounted to less than 4%, but based on the results of intravenous administration in dogs, amounts excreted in the bile may be assumed to be similar to that via urine (Hespe & Meier, 1980). Total radioactivity in major organs and tissues were generally low (Anonymous, 1979; Blankwater & Hespe, 1979).

Identities of natamycin-related metabolites were not investigated.

Livers of rats treated with oral doses of natamycin at 0.3, 1, 3 and 10 mg/kg bw per day for six days were isolated and the microsomes prepared and used to determine the activities of several cytochrome P450 enzymes (CYP). The lowest dose of natamycin (0.3 mg/kg bw per day for six days) had no significant effects on the activities of the hepatic cytochrome enzymes studied. For the 1, 3 and 10 mg/kg bw doses, significant dose-dependent decreases were observed in the total hepatic cytochrome content and in the activities of aniline hydroxylase (associated with CYP2E1), aminopyrine *N*-demethylase, MROD (associated with CYP1A2), EROD (associated with CYP1A1), and PROD (associated with CYP2B1/2) enzymes. Natamycin also produced a significant decrease in the 12- and 11-hydroxylation of lauric acid (associated with CYP4A subfamily) (Martinez et al., 2013).

Toxicological data

Acute toxicity of natamycin was determined after oral (rat LD_{50} > 2000 mg/kg bw), dermal (rat LD_{50} > 5050 mg/kg bw) and inhalation (rat LC_{50} > 2.39 mg/L) exposure (Crutchfield, 2008; Kuhn 2008a,b). Natamycin induced slight and transient irritation in skin and eyes (Kuhn, 2008c,d). In a mouse local lymph node assay (LLNA), no induction of skin sensitization was seen (Kuhn, 2008e).

Short-term toxicity studies with oral administration were conducted in the rat and dog. Mainly body weights and feed intakes were affected in rats and dogs; diarrhoea was also observed in dogs.

In a 13-week dietary toxicity study, groups of male and female rats received diets containing natamycin at concentrations of 0, 125, 500 or 2000 ppm (equal to 0, 11, 42 and 204 mg/kg bw per day for males and 0, 12, 48 and 238 mg/kg bw per day for females, respectively; when considering feed scatter, the substance intakes were 10–30% lower). The NOAEL was 500 ppm (equal to 42 mg/kg bw per day) based on reduced body weights and clinical chemistry findings such as changes in alanine aminotransferase, urea, inorganic phosphorous, cholesterol and total protein at 2000 ppm (equal to 204 mg/kg bw per day) (Otterdijk, 2003).

In a 90-day dietary toxicity study, groups of two male and two female dogs received diets containing natamycin at concentrations of 0, 375 or 750 ppm (equivalent to 0, 12 and 25 mg/kg bw per day). The NOAEL was 375 ppm (equivalent to 12 mg/kg bw per day) based on lower body weight gains and increased number of days with diarrhoea at 25 mg/kg bw per day (Van Eeken et al., 1984).

In a two-year dietary toxicity study, groups of three male and three female dogs received diets containing natamycin at concentrations of 0, 125, 250 or 500 ppm (equivalent to 0, 3.1, 6.3 and 12.5 mg/kg bw per day). The NOAEL was 250 ppm (equivalent to 6.3 mg/kg bw per day) based on lower body weight gains in males at 12.5 mg/kg bw per day (Fogleman, 1963).

No reports on long-term toxicity or carcinogenicity studies in mice were available to the Meeting.

In a long-term toxicity and carcinogenicity study, groups of 35 male and 35 female rats received diets containing natamycin at concentrations of 0, 125, 250, 500 or 1000 ppm (equivalent to 0, 6, 12.5, 25 or 50 mg/kg bw per day) for up to two years. Body weights and feed consumptions were lower in the high-dose groups than in control groups. Due to the low number of animals per dose group and sex when compared with OECD TG 453 or 451, and the gross and microscopic examination of selected animals only, the results of this study do not contribute to the toxicological assessment of natamycin (Levinskas et al., 1963a).

Considering the limitations in the available database on carcinogenicity, no conclusions can be drawn on the carcinogenic potential of natamycin.

Natamycin was tested for genotoxicity in a range of assays, both in vitro and in vivo. Natamycin tested negative in an Ames test and in an in vitro chromosomal aberration study (Meerts, 2002; Verspeek, 2002), but increases in chromosomal aberrations and micronuclei were reported elsewhere (Rencuzogullari et al., 2009). It is noted that no report on in vitro mammalian cell gene mutation assay was available. A summary report on the assessment of the veterinary drug natamycin listed GLP-compliant studies on mutagenicity that included Ames test, mouse LLNA and chromosomal aberration assay in Chinese hamster ovary cells, which gave negative results (EMEA, 1998); however, the Meeting had no access to these studies. Follow-up in vivo studies on clastogenicity/aneugenicity, which gave both positive (Rasgele & Kaymak, 2013a,b) and negative results (Cox, Bailey & Morgareidge, 1973), had serious limitations.

The Meeting concluded that the available database on the in vivo and in vitro genotoxicity of natamycin was inadequate to draw a clear conclusion on genotoxicity.

In view of the limitations in the available database on carcinogenicity and genotoxicity, the Meeting determined that no conclusions can be drawn on the carcinogenic risk to humans from the diet.

Groups of male and female rats were fed diets containing natamycin at concentrations adjusted to maintain target dose levels of 0, 5, 15, 50 and 100 mg/kg bw per day (achieved dose levels were not reported) for three generations. The NOAEL for parental toxicity was 15 mg/kg bw per day based on

lower body weights at 50 mg/kg bw per day. The NOAEL for offspring toxicity was 5 mg/kg bw per day based on lower pup weights at 15 mg/kg bw per day. The NOAEL for reproductive toxicity was 50 mg/kg bw per day based on low pre- and postnatal survival at 100 mg/kg bw per day (Cox, Bailey & Morgareidge, 1973).

Groups of female rats from the second litters of the F_1 generation of a three-generation study of reproductive toxicity were reared to maturity on control diets and mated with untreated males. The F_{1b} females were given the same dose of natamycin as their parents (0, 5, 15, 50 or 100 mg/kg bw per day) by gastric intubation during gestation days 6–15; they were killed and examined on gestation day 20. The NOAELs for maternal toxicity and embryo/fetal toxicity were 100 mg/kg bw per day based on the absence of adverse effects up to the highest dose level tested.

In a developmental toxicity study, groups of female rabbits were treated with aqueous suspensions of natamycin at doses of 0, 5, 15 or 50 mg/kg bw per day via gavage during gestation days 6–18; they were killed and examined on gestation day 29. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on the absence of adverse effects up to the highest dose level tested. The NOAEL for embryo/fetal toxicity was 5 mg/kg bw per day based on the increased incidence of extra sternebrae and decreases in pup body weight of equivocal toxicological relevance at 15 mg/kg bw per day (Knickerbocker & Re, 1978, 1979).

The Meeting concluded that the available studies with natamycin did not indicate teratogenic potential.

No reports from specific neurotoxicity studies were available to the Meeting. The submitted 90-day rat study (Otterdijk, 2003) did not indicate neurotoxic potential based on the absence of clinical signs.

The Meeting concluded that the limited available studies with natamycin did not show evidence of neurotoxicity.

No reports from specific immunotoxicity studies were available to the Meeting.

Microbial aspects

Data from in vitro MICs, susceptibility and selectivity of natamycin resistance were available.

The Meeting concluded that the amounts of natamycin, as residues, were unlikely to induce resistance in gastrointestinal microbiota or colonization barrier disruption.

The Meeting considered that the limited available database, which did not raise specific health concerns, needs to be balanced against the long history of use and the data necessary for a thorough assessment of pesticide residues. The Meeting considered the available data insufficiently robust for the purpose of establishing health-based guidance values because of the limitations of the present database on natamycin (design of the animal studies, limited number of animals, unclear genotoxicity results, lack of adequate carcinogenicity studies) and because of the inadequate descriptions in some of the studies. The Meeting was aware that further toxicological data on natamycin were available to other agencies.

The Meeting concluded that the available database on natamycin was inadequate to characterize the potential hazards to the general population, including fetuses, infants and children, from natamycin residues from its use as a pesticide.

Human data

No effects were reported on the health of workers involved in the manufacture or use of natamycin (J.S. Moore, Technology Sciences Group, personal communication, 2017). No information on accidental or intentional poisoning in humans were available.

No effects were reported in persons who received dermal or ocular natamycin treatment (Grupper, undated, 1967; Malten 1967, 1968). Nausea, vomiting and diarrhoea were described in patients treated with oral natamycin (Newcomer et al., 1960).

Toxicological evaluation

The Meeting did not establish an ADI or an acute reference dose (ARfD) due to the inadequate database available to the Meeting.

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

Information on the genotoxic potential, information on the carcinogenic potential, results from epidemiological, occupational health and other such observational studies of human exposure

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Indications of low exposure
Dermal absorption	No data
Distribution	No conclusive data
Potential for accumulation	No conclusive data
Rate and extent of excretion	No conclusive data, excretion mainly via faeces and in lower amounts via urine
Metabolism in animals	No data
Toxicologically significant compounds in animals and plants	No data
Acute toxicity	
Rat, LD_{50}, oral	> 2000 mg/kg bw
Rat, LD_{50}, dermal	> 5050 mg/kg bw
Rat, LC_{50}, inhalation	> 2.39 mg/L (4 h exposure, nose-only)
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Slightly irritating
Mouse, dermal sensitization	Non-sensitizing (LLNA)
Short-term studies of toxicity	
Target/critical effect	Low body weight gain, diarrhoea
Lowest relevant oral NOAEL	6.3 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	No data
Lowest relevant NOAEL	No data
Carcinogenicity	No data to conclude on the carcinogenic potential
Reproductive toxicity	
Target/critical effect	Low body weight gain in parental animals, low pre- and postnatal survival, low pup weight
Lowest relevant parental NOAEL	15 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	5 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	50 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Low pup weight, increased incidences of extra sternebrae
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rabbit)[a]
Lowest relevant embryo/fetal NOAEL	5 mg/kg bw per day (rabbit)

[a] Highest dose tested.

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Appendix 1: Literature search

The authors conducted a literature search in several databases (Table 1). Articles that were not obviously relevant for a toxicological or human health evaluation were excluded from the results list based on their titles and/or abstracts. For the remaining references, the full articles were retrieved and further assessed for relevance. Articles describing research in plants or animals / test organisms not typically used for human health risk assessment were excluded. Similarly, articles focusing mainly on the therapeutic use of natamycin were also excluded, particularly in the cases where no details on the examinations or findings relating to toxicological / adverse drug reactions were reported. Relevant articles were included in the evaluation and are described in the appropriate sections.

Table A.1. Details on literature search and its results (date: 03 April 2017)

Database	Search term	Number of hits
PubMed	User query: ((((((((((mouse) OR rat) OR guinea pig) OR rabbit) OR dog) OR woman) OR man) OR (urine OR urinary)) OR plasma) OR blood) OR (worker OR workers) OR patient) AND (((natamycin) OR pimaricin) OR delvocid)[all]) Query translation by search engine: ((((((((((“mice”[MeSH Terms] OR “mice”[All Fields] OR “mouse”[All Fields]) OR (“rats”[MeSH Terms] OR “rats”[All Fields] OR “rat”[All Fields])) OR (“guinea pigs”[MeSH Terms] OR (“guinea”[All Fields] AND “pigs”[All Fields]) OR “guinea pigs”[All Fields] OR (“guinea”[All Fields] AND “pig”[All Fields]) OR “guinea pig”[All Fields])) OR (“rabbits”[MeSH Terms] OR “rabbits”[All Fields] OR “rabbit”[All Fields])) OR (“dogs”[MeSH Terms] OR “dogs”[All Fields] OR “dog”[All Fields])) OR (“women”[MeSH Terms] OR “women”[All Fields] OR “woman”[All Fields])) OR (“men”[MeSH Terms] OR “men”[All Fields] OR “man”[All Fields])) OR ((“urine”[Subheading] OR “urine”[All Fields] OR “urine”[MeSH Terms]) OR (“urinary tract”[MeSH Terms] OR (“urinary”[All Fields] AND “tract”[All Fields]) OR “urinary tract”[All Fields] OR “urinary”[All Fields]))) OR (“plasma”[MeSH Terms] OR “plasma”[All Fields])) OR (“blood”[Subheading] OR “blood”[All Fields] OR “blood”[MeSH Terms])) OR (worker[All Fields] OR (“manpower”[Subheading] OR “manpower”[All Fields] OR “workers”[All Fields])) OR (“patients”[MeSH Terms] OR “patients”[All Fields] OR “patient”[All Fields])) AND ((“natamycin”[MeSH Terms] OR “natamycin”[All Fields]) OR (“natamycin”[MeSH Terms] OR “natamycin”[All Fields] OR “pimaricin”[All Fields]))	367
Web of science	*Query #1:* TS=(natamycin OR pimaricin OR delvocid) *Query #2:* TS=(rat) or TS=(mouse) or ts=(dog) or ts=(rabbit) or ts=(guinea pig) or ts=(man) or ts=(woman) or ts=(urine or urinary) or ts=(plasma) or ts=(blood) or ts=(worker or workers) or ts=(patient) *Query #3:* #2 AND #1 Indexes=SCI-EXPANDED, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED, IC Timespan=All years	Query #1: 726 Query #2: 9 985 321 Query #3: 216
	Total number of hits after removal of duplicates:	415
	Hits for which the full articles were retrieved:	47
	Hits with retrieved full articles excluded after detailed assessment:	16
	Number of references included in the evaluation:	31

OXAMYL

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Explanation

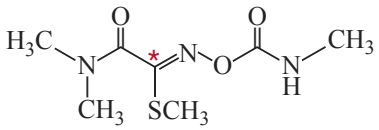
Oxamyl is the (ISO)-approved common name for *N*,*N*-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio)acetamide (International Union of Pure and Applied Chemistry [IUPAC] name), with the Chemical Abstracts Service number 23135-22-0. Oxamyl (Fig. 1) is a carbamate insecticide that acts by inhibiting acetylcholinesterase (AChE) activity. It was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1980, 1983, 1984 and 1985 (Annex 1, references 32, 40, 42 and 44). An acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) was established in 1984 on the basis of a no-observed-adverse-effect level (NOAEL) of 2.5 mg/kg body weight (bw) per day in a two-year feeding study in rats, supported by a NOAEL of 2.5 mg/kg bw per day in a two-year feeding study in dogs. In 2002, the previous ADI was withdrawn and an ADI of 0–0.009 mg/kg bw and an acute reference

dose (ARfD) of 0.009 mg/kg bw were established on the basis of the NOAEL of 0.09 mg/kg bw per day in a human volunteer study (Annex 1, reference 95).

Oxamyl was evaluated by the present Meeting within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). The present Meeting reviewed all previous studies, and new data have been included in the monograph.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with national or international test guidelines, unless otherwise specified. The study on human volunteers was conducted in accordance with the principles expressed in the Declaration of Helsinki or equivalent ethical standards.

Figure 1. Structure of oxamyl



* Denotes position of ^{14}C radiolabel

Evaluation for acceptable daily intake

Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Intraperitoneal route

Mouse

Swiss Webster mice (5 males/group) were treated by intraperitoneal injection with [^{14}C]oxamyl (purity: > 97%, position of radiolabel not indicated) in saline at a dose of 1.2 mg/kg bw. Urine and faeces samples were collected at 6, 12, 24, 48, 72 and 96 hours after dosing. Excretion was very rapid: urinary and faecal excretion were 73% and 3% of the administered dose by six hours; 84% and 5% by 24 hours and 89% and 8% after 96 hours, respectively. By 96 hours, the concentrations of radiolabel (as oxamyl equivalents) in tissues were reported to be low, from 11 ng/g in the testes to 37 ng/g in liver. Only a summary of the results of this study, as published in the open literature, was provided. The original data were not available (Chang & Knowles, 1979).

(b) Oral route

Rat

The kinetics and metabolism of oxamyl were studied in CD rats according to Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Guideline 85-1. In a pilot experiment, one male and one female rat received [1-^{14}C]-labelled oxamyl (purity: 98%) in 0.1 mol/L sodium acetate containing 1% carboxymethylcellulose, at a single oral dose of 1 mg/kg bw. Urine was collected at 0–6 and 6–24 hours and daily thereafter for up to 168 hours. Faeces was collected at daily intervals. Excretion of $^{14}CO_2$ in expired air was assessed over the 168 hours. The rats were killed, and the radioactivity in the carcass was measured. About 84% and 1% of the administered radioactivity was excreted within 24 hours in urine and faeces, respectively. After 168 hours, a total of 93% (male) and 95% (female) was excreted in urine, 2% in faeces and 0.4–0.5% in expired air; 4% of the administered radioactivity remained in the carcass.

In the main study, rats (5/sex) received [1-^{14}C]-oxamyl (purity: 96%) at a single oral dose of 1 mg/kg bw. Urine was collected at 0–6 and 6–24 hours and daily thereafter for up to 168 hours; faeces was collected at daily intervals. The concentration of radioactivity in expired air was not measured. At 168 hours, animals were killed, blood was sampled and heart, lungs, liver, kidneys, spleen, gastrointestinal tract, brain, ovaries/testes, skin, muscle, fat and bone were dissected. The concentration of radioactivity in blood, tissues and remaining carcass were measured. Approximately 80% of the administered radioactivity was excreted in urine and 1% in faeces within 24 hours. After 168 hours, total urinary excretion was 95% and faecal excretion was 3%. At scheduled kill, the highest concentrations of radioactivity were found in whole blood (0.08–0.1 µg/g, expressed as oxamyl equivalents). In heart, liver, kidneys, lungs, spleen and gastrointestinal tract, levels ranged from 0.04 to 0.09 µg/g. Levels in

other tissues were less than 0.03 µg/g. No marked differences in tissue levels between male and female rats were observed.

In a whole-body autoradiography experiment, one male and one female rat received a single oral dose of [^{14}C]oxamyl (purity: 96%) at a single dose of 1 mg/kg bw. After 168 hours the animals were killed and sagittal sections were cut at six levels. The autoradiograms of the male as well as the female rat showed highest concentrations of radioactivity in gastric mucosa, stomach content, kidneys, ureter, blood, hair follicles, liver and lungs. The authors considered that the radioactivity on fur was the result of urinary contamination, and the Meeting endorsed that view.

As part of the main study, male rats received a single oral dose of 2 (two animals) or 100 mg/kg bw (10 animals) of methyl 2-(dimethylamino)-*N*-hydroxy-2-oxo-[1-^{14}C] ethanimidothioate ([^{14}C]DMTO, IN-A2213 or oxamyl oxime; purity: 97%), one of the main metabolites of oxamyl. Urine was collected at 8, 24 and 48 hours and faeces at 24 and 48 hours. Within 48 hours, 100–102% of the dose was excreted in urine, with 93–100% within 24 hours; 2–3% was excreted in faeces (Hawkins et al., 1990).

1.2 Biotransformation

(a) In vitro

Mouse

Following incubation for two hours of [^{14}C]oxamyl with mouse liver homogenates or subcellular liver fractions, most of the radioactivity represented parent compound. Minor concentrations of DMTO, (dimethyamino)oxoacetic acid (DMOA or IN-D2708), dimethylcarbonocyanidic amide (DMCF or IN-N0079), *N*-methyloxamic acid (MOA) and methyl *N*′-methyl-*N*-[(methylcarbamoyl)oxy]-1-thiooxamimidate (DMO) were detected. Degradation of oxamyl was found in nuclear, mitochondrial and microsomal plus soluble liver fractions. No individual data were presented (Chang & Knowles, 1979).

Rat

Freshly prepared ChR-CD rat (sex not specified) liver microsomes were incubated with 0.3, 1 or 2 mg of [^{14}C]oxamyl or with 0.3, 1 or 5 mg of [^{14}C]-labelled DMCF, DMTO or its glucose conjugate metabolite A (metabolite IN-QKT34, or IN-A2213 glucoside, or DMTO glucoside; see Fig. 2), for two hours at 37 °C to determine the metabolic pathway of oxamyl in vitro. Aliquots of the incubations were extracted with ethyl acetate. The ethyl acetate and aqueous phase were radioassayed by liquid scintillation counting, and metabolites were identified by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography with mass spectrometry (GC-MS).

After incubation of 1 or 2 mg of oxamyl with rat liver microsomal fractions, the parent compound was the major fraction found (59–60%), followed by DMCF (17–20%), DMTO (10–13%) and DMOA (7%) (for identification of metabolites see Fig. 2). Small amounts of DMO (about 4%) and methyl *N*-hydroxy-*N*′-methyl-1-thiooxamimidate (MTO) (about 1%) were also found.

A control incubation, without microsomes, produced only DMTO, at levels comparable with those found after incubation in the presence of liver microsomes. This suggests that the hydrolysis is not mediated by liver enzymes. About 0.3 mg of [^{14}C]oxamyl was degraded to a slightly larger extent, producing the same metabolite pattern, but with a slightly larger fraction of DMCF. Incubation with 0.3–5 mg of DMCF resulted in the formation of DMOA (5–20%, depending on the amount incubated). DMTO was not metabolized in vitro, and metabolite A (DMTO glucoside) was metabolized only to a limited extent (< 10%). Data of the in vitro experiments together with those of the in vivo experiments (see section 1.2b) result in the metabolism scheme of Fig. 2 (Harvey & Han, 1978).

In a second study, rat liver homogenates in the presence of cofactor nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) were incubated for 2–2.5 hours at 37 °C with 0.5–1 mg of [^{14}C]oxamyl, to determine the metabolic pathway in vitro. The results obtained were similar to those of Harvey & Han (1978). Differences in the amount of the metabolites extracted were observed. However, no individual data were presented and no purity of oxamyl was provided. Based on the limitations of the study, results were considered to be only qualitatively useful (Belasco, 1979).

(b) In vivo

Mouse

Swiss Webster mice (5 males/group) received an intraperitoneal injection of [^{14}C]oxamyl (purity: > 97%, position of radiolabel not indicated) in saline at a dose of 1.2 mg/kg bw. Urine samples were collected at 6, 12, 24, 48, 72 and 96 hours after dosing. The urine samples were extracted with ethyl acetate to obtain an aqueous phase and compounds soluble in organic solvents. Following ethyl acetate extraction, most of the radiolabel remained in the aqueous phase (from 75% at 6 hours to 92% at 96 hours). The metabolites in the aqueous fraction were not identified, although about 7% of the radioactivity appeared to represent glucuronidated and sulfated metabolites. The concentration of radiolabel in the fraction soluble in organic solvents decreased from 25% at 6 hours to 8% at 96 hours. In this fraction, 16% of radioactivity was associated with the parent compound. Five metabolites were identified in this phase by TLC (for structures, see Fig. 2). The major metabolite was DMTO (44%), and others were DMOA, DMCF, DMO,MOA, with several unknowns (percentages not indicated) (Chang & Knowles, 1979).

Rat

Experiments were conducted in ChR-CD rats to determine the toxicokinetics of oxamyl in vivo (Table 1). The excretion of oxamyl was investigated in glass metabolism cages. Urine, faeces and exhaled air were analysed for radiolabel by liquid scintillation counting and metabolites were identified by TLC and GC-MS, with or without extraction with ethyl acetate or methanol (faeces were extracted with distilled water before analysis). At terminal kill, organs and tissues were isolated, freeze-dried and combusted before quantification of radioactivity. The tissue samples were also analysed by combined HPLC and GC-MS (with or without acid hydrolysis or enzymatic degradation).

The experiments showed similar kinetic patterns, regardless of which ^{14}C-labelled component was administered. Rats given diets containing 50 mg/kg bw of oxamyl, metabolite A or DMCF excreted about 60% of the administered radioactivity in urine and 5–6% in faeces, whereas the rat given the diet containing 150 mg/kg bw of oxamyl excreted 50% in urine and 20% in faeces; 70–80% was recovered as polar conjugates (not further identified) of the metabolites DMTO, DMO, DMOA and MOA.

Of the radioactivity in the urine of the rat treated with [^{14}C]metabolite A, 45% was accounted for by metabolite A itself and 19% appeared to be conjugates of metabolites DMTO and DMO, the remaining radioactivity was associated with high polar water-soluble conjugates, most of which were conjugates of metabolites DMOA and MOA.

Table 1. Design of studies of the metabolism of [^{14}C]oxamyl, [^{14}C]metabolite A and [^{14}C]DMCF in rats in vivo [a]

Pretreatment	Dose in mg/kg bw (purity, specific activity), vehicle, mode of administration
Unlabelled oxamyl at 50 mg/kg feed for 32 days	2.54 mg/kg bw of [^{14}C]oxamyl (purity not given, 0.138 MBq) in peanut oil, by gavage
Unlabelled oxamyl at 150 mg/kg feed for 18 days	4.57 mg/kg bw of [^{14}C]oxamyl (purity not given, 0.20 MBq) in peanut oil, by gavage
Unlabelled metabolite A at 1540 mg/L drinking-water for 8 days	2.42 mg/kg bw of [^{14}C]metabolite A (> 99% pure, 0.139 MBq) in water, by gavage
Unlabelled DMCF at 450 mg/kg feed for 7 days	3.89 mg/kg bw of [^{14}C]DMCF (> 99% pure, 0.395 MBq) in water, by gavage

bw: body weight; DMCF: dimethylcarbonocyanidic amide; metabolite A: metabolite IN-QKT34 (or IN-A2213 glucoside or DMTO glucoside)

Source: Harvey & Han (1978)

[a] One male rat per treatment (sex of that given 150 mg/kg feed not stated but presumed to be male); single radiolabelled dose; urine, exhaled air and faeces collected at 0–24, 24–48 and 48–72 hours; terminal kill at 72 hours.

In the urine of the [^{14}C]DMCF-treated rat, 15% of the radioactivity was attributed to conjugates of DMOA and 7% to MOA.

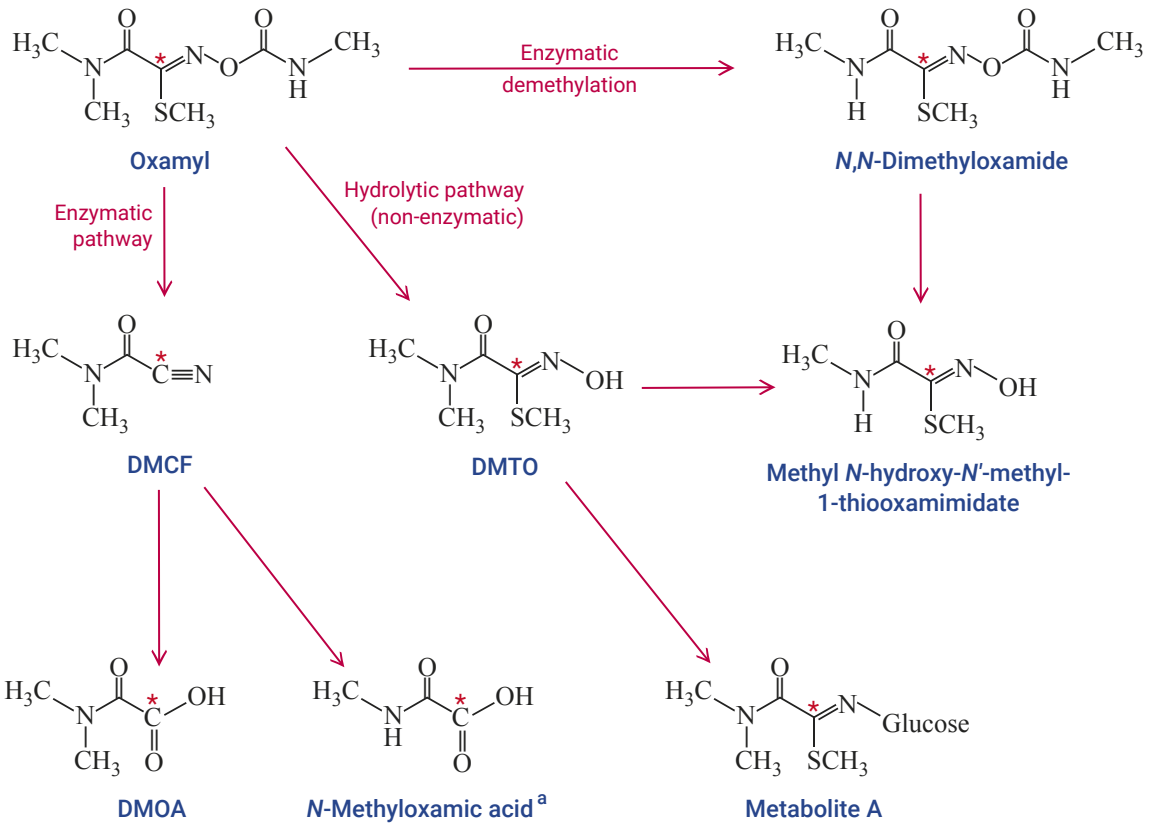
In the rats given oxamyl, about 20% of the administered radioactivity was recovered from the tissues, mainly in skin and hair, carcass, gastrointestinal tract, liver and blood. No non-polar metabolites of oxamyl or oxamyl itself, or conjugates thereof, were found in the tissues of oxamyl-treated rats. About half of the radioactivity found in the skin, hair, blood and liver was incorporated into amino acids (Harvey & Han,1978).

The purity of the non-labelled oxamyl, metabolite A and DMCF was not reported. Moreover, all results were obtained in single rats and none of the data were obtained in naive rats. Because of these limitations, results of the present study were considered to be only qualitatively useful.

CD rats (5/sex) received [1-^{14}C]oxamyl (purity: 96%) at a single oral dose of 1 mg/kg bw. Metabolites were analysed in urine collected over 0–24 and 24–48h. An additional 10 male rats received 100 mg/kg bw of DMTO (oxamyl oxime), one of the main metabolites of oxamyl. Metabolites in urine were identified by TLC, HPLC and mass spectrometry. The major urinary metabolite of oxamyl, representing about 35% of the administered dose in urine, was a glucuronide of DMTO. Oxamyl and DMTO were present at 9% and 16% of the administered dose, respectively. Three other metabolites in the urine, representing about 20% of the administered radioactivity could not be identified. After administration of DMTO, a similar metabolite profile in urine was observed. No marked sex differences were observed in the relative proportions of metabolites (Hawkins et al., 1990).

Studies of biotransformation in vitro and in vivo showed that oxamyl is metabolized in rats and mice by two major pathways, hydrolysis to the oxime (DMTO) and enzymatic conversion to DMOA. A proposed metabolic pathway is presented in Fig. 2.

Figure 2. Metabolic pathway of oxamyl in rats and mice



* Denotes position of ^{14}C label

DMCF: dimethylcarbonocyanidic amide; DMOA: *N*,*N*-dimethyamino(oxo)acetic acid;
DMTO: methyl 2-(dimethylamino)-*N*-hydroxy-2-oxo-ethanimidothioate;
Metabolite A, DMTO glucoside; MTO: methyl *N*-hydroxy-*N*′-methyl-1-thiooxamimidate

[a] In rats, found only in vivo.

Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The acute toxicity of oxamyl is summarized in Table 2.

Table 2. Summary of acute toxicity studies with oxamyl

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) / LC_{50} (mg/L)	Reference
Rat	ChR-CD	M + F	Oral	97.1	2.5	Hinckle (1980)
Rat	Crl:CD(SD)	F	Oral	98	2.5	Carpenter (2009)
Rat	Crl:CD	M + F	Inhalation (4 h) [a]	98	0.05–0.065	Kelly (2001)
Rat	ChR-CD	M	Inhalation (4 h) [b]	95	0.064	Tayfun (1969a)
Rat	ChR-CD	M + F	Inhalation (1 h) [b]	95	0.120	Tayfun (1969b)
Rabbit	NZW	M + F	Dermal	97.1	>2000	Brock (1988a)

bw: body weight; F: females; M: males; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; MMAD: mass median aerodynamic diameter

[a] MMAD 3.2–4.2 µm.

[b] MMAD 3.5 µm.

In a study performed according to Environmental Protection Agency (USEPA) Guideline 40 CFR163.81-1, oxamyl was administered by gavage to ChR-CD rats at doses of 1–5 mg/kg bw. Deaths occurred in males at 2.0 mg/kg bw and above and in females at 2.4 mg/kg bw. Signs of toxicity included tremors, fasciculations, exophthalmus, salivation, chromodacryorrhoea, piloerection, lachrymation and weight loss.

The oral median lethal dose (LD_{50}) for oxamyl was 2.5 mg/kg bw for female rats (Hinckle, 1980).

In an acute oral toxicity study, oxamyl (purity: 98%) was administered by gavage to two fasted Crl:CD(SD) female rats at a dose of 1 mg/kg bw, to three fasted female rats at a dose of 2.5 mg/kg bw, and to two fasted female rats at a dose of 6.3 mg/kg bw. The animals were dosed one at a time at a minimum of 48-hour intervals. Animals were observed for clinical signs of toxicity, body weight effects and mortality for up to 14 days after dosing. All animals were examined grossly for evidence of organ or tissue damage. USEPA acute toxicity software (AOT425StatPgm) was used to determine the dose progression and to estimate the LD_{50}. Death occurred in 1/3 rats dosed at 2.5 mg/kg and in 2/2 rats dosed at 6.3 mg/kg. Clinical signs of toxicity were observed in all rats and included ataxia, clear oral discharge, red nasal discharge, high or low posture, tremors, clear ocular discharge, mydriasis, salivation, stained skin/fur, wet fur, hyperreactivity, absent faeces or prostrate posture. With the exception of stained skin/fur, no clinical signs were observed after test day 5. No body weight losses occurred in surviving rats. No gross lesions were present in the rats at necropsy.

The oral LD_{50} for oxamyl was 2.5 mg/kg bw for female rats (Carpenter, 2009).

In a study performed according to USEPA Office of Prevention, Pesticides & Toxic Substances (OPPTS) Guideline 870.1300 and Organisation for Economic Co-operation and Development guideline (OECD TG) 403, Crl:CD rats (5/group) were exposed nose-only for four hours to oxamyl at nominal concentrations of 0.05–0.12 mg/L. Deaths occurred at all concentrations. Clinical signs during exposure were a diminished response to an alerting stimulus, red nasal discharge, gasping and salivation. During the first week after exposure, lethargy, decreased muscle tone, tremors, spasms, fasciculations, abnormal posture and gait, abnormal hindlimb gait, high carriage and ataxia, and (reversible) weight loss were observed (Kelly, 2001).

Rats (6 males/dose) were exposed head-only to oxamyl for four hours at actual concentrations of 0.02–0.09 mg/L. Deaths occurred at concentration of 0.053 mg/L and higher. Clinical signs during exposure were intensive salivation, facial fasciculations, exophthalmos, lachrymation, red discharge from the nose and difficulty in breathing. Post-exposure minor weight loss and occasionally dilated pupils were observed (Tayfun, 1969a).

Rats (6/group) were exposed for one-hour head-only to oxamyl at actual concentrations of 0.1–0.21 mg/L. Deaths occurred in males at concentrations greater than 0.16 mg/L; females died at all doses. Clinical signs during exposure were facial fasciculations, exophthalmos, lachrymation, red discharge around the nose and eyes, salivation and gasping. Pallor and severe weight loss were observed on the first day post exposure (Tayfun, 1969b).

In a study performed according to USEPA Guideline 81-2, oxamyl (purity: 98%) was applied at 2000, 3500 or 5000 mg/kg bw to the skin of rabbits. The application site was occluded for 24 hours. Two of five males at 3055 mg/kg bw, two of five males at 5000 mg/kg bw, one of five females at 2000 mg/kg bw and one of five females at 3500 mg/kg bw died. The clinical signs were slight (reversible) weight loss, erythema and oedema. Gross pathological findings in dead animals were consistent with inhibition of AChE (Brock, 1988a).

(b) Dermal irritation

In a study of primary dermal irritation performed according to OPPTS Guideline 870.2500 and OECD TG 404, the shaved skin of New Zealand White rabbits (6 males) was exposed to 0.5 g of technical oxamyl (purity: 98%), moistened with water and kept under semi-occlusion for four hours. At 1, 24, 48 and 72 hours after the four-hour exposure period, the skin was scored for dermal effects according to the Draize scheme. Erythema of score 1 was observed in one rabbit at 1 and 24 hours after exposure. No clinical signs were observed in any of the rabbits.

Oxamyl was considered not irritating to the skin (Ladics, 2001a).

(c) Ocular irritation

In a study of primary ocular irritation performed according to OPPTS Guideline 870.2400 and OECD TG 405, adult HM:(NZW)fBR New Zealand White rabbits (6 males) received an instillation of 24 mg technical oxamyl (purity: 98%) into the conjunctival sac of the right eye. The eyes were examined at 1, 24, 48 and 72 hours and scored for irritation according to the Draize method. Slight irritation (score 1 for iritis in five animals, score 1 or 2 for conjunctival redness in four animals and score 1, 2 or 3 for discharge) was observed on the day of treatment. In all treated eyes, the pupil was constricted and did not react to light. Other observed clinical signs on the day of treatment were shivering, salivation, effects on balance, rapid and irregular breathing and lung noise. After 24 hours, the treated eyes of 5/6 animals were normal. At 48 hours the eyes of all animals were normal. The compound is not considered irritating to the eyes (Ladics, 2001b).

(d) Dermal sensitization

When oxamyl (purity: 96.9%) was administered according to the recommended test procedures by the Magnusson-Kligman maximization and Buehler methods, all guinea pigs died following intradermal injection. Following topical treatment, animals either died or showed significant clinical signs of AChE inhibition. The study was discontinued due to animal welfare concerns. The animals survived only when applied at half the maximum dose rate (0.5 mL of a 50% dilution in water) according to the Buehler method, although clinical signs of AChE inhibition were still observed (Ladics, 1999).

(e) Phototoxicity

In a phototoxicity assay performed according to the OECD TG 432, Balb/c 3T3 cells cultured in 96 well plates were incubated with oxamyl (purity: 98%) at concentrations of 1000, 316.5, 100, 31.6, 10, 3.16, 1.0 and 0.316 µg/mL in three experiments, in replicates of six. The test substance was soluble at 1000 µg/mL,

the maximum concentration specified by the test guideline. The positive control, chlorpromazine, was tested at eight concentration levels, in the presence and absence of ultraviolet (UV) A irradiation. After approximately 22 hours in culture, cells were analysed for cytotoxicity by neutral red uptake.

Under the conditions of this study, no phototoxic effect was observed with oxamyl following UVA irradiation, as determined by the mean photo effect. Photo irritation factor values in two of the three assay runs (runs 2 and 3) were between 2 and 5; however, compounds in this range generally do not warrant further photosafety evaluations. No inherent toxicity or phototoxicity to Balb/c 3T3 cells was observed at any concentration of oxamyl in this assay.

Oxamyl was not phototoxic following UVA irradiation when tested at concentrations up to 1000 µg/mL (Markell, 2015).

2.2 Short-term studies of toxicity

(a) Oral administration

Rat

In a limited 10-day oral toxicity study, young adult ChR-CD rats (six males) received oxamyl in 1% acetone and 99% peanut oil by gavage at a dose of 2.4 mg/kg bw for 5 days/week for two weeks. Three rats were killed four hours after the last dose, and the remaining three after a 14-day recovery period. Oxamyl caused weight loss during the first two days of treatment in the first week and on day 1 of the second week. Clinical signs were fasciculations, slight pallor and salivation. A mild inflammation of the stomach was observed in all animals at both kill times. Stomach inflammation was also observed in control animals, but this was less severe (Fretz & Sherman, 1968). The report provided only a summary of the observed effects. No group or individual data were provided.

Sprague Dawley rats (16/sex per group) received diets containing oxamyl (purity unknown) at a concentration of 0, 50, 100 or 150 ppm (equal to 0, 3.9, 8.4 and 14.6 mg/kg bw per day for males and 0, 4.3, 9.2 and 14 mg/kg bw per day for females, respectively) for 91–95 days. Rats at 150 ppm initially received a diet containing 500 ppm oxamyl for four days followed by 3 days of control diet. Blood samples were taken prior to the start of the study, after 1 and 2 months of treatment and at study termination for examination of a limited number of haematological and blood chemistry parameters and urine analysis. After 91–95 days of feeding, 10 animals/sex per dose were killed for pathological examination.

The animals receiving feed containing 500 ppm oxamyl displayed fasciculation after two days; by day 4, fasciculation, ruffled fur, mild diarrhoea, bulging eyes, lachrymation, spillage and weight loss were observed, and the dose was lowered to 150 ppm. One male rat of the 500 ppm group was found dead on day 5. Rats at 100 and 150 ppm had reduced body weight and weight gain. Feed consumption was increased at 50 ppm and decreased at 150 ppm. Analysis of urine at 150 ppm showed increased proteinuria and occult blood. Females at 100 ppm had decreased absolute kidney, liver and lung weights, and males at 100 and 150 ppm had decreased absolute weight of kidney, heart, thymus and spleen. Males and females at 150 ppm had decreased liver weight and increased stomach weight. No toxicologically relevant effects were observed at 50 ppm (Snee, 1969).

Because of the omission of a number of results in the report, for example, relative organ weights, blood chemistry and histopathological data (including AChE activity), a NOAEL could not be identified.

Dog

Beagle dogs (4/sex per group) were fed diets containing oxamyl (purity unknown, assumed 100%) at levels of 50, 100 and 150 ppm for 13 weeks (equal to 1.5, 2.8 and 5.0 mg/kg bw per day for males and 1.3, 2.6 and 4.2 mg/kg bw per day for females, respectively). Clinical observations were performed daily; body weight and feed consumption were measured weekly; haematology, clinical chemistry and urine analysis were performed at study initiation and at weeks 4 and 13. Cholinesterase (ChE) activity

was not measured. The animals were killed and macroscopically and histopathologically examined. No treatment-related effects were observed on any of the parameters investigated.

The NOAEL was 150 ppm (equal to 4.2 mg/kg bw per day), the highest dose tested (Holsing, 1969).

Beagle dogs (4/sex per group) received diets containing oxamyl (purity: 95%) at a concentration of 0, 50, 100 or 150 ppm (equal to 0, 1.3, 2.8 and 4.3 mg/kg bw per day for males and 0, 1.2, 3.0 and 4.2 mg/kg bw per day for females, respectively) for two years. Body weight and feed consumption were measured weekly; clinical signs were assessed daily; and haematology, clinical chemistry and urine analysis were performed pretest and at 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 months after treatment initiation. AChE and aliesterase activities in whole blood were assessed pretest and at 1, 6, 12 and 24 months after treatment initiation. After one year of treatment, 1 dog/sex in the control group and the 150 ppm group were killed for histopathological examination. The remaining animals were killed after two years. All animals were grossly examined. Microscopic examination of a complete range of organs and tissues was performed on animals of the control and 150 ppm groups. The liver, kidneys and testes of animals at 50 and 100 ppm were also examined microscopically.

No treatment-related effects on body weights, feed consumption, clinical signs or AChE and aliesterase activity were observed. The time between feeding and blood sampling for determination of ChE and aliesterase activities was not indicated. Throughout the treatment period, haemoglobin levels, haematocrit and red blood cell (RBC) count were reduced at most time points in animals at 150 ppm. Increases in cholesterol and in alkaline phosphatase activity were found in the 150 ppm group, particularly in males. Increases in cholesterol were found in the 50 and 100 ppm groups. Occasional differences in other haematological and biochemical parameters were observed. Group differences in pretest values appeared to exist for some haematological and urine chemistry parameters. However, since no statistical analyses of the data were performed, the significance of the differences before and during treatment with oxamyl cannot be established. Data of control and 150 ppm groups after one year of treatment are based on only 3 animals/group (Sherman, 1972).

Although the 1984 JMPR based the ADI on this study, the 2002 Meeting concluded that a NOAEL could not be identified due to the shortcomings in this study (Annex 1, reference 95).

In a study performed according to USEPA Guideline 83-1, beagle dogs (5/sex per group) received diets containing oxamyl (purity: 97.1%) at a concentration of 0, 50, 150 or 250 ppm (equal to 1.6, 4.6 and 8.0 mg/kg bw per day for males and 0, 1.5, 4.5 and 7.8 mg/kg bw per day for females, respectively) for 382–386 days. The doses were chosen on the basis of the results of two range-finding studies. Blood was sampled for haematological and blood chemistry examinations pretest and at treatment days 81, 181, 264 and 356. To determine ChE activity, blood was sampled three hours post feeding at days 7, 34, 88, 187, 270 and 363. The dogs were killed at days 382–386.

One female at 250 ppm died on test day 348; the cause(s) of death could not be established. Body weight, weight gain and feed efficiency were dose-dependently decreased in males in all treatment groups and in females at 150 and 250 ppm. Feed consumption was decreased in both sexes at 150 and 250 ppm. No effects on haematology and blood chemistry were observed. Increases in relative brain and kidney weights were found in males at 250 ppm. In both sexes at 150 and 250 ppm, tremors, salivation and increased incidences of vomiting, diarrhoea and mucoidal stool were observed. Tremors were also observed in females at 50 ppm. The effects on AChE activity are summarized in Table 3.

Erythrocyte AChE activity did not correlate well with the plasma AChE activity. A statistically significant decrease in erythrocyte AChE activity in males at 250 ppm was found only at six months of treatment. The authors considered that all the decreases in AChE activity in the brain in males and in the caudate nucleus in females at 150 and 250 ppm were biologically significant.

The NOAEL could not be identified because of the occurrence of tremors in females at 50 ppm and decreased body weights and decreased AChE activity in brain of males at 50 ppm. The lowest-observed-adverse-effect level (LOAEL) was 50 ppm (equal to 1.5 mg/kg bw per day) (Mebus, 1990).

Table 3. Effects of oxamyl treatment on AChE activity in a one-year dog study

	% change in acetylcholinesterase (AChE) activity per dose level					
	50 ppm		150 ppm		250 ppm	
Tissue / organ	M	F	M	F	M	F
Plasma (terminal)	−32*	−8	−48*	−37	−58*	−45*
Erythrocyte (terminal)	4	22	3	1	−6	2
Brain						
Caudate nucleus	−31*	0	−47*	−24	−30*	−30
Cerebellum/medulla	−17	3	−38*	−22	−28	−14
Cerebrum	−23	−10	−46	−16	−34	−15

ppm: parts per million; *: significantly different from control at 5% (Dunnett test) *Source*: Mebus (1990)

In a study performed according to USEPA Guideline 83-1, beagle dogs (5 males/group) received diets containing oxamyl (purity: 97.1%) at a dose level of 0, 12.5, 20, 35 or 50 ppm (equal to 0, 0.37, 0.58, 0.93 and 1.36 mg/kg bw per day) for 52 weeks. The stated purpose of the study was to determine the NOAEL for inhibition of AChE activity. Animals were observed twice daily for mortality, moribundity and clinical signs. Body weights and feed consumption were measured weekly for the first 15 weeks and every fourth week thereafter. Exophthalmic examination was performed before the start of the treatment and at weeks 26, 39 and 53. Blood samples for haematological and blood chemistry examinations, including erythrocyte and plasma AChE activities, were taken pretest, at treatment weeks 13, 26 and 39 and at scheduled kill. At scheduled kill, organs were weighed and examined macroscopically and brain tissue was dissected to determine AChE activity.

No deaths occurred and there were no treatment-related effects on body weight, feed consumption; clinical signs; haematological, blood chemistry or ophthalmic end-points; organ weights and macroscopic appearance; or AChE activity in brain (caudate nucleus, cerebellum/medulla, cerebrum) or plasma.

Based on the lack of effects, the study author established a NOAEL of 50 ppm (equal to 1.36 mg/kg bw per day) (Dickrell, 1991).

At the highest dose, erythrocyte AChE activity was decreased by 21% at two weeks before treatment, by 30% in week 13 and by 22% at week 53 of treatment. The toxicological relevance of this decrease is unclear, and it is difficult to determine whether 50 ppm is the NOAEL or LOAEL.

Combining the two one-year studies, the Meeting established an overall NOAEL in dogs of 35 ppm (equal to 0.93 mg/kg bw per day).

(b) Dermal application

Rabbit

In a study performed according to USEPA Guideline 82-2, New Zealand White rabbits (5/sex per group in the low- and mid-dose groups; 10/sex group in the control and high-dose groups) received daily dermal applications of 5 mL of oxamyl (purity: 97.2%) in distilled water at doses of 0, 2.5, 50 or 250 mg/kg bw per day to the clipped skin of the back; the test sites were kept under an occlusive wrapping for 22 days. (The doses were based on the results of a range-finding study.) The test substance was washed off the treated skin with warm water six hours after application, and the test site was observed for dermal irritation (scoring according to the Draize scheme). The animals were observed for clinical signs or signs of dermal toxicity each morning. After 22 days, rabbits (5/sex per group) were killed; the remaining control and high-dose rabbits were allowed a recovery period of 14 days before scheduled kill. Blood was sampled for haematology, clinical chemistry and AChE activity end-points pretest, one hour after the end of treatment on day 22, and on day 36 (control and high-dose group only). Brain AChE activity was determined at day 22 and 36.

Three high-dose bucks died during the study. The causes of death could not be established but were considered not treatment related. On day 22, a dose-related decrease in AChE activity was found in plasma, erythrocytes and brain at 50 and 250 mg/kg bw per day. There were no significant sex differences

in AChE activities. The decreases in AChE activity in males and females at 50 mg/kg bw were 51% and 45% in plasma, 18% and 15% in erythrocytes and 31% and 25% in brain, respectively; the decreases in AChE activity in males and females at 250 mg/kg bw per day were 73% and 75% in plasma, 36% and 30% in erythrocytes and 63% and 66% in brain, respectively. No significant decreases in AChE activity were observed at 250 mg/kg bw after the 14-day recovery period. An unidentified eosinophilic substance was found in the duodenal submucosa of five animals at 250 mg/kg bw. No treatment-related effects on body weight, feed intake and clinical signs were observed. Occasional statistically significant effects on dermal irritation, haematological parameters, clinical chemistry, organ weights, and macroscopic and microscopic examination parameters were considered not toxicologically relevant.

The NOAEL was 2.5 mg/kg bw per day based on the effects on brain AChE activity at 50 mg/kg bw (Brock, 1988b).

In a dermal toxicity study performed according to OECD TG 410 and USEPA Guideline 82-2, oxamyl (purity: 96.9%) in deionized water was applied to the shaved intact skin of HM:(NZW)fBR rabbits (6/group) under a semi-occlusive dressing for six hours/day for 21 consecutive days, at a dose of 0, 25, 40, 50 or 75 mg/kg bw per day. The animals were checked daily for clinical signs and skin irritation, twice per week for body weight and weekly for feed consumption. Blood was sampled pretest and one hour after the end of the last exposure period on day 21 to measure plasma and erythrocyte AChE activity. Animals were killed for gross pathological examination. Brains were collected for assessment of AChE activity.

No compound-related effects on body weight, weight gain, feed consumption and clinical signs were observed. Females at 75 mg/kg bw per day showed decreases in AChE activity by 11% in brain, 24% in erythrocytes and 29% in plasma. The decrease in AChE activity in erythrocytes did not reach statistical significance. In males, the decreases in AChE activity were less than 20% and did not reach statistical significance.

Based on the absence of clinical signs and statistically significant decreases in brain AChE activities, the NOAEL was 75 mg/kg bw per day, the highest dose tested (Malley, 1999).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a two-year study of toxicity and carcinogenicity, CD-1 mice (80/sex per group; aged 4.5 weeks) received diets containing oxamyl (purity: 97.1%) at a concentration of 0, 25, 50 or 75 ppm (equal to 0, 4.2, 8.7 and 13.5 mg/kg bw per day for males and 0, 5.2, 10.8 and 16.8 mg/kg bw per day for females, respectively). The high-dose animals received a diet containing oxamyl at 100 ppm for the first six weeks, but because 13 males and 11 females died during this period, the dose level was reduced to 75 ppm. Mice (eight males, eight females) from the same shipment group were added to the high-dose group to replace the dead animals, and to the mid-dose group (one male, four females) and low-dose group (one female) to replace the animals found dead. Animals were examined twice daily for mortality, signs of toxicity and behavioural changes, and palpated weekly for masses. Individual body weights and feed consumption were assessed weekly at weeks 1–29, every other week at weeks 29–53 and monthly thereafter. Blood for haematological analysis was sampled from the orbital sinus at weeks 4, 13, 26, 52, 78 and 104 from animals (10/sex per dose) randomly selected at each time. Surviving animals were killed by carbon dioxide asphyxiation and necropsied. Liver, kidney, testes, brain, brain stem and heart weights were weighed. Specimens of a full range of tissues were collected for microscopic examination. AChE activity was not examined.

During the first six weeks, mortality rates were high in males at 100 ppm and in females at 50 (9/80) and 100 ppm groups. After reduction of the highest dose to 75 ppm, long-term survival was not affected by treatment. Males at 50 ppm had reduced body weights throughout the study, and males at 75 ppm had reduced body weights during the first 28 weeks of the study and occasional decreases in body weights from week 29. On average, the body weight decreases in males at 50 and 75 ppm were approximately 4–6% compared to control. Males at 50 and 75 ppm also had consistently reduced feed consumption throughout the study. Those at 25 ppm showed sporadic reduction in feed consumption during the first 37 weeks of the study, but this was consistent for the remainder. Females at 50 and 75 ppm showed significant but sporadic decreases in body weight throughout the study; significant

reductions in feed consumption occurred sporadically at 25, 50 and 75 ppm throughout the study, with no clear dose-related pattern. No treatment-related clinical signs of toxicity were recorded.

There were no dose-related changes in haematological parameters, although significant decreases in erythrocyte count, haemoglobin concentration and erythrocyte volume fraction were observed at 4 weeks in males at the highest dietary concentration, which was 100 ppm at that time. These parameters were not similarly affected at other time points, when animals had lower dietary intakes. No unusual cell types were seen in peripheral blood. Other significant differences were observed sporadically, but as they were not dose dependent, they were considered not treatment related. At scheduled kill, absolute liver weight was decreased in males at 50 ppm and relative kidney weight was slightly increased in males at 75 ppm. No other effects on organ weights were observed.

Oxamyl was not carcinogenic in this mouse strain at the doses used in this study. The NOAEL was 25 ppm (equal to 5.2 mg/kg bw per day) based on early mortality in females at 50 ppm (equal to 10.8 mg/kg bw per day) (Adamik, 1981; Tompkins, 1990).

Rat

In a two-year dietary toxicity study, ChR-CD rats (36/sex per group) were given diets containing oxamyl at a concentration of 0 (two groups), 50, 100 or 150 ppm (equal to 0, 2.3, 4.9 and 7.4 mg/kg bw per day for males and 0, 2.8, 6.2 and 9.3 mg/kg bw per day for females, respectively). Individual body weights and feed consumption (on a per group and per sex basis) were measured weekly for the first seven months, biweekly from month 7 to 12, and monthly thereafter. Haematological and urinary end-points and alkaline phosphatase and alanine aminotransferase activities were measured in whole blood from animals (10/sex) at 100 and 150 ppm after 1, 3, 6, 9, 12, 18 and 24 months of feeding. Whole blood AChE activity was assessed at 4 and 8 days and at 1, 6, 12 and 24 months in animals (10/sex) at 0, 100 (1 and 6 months only) and 150 ppm. Whole blood aliesterase activity was measured in these animals at 1, 12 and 24 months. After one year (6 rats/sex per dose) and two years (all surviving animals) were killed for gross and microscopic examination. Histopathological data from the control and 150 ppm groups are presented below.

A dose-related decrease in body weight was observed in both sexes at 100 and 150 ppm; feed consumption in animals at 150 ppm was slightly reduced. No effect on feed efficiency was observed. Decreases in whole blood AChE activity were observed at 150 ppm shortly after treatment initiation, in females at day 4 (19% inhibition) and in males at day 8 (33% inhibition). Aliesterase activity was not affected. No treatment-related clinical signs, deaths or effects on haematological end-points, alkaline phosphatase or alanine aminotransferase activity and histological appearance were observed. Treatment-related changes (> 10%) in relative organ weights were observed in both sexes. After one year, increased relative weights of brain, heart, lungs and stomach were observed in female rats at all dietary concentrations; decreases in relative liver weights were observed in males at 100 and 150 ppm and in females at all concentrations. After two years, the relative weights of brain, lungs, testes and adrenals were increased in males at 150 ppm; in females, the relative weights of adrenals (150 ppm only, not dose-related) and brain, heart, lungs, kidneys and stomach (at all dietary concentrations) were dose-dependently increased. The relative spleen weights were increased in females at 50 and 100 ppm. The increases in relative organ weights were considered to be secondary to the effects of oxamyl on body weight. No increase in tumour incidence was observed in the oxamyl-treated groups. The results were not statistically analysed (Sherman, 1972).

Although the 1984 JMPR based the ADI on this study, the 2002 Meeting concluded that a NOAEL could not be identified due to the shortcomings in this study (Annex 1, reference 95).

In a two-year study of toxicity and carcinogenicity conducted according to USEPA Guideline 83-5, Sprague Dawley rats (62/sex per group) were given diets containing oxamyl (purity: 97.1%) at a concentration of 0, 25, 50, 100 or 150 ppm (equal to 0, 0.99, 2.0, 4.2 and 7.0 mg/kg bw per day for males and 0, 1.3, 2.7, 6.7 and 11 mg/kg bw per day for females, respectively). Urine and blood samples were collected from 7–10 rats/sex at 1 (analysis of plasma and erythrocyte AChE activity only), 3, 6, 12, 18 and 24 months. Except for the one-month measurement, the rats were fasted for at least 16 hours before the blood was sampled. Urine samples were collected during the fasting period. Ophthalmological examinations were carried out pretest and at 358 and 728 days of treatment. At day 378, 10 rats/sex per group underwent interim kill. The remaining rats were killed on

day 728 for a full pathological examination. Portions (not specified) of brains of 7–10 rats/sex per dose selected for haematological examination were used to determine brain AChE activity.

A dose-dependent reduction in mortality was observed. Body weight and weight gain were reduced in rats at 100 and 150 ppm (Table 4). In these groups, feed consumption was slightly decreased in males and slightly increased in females. These animals also showed increased incidences of hyperreactivity, alopecia, skin sores and swollen legs or paws. Slightly increased incidences of swollen legs or paws were also observed in males at 25 and 50 ppm. Males at 100 ppm had a higher incidence of masses. A treatment-related increase in percentage of rats with pale ocular fundi was observed, but in the absence of treatment-related microscopical ocular lesions related to the pale ocular fundi, this increase was considered not toxicologically relevant. Female rats at 150 ppm had an increased incidence in photoreceptor cell atrophy, which the authors considered to be secondary to the nutritional status of this group. Rats at 50, 100 and 150 ppm showed small decreases in erythrocyte count and increases in Na, K and Cl concentrations; however, since the effects were small and within the range of biological variation, they were considered not toxicologically relevant. Rats at 100 and 150 ppm had increased absolute brain weights; this was considered to be related to the brain compression induced by increased incidence of pituitary tumours in the control groups, a common finding in ageing rats. In the absence of treatment-related histopathological findings, other effects on absolute and relative organ weights were considered to be secondary to the effect of oxamyl on body weight. Males and females at 150 ppm had increased incidences of myeloid hyperplasia of bone marrow, and males showed extramedullary haematopoiesis in spleen. No treatment-related increase in the incidence of any tumour was observed. One month after treatment initiation, dose-related and statistically significant inhibition of plasma AChE activity was observed in all treatment groups. No other treatment-related effects on plasma, erythrocyte or brain AChe activities were found.

The study author considered the NOAEL to be 50 ppm, based on the decreased body weight and inhibition in plasma AChE at 100 ppm (Malley, 1991). The 2002 Meeting noted that clinical chemistry and determination of blood and brain AChE activities were carried out after a fast of at least 16 hours in only 7–10 rats/sex per dose, except at the one-month determination in plasma. As AChE activity probably recovered during the fast, no conclusions about the effect of oxamyl on this parameter could be drawn. The Meeting considered that this study could not be used to identify a NOAEL for inhibition of AChE activity. The Meeting identified a NOAEL of 50 ppm (equal to 2.0 mg/kg bw per day) based on effects on body weight, body weight gain and clinical signs at 100 ppm (equal to 4.2 mg/kg bw per day).

Table 4. Body weight and body weight gain changes in a 2-year toxicity and carcinogenicity study with oxamyl in rats

	Weight (g) per dose									
	Males					Females				
Parameter / day	**0 ppm**	**25 ppm**	**50 ppm**	**100 ppm**	**150 ppm**	**0 ppm**	**25 ppm**	**50 ppm**	**100 ppm**	**150 ppm**
Mean body weight										
Day 372	827	802 (−3)	833 (+1)	756* (−9)	653* (−21)	465	456 (−2)	448 (−4)	372* (−20)	342* (−26)
Day 680	792	793 (0)	882 (+11)	785 (−1)	686* (−13)	543	586 (+8)	556 (+2)	470* (−13)	385* (−29)
Day 722	760	743 (−2)	877 (+15)	765 (+1)	666 (−12)	555	606 (+9)	506 (−9)	471* (−15)	389* (−30)
Mean body weight gain										
Day 0–91	427	419 (−2)	412 (−4)	345* (−19)	310* (−27)	167	164 (−2)	158 (−5)	134* (−20)	134* (−20)
Day 0–372	681	659 (−3)	687 (+1)	612* (−10)	510* (−25)	330	322 (−2)	317 (−4)	240* (−27)	209* (−37)
Day 0–722	616	604 (−2)	734 (+19)	622 (+1)	519 (−16)	425	468 (+10)	375 (−12)	338* (−20)	258* (−39)

ppm: parts per million; *: $P < 0.05$ (Dunnett test) *Source*: Malley (1991)

2.4 Genotoxicity

Oxamyl has been tested in a range of in vitro and in vivo genotoxicity tests. The results are summarized in Table 5.

Table 5. Results of studies on the genotoxicity of oxamyl

End-point	Test object	Concentration [a]	Purity (%)	Results	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	50–10 000 µg/plate (± S9)	97.1	Negative	Arce (1987)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA1535 *Escherichia coli* WP2uvrA (pKM101)	5–5000 µg/plate (± S9)	96.9	Negative	Gladnick (1999)
Chromosomal aberrations	CHO cells	0.7–100 µg/mL for 8.5–10 h (−S9) [b]	NR	Negative	Galloway (1982) [c]
		2.3–700 µg/mL for 2 h (+S9) [d]		Negative	
Gene mutation	CHO cells	50–1200 µmol/L for 18–19 h (−S9) [b]	97.1	Negative	Rickard (1987)
		25–700 µmol/L for 5 h (+S9) [b]			
Gene mutation	CHO cells	50–300 µg/mL for 5 h (−S9) [e]	96.9	Negative	San & Clarke (2000)
		150–500 µg/mL for 5 h (+S9) [e]		Negative	
Chromosomal aberrations	Human lymphocytes	10–50 µg/mL for 4 h (−S9)	96.9	Negative	Gudi & Schadly (2000)
		50–200 µg/mL for 20 h (−S9)		Negative	
		100–300 µg/mL for 4 h (+S9)		Negative	
Unscheduled DNA synthesis	Rat hepatocytes	0.01–10 000 µmol/L for 18 h [f]	97.1	Negative	Vincent (1987)
Micronucleus test	Mouse bone marrow erythrocytes	1, 2 and 3 mg/kg bw [g]	98.2	Negative	Donner (2002)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; NR: not reported; S9: 9000 × *g* supernatant fraction from rat liver homogenate (metabolic activation)

[a] Positive and negative (solvent) controls were included in all studies.

[b] Concentration-related cytoxocity was observed in tests with metabolic activation (19% survival at 700 µmol/L) and without metabolic activation (17% survival at 1200 µmol/L).

[c] Statements of adherence to good laboratory practice (GLP) and quality assurance (QA) were not included.

[d] Dose-related cytotoxicity of oxamyl was observed at concentrations of ≥ 23 µg/mL without metabolic activation and at concentrations of ≥ 70 µg/mL with metabolic activation.

[e] Cloning efficiency was < 50% of solvent control at oxamyl concentrations of 150 µg/mL and 250 µg/mL without and with metabolic activation respectively.

[f] Cytotoxicity was observed at oxamyl concentrations of 5 and 10 mmol/L.

[g] Groups of 10 Crl:CD mice were administered a single dose of oxamyl at 0, 1, 2 or 3 mg/kg bw by gavage. Clinical signs indicative of inhibition of acetylcholinesterase (AChE) activity were observed at the highest dose in both sexes. There were no statistically significant increases in micronucleated polychromatic erythrocyte (MNPCE) frequency at any time point at any dose level in male mice exposed to oxamyl. Statistically significant increases in MNPCE frequency were observed in female mice at 2 or 3 mg/kg bw killed at the 48-hour time point only. However, the frequencies were within laboratory historical control ranges; the statistical significance could be attributed to a lower concurrent negative control in comparison to the 24-hour control females and the 24- and 48-hour control males, and the frequencies were similar to the MNPCE frequencies observed in the 24-hour treated female mice and 24- and 48-hour treated males. Clinical signs suggestive of AChE inhibition were observed at 3 mg/kg bw approximately 0–2 hours after dosing only. At 2 mg/kg bw, transient mild clinical signs were observed. None of the observed clinical signs of abnormality persisted beyond two hours after dosing.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a three-generation study of reproductive toxicity, ChR-CD rats (16/sex per group) were fed diets containing oxamyl (purity: 95%) at a concentration of 0, 50, 100 or 150 ppm (equivalent to 0, 3.3, 6.7 and 10 mg/kg bw per day). Over a 15-day period, each female was mated with three males of the same treatment group (5 days/male). One week after weaning of the first litters (F_{1A}), the F_0 females were again mated to produce F_{1B} litters. F_{1B} animals remained on the diet and, at 110 days of age, were mated to produce F_{2A} and subsequently F_{2B} litters. Animals from the F_{2B} litters were used to produce the F_{3A} and F_{3B} litters. Litters containing more than 10 pups were reduced to 10 on day 4 after birth. The number of pregnancies, litter size at birth and days 4, 12 and 21 days of age, and pup body weights were assessed. Two F_{3B} rats/sex from five control litters and the 150 ppm group were examined histologically. Randomly selected F_{3B} animals (10/sex) from the control and 150 ppm groups remained on their respective diets for eight weeks, while another 10/sex were transferred from the control to 150 ppm and vice versa for the same duration.

Oxamyl had no effects on the number of pregnancies or gestation and fertility indexes. Dose-dependent reductions in litter size and body weights of weanlings at 100 and 150 ppm were observed consistently throughout the study; body weights of weanlings at 50 ppm were slightly reduced. Occasional reductions in viability and lactation indexes were observed in rats at 100 and 150 ppm. In the cross-feeding study of the F_{3B} weanlings, the reductions in body weight in weanlings at 150 ppm appeared reversible within eight weeks, while control weanlings subsequently given the 150 ppm diet showed reduced body weight gain. F_{3B} weanlings at 150 ppm had slightly increased relative kidney weights and relative testis weights (at 100 and 150 ppm). No treatment-related gross or histopathological abnormalities were observed in the F_{3B} animals at 150 ppm (Sherman, 1972).

The 2002 Meeting noted that mating each female with three males might have obscured observations of effects of oxamyl on male fertility. Furthermore, the description of the study is very brief, and no individual data were presented for most parameters; no statistical analysis of the data was performed.

In a two-generation study of reproductive toxicity performed according to FIFRA Guideline 40 CFR 160, reference 83-4, Sprague Dawley rats (30/sex per group) received diets containing oxamyl (purity: 97.1%) at a concentration of 0, 25, 75 or 150 ppm (equal to 0, 1.4, 4.2 and 8.7 mg/kg bw per day for males and 0, 1.7, 5.4 and 12.2 mg/kg bw per day for females, respectively). F_0 rats were mated at a 1:1 ratio, 74 days after the beginning of the treatment. F_1 rats (24/sex per dose) were given the corresponding experimental diet for at least 105 days after weaning, and subsequently mated in a 1:1 ratio to produce the F_2 generation. Treatment of F_1 rats continued until weaning of the F_2 generation.

A single F_0 (P) female at 150 ppm died on day 115. Of the three F_1 males at 150 ppm that died, one died on day 6 from undetermined causes; the one that died on day 35 had atrophy of thymus and liver, and the one that died on day 168 had atrophy of the spleen. Given the lack of correlation between treatment administration and the day of death and observations at death, it is unlikely the deaths were compound related. Body weight, weight gain and feed efficiency were reduced in F_0 and F_1 parental animals at 75 and 150 ppm. Feed consumption was decreased in F_0 and F_1 parental males at 75 and 150 ppm and increased in F_1 parental females at 150 ppm. F_1 males at 150 ppm showed increased reactivity and females had a higher incidence of alopecia. In F_0 and F_1 males at 75 and 150 ppm, the relative testes weight was increased. The number of F_1 and F_2 pups per litter was decreased at 150 ppm, and the viability of these pups and litter survival were decreased during the first four days after birth. Pup weights were decreased at 75 and 150 ppm. The percentage of pups with a small bodies was increased in F_1 animals at 150 ppm and in F_2 animals at 75 and 150 ppm. The number of pups with no milk spot was increased in F_1 animals at 150 ppm.

Brain, erythrocyte and plasma AChE activities were not measured.

The NOAEL for parental toxicity was 25 ppm (equal to 1.4 mg/kg bw per day) based on the decreases in body weight, body weight gain, feed consumption and feed efficiency and increase in relative testis weight. The NOAEL for offspring toxicity was 25 ppm (equal to 1.7 mg/kg bw per day) based on reduced pup weight. The NOAEL for reproductive toxicity was 75 ppm (equal to 5.4 mg/kg bw per day) based on reduced number of pups per litter (Hurtt, 1990).

(b) Developmental toxicity

Rat

Pregnant ChR-CD rats (26–28/group) were given diets containing oxamyl (purity: 95.0%) at a concentration of 0, 50, 100, 150 or 300 ppm (equal to 0, 4.5, 8.2, 11.6 and 20.5 mg/kg bw per day) on gestation days 6–15.

Dams at 100 ppm and above had dose-related decreases in body weight and feed consumption. At scheduled kill on gestation day 20, no macroscopic changes in tissues and organs were observed. Oxamyl did not affect the number of implantation sites, resorptions or dead or live fetuses; nor did it affect embryonic weight and crown–rump length. No treatment-related increases in skeletal malformations and anomalies were observed. The description of the study was very brief, and no individual data were presented (Culik & Sherman, 1971).

In a developmental toxicity study performed according to USEPA Guideline 83-3, Sprague Dawley rats (25 females/group) received oxamyl (purity: 97.2%) at doses of 0, 0.20, 0.50, 0.80 or 1.5 mg/kg bw per day in distilled water by gavage on gestation days 7–16. The dams were killed on gestation day 22, fetuses were removed, and dams and fetuses were examined to determine maternal toxicity and reproductive and developmental effects. Brain, erythrocyte and plasma AChE activities were not measured.

The body weight gain of dams was reduced dose-dependently (by 8.6%, 21% and 30% at 0.5, 0.8 and 1.5 mg/kg bw per day, respectively), and the feed consumption of dams at 0.8 and 1.5 mg/kg bw per day was decreased. Dams at 1.5 mg/kg bw showed clinical signs of AChE inhibition (tremors, salivation, ocular discharge, wet perineum, wet body and diarrhoea), and 4/25 females at 0.8 mg/kg bw per day had tremors. Small but significant reductions in fetal weight were observed (by 3.9%, 6.8% and 6.9% at 0.5, 0.8 and 1.5 mg/kg bw per day, respectively). Treatment with oxamyl caused no irreversible structural changes in the fetuses.

The NOAEL for maternal toxicity was 0.5 mg/kg bw per day, based on decreases in body weight gain and feed consumption, and tremors at 0.8 mg/kg bw per day. The NOAEL for fetal toxicity was 0.5 mg/kg bw per day, based on decreased fetal body weight at 0.8 mg/kg bw per day (Rickard, 1988; Munley, 1998).

Rabbit

Artificially inseminated New Zealand White rabbits (17/group) were given oxamyl (purity: 97.1%) at a dose of 0, 1, 2 or 4 mg/kg bw per day by gavage from gestation day 6 to 19. On day 29, all animals were killed, and the fetuses were removed. Brain, erythrocyte or plasma AChE activity were not measured.

The pregnancy rate were 17/17, 15/17, 15/17, 13/17 at 0, 1, 2 and 4 mg/kg bw per day, respectively. One animal in each of the 1 and 4 mg/kg bw per day group died, probably because of tracheal intubation. Maternal body weight gain of does at 1 mg/kg bw per day was reduced by 29%, and that of does at 2 and 4 mg/kg bw per day was significantly reduced during treatment, by 62% and 67%, respectively. The ovarian and uterine weights with and without fetuses of does at 2 mg/kg bw per day were slightly reduced. Fetal viability was slightly reduced in does at 4 mg/kg bw per day, and two had 100% resorption. One female at 2 mg/kg bw per day had one dead fetus. No treatment-related developmental toxicity was observed, and no irreversible structural changes were found.

The NOAEL for maternal toxicity was 1 mg/kg bw per day based on decreased weight gain at 2 mg/kg bw per day. The NOAEL for fetal toxicity was 2 mg/kg bw per day based on increased percentage of resorptions at 4 mg/kg bw per day (Hoberman, 1980).

2.6 Special studies

(a) Neurotoxicity

Single-dose delayed neurotoxicity

In a study of delayed neurotoxicity, groups of five 1-year- old white Leghorn hens received a single instillation of oxamyl (purity unknown) as a 1% suspension in water, directly into the crop, at a dose of 20 or 40 mg (the acute lethal dose, as determined in a pilot experiment). A few minutes before dosing, the hens received atropine at 0.5 mg/kg bw by intramuscular injection. Treatment with oxamyl caused sudden depression, lethargy, slight ruffled feathers, slight respiratory difficulty, ataxia and impaired coordination immediately after dosing. The respiratory signs disappeared within 30 minutes, but depression and nervous signs continued for 12 hours. All the hens survived for up to 28 days after treatment.

In the pilot experiment, administration of oxamyl at 40 mg without prior treatment with atropine caused the same clinical signs to a very severe degree, and resulted in death of the hens within five minutes. Treatment with oxamyl (either in or not in combination with atropine) did not cause any gross or histopathological changes (the sciatic nerve was included in the pathological examination) (Lee, 1970).

The study was considered outdated and of limited value. Owing to the poor description of the study design, its compliance with OECD TG 418 could not be ascertained.

Reversibility

Crl:CD BR rats (40/sex per group) received technical grade oxamyl (purity: 98.3%) in deionized water by gavage at a single dose of 1 mg/kg bw. All the rats were examined for clinical signs at 30 minutes after dosing, and 10 rats/sex were examined for clinical signs again 2, 3 and 4 hours after dosing, with blood being sampled. The animals were then killed, and their brain collected to measure AChE activity.

No mortality occurred. Thirty minutes after administration of oxamyl, tremors were observed in males (38/40) and females (36/40). At 30 minutes after dosing, ChE activity was reduced in males and females, respectively, by 57% and 50% in plasma, 58% and 61% in erythrocytes and 45% and 48% in whole brain. At 2, 3 and 4 hours after oxamyl treatment, no clinical signs were observed and deviations from control AChE values were 13% or less. There were no obvious differences in levels of AChE activity over the 2–4 hour period (Malley, 1997a).

Acute neurotoxicity

In a study performed according to USEPA Guideline 81-8, Crl:CD BR rats (42/sex per group) were given oxamyl (purity: 98.3%) by gavage in deionized water at a single dose of 0.1, 1 or 2 mg/kg bw for males and 0.1, 0.75 or 1.5 mg/kg bw for females. Clinical signs, body weight, body weight gain and feed consumption were assessed periodically in 12 rats/sex per dose. These animals were administered a functional observational battery (FOB) motor activity test before treatment and 30–60 minutes and 7 and 14 days after treatment. Of these rats, six were killed on day 15 and examined grossly. Brain, spinal cord, nerve and muscular tissues from animals in the control group and at the highest dose were examined histologically for neuropathological effects. AChE activity was determined in brain tissue, plasma and erythrocytes from 10 rats/sex per dose 30–60 minutes and 1 and 14 days after dosing. In addition, plasma and erythrocyte AChE activity was assessed prior to dosing in the animals killed after 30–60 minutes.

On the first day after treatment, body weight gain and feed consumption were reduced in mid- and high-dose males and body weight gain was reduced in high-dose females. One high-dose male died on the day of administration. Almost all mid- and high-dose animals displayed tremors, and dose-dependent increases in salivation, low posture and wet perineum (females only) were observed. Other effects related to AChE inhibition in these groups were decreased grip strength and responses to tail pinch, decreased coordination, urination and defecation, decreased reaction to approach and touch, decreased vocalization during handling, and increases in foot splay; they also showed soiled fur, lachrymation, dilated pupils, slow righting reflex, abnormal gait, impaired locomotion, low arousal, splayed limbs

and laboured breathing. Furthermore, decreases in palpebral closure and in mean and total number and duration of movements, and increases in curled-up posture and docile behaviour were observed at mid and high dose. Effects on clinical signs, FOB and motor activity were observed 30–60 minutes after application. No clinical signs or behavioural effects were seen at low dose. Behavioural effects observed occasionally on days 7 and 14 after treatment and on AChE activity on days 1 and 14 after treatment were considered incidental, as the Malley (1997a) study on reversibility (section 2.6a) showed that the effects of oxamyl were short lasting.

Large reductions in AChE activity were observed in brain and blood 30–60 minutes after administration of the mid and high doses (see Table 6). A statistically significant 25% reduction in AChE activity in the cerebellum of females at 0.1 mg/kg bw was considered not toxicologically relevant because significant inhibition of AChE activity was not observed in other brain structures, in the half brain preparation, or in erythrocytes or plasma of males and females at this dose. Macroscopic and microscopic neuropathological examination revealed no treatment-related toxicologically relevant effects. As the doses used for males and females were different, it is difficult to assess whether this was a sex difference in sensitivity to the neurotoxic effects of oxamyl.

Table 6. Acute neurotoxicity effects of oxamyl on AChE activity in rats in an acute neurotoxicity study

	% change in acetylcholinesterase (AChE) activity per dose					
	Males			Females		
Tissue	0.1 mg/kg bw	1.0 mg/kg bw	2.0 mg/kg bw	0.1 mg/kg bw	0.75 mg/kg bw	1.5 mg/kg bw
Blood						
Plasma	−10	−60*	−77*	+9	−38*	−72*
Erythrocyte	−7	−57*	−63*	+13	−55*	−70*
Brain						
Cortex	−19	−56*	−71*	−15	−59*	−68*
Hippocampus	−10	−38*	74*	−15	−40*	−71*
Midbrain	+3	−40*	−60*	−8	−50*	−70*
Cerebellum	+4	−26*	−70*	−25*	−51*	−74*
Half brain	−2	−47*	−66*	−1	−46*	−67*

bw: body weight; *: significantly different from control

Source: Malley (1997b)

The NOAEL was 0.1 mg/kg bw based on AChE inhibition in brain, plasma and erythrocytes, a variety of clinical signs and effects in the FOB at 0.75 mg/kg bw (Malley, 1997b).

Crl:CD rats (10/sex per group) were exposed for four-hour, nose-only to a single dose of oxamyl (purity: 96.9%) at an actual concentration of 0.0049 or 0.024 mg/L. The mass median aerodynamic diameter (MMAD) for the aerosol tested ranged from 0.85 to 1.2 µm. A control group was exposed to air only. Rats were observed for clinical signs of toxicity and mortality during exposure. Immediately after exposure, the animals were killed, and blood and brain (whole) samples were collected for AChE analysis.

No deaths occurred during the study. Most rats at 0.024 mg/L showed tremors and lethargy immediately after exposure. At 0.0049 mg/L, clinical signs were mild and similar in incidence to controls. In animals at 0.024 mg/L, a significant decrease in plasma, erythrocyte or whole brain AChE activity was observed, from 67% to 76% (Table 7). Animals in this group showed clinical signs correlated to AChE inhibition.

Table 7. Effects of oxamyl on AChE activity in a four-hour nose-only inhalation study

Tissue	% change in AChE activity per inhalation dose			
	Males		Females	
	0.024 mg/L	0.004 9 mg/L	0.024 mg/L	0.004 9 mg/L
Blood				
Plasma	−72*	−12*	−76*	−6
Erythrocyte	−72*	−28*	−73*	−29*
Brain	−68*	−15*	−67*	−9*

AChE: acetylcholinesterase; *: significantly different from control *Source*: O'Neill (2001)

Significant decreases in AChE activity were recorded in both sexes at 0.004 9 mg/L, with minimal decreases in brain and plasma, but decreases in erythrocyte AChE activity at 28% and 29% in males and females, respectively. Brain AChE activity was marginally affected at 0.004 9 mg/L.

A NOAEL could not be determined (O'Neill, 2001).

The relative sensitivity of pre-weanling rat pups (lactation day 11) and young adult rats to inhibition and recovery of AChE activity was studied in male and female Crl:CD®(SD)IGS BR rats administered pure oxamyl (purity: 98%) in NanoPure® water by single-dose oral gavage. Three subsets of experiments were performed: Subset 1, to evaluate the peak inhibition and recovery of AChE activity in pups; Subset 2, to determine the dose response of AChE activity inhibition at the time of peak inhibition in pups; and Subset 3, to evaluate dose responses at peak inhibition and at recovery in young adult rats. For Subset 1, doses in male and female rats were 0 or 0.1 mg/kg bw. For Subset 2, doses in male and female rats were 0, 0.075, 0.1, 0.125 or 0.15 mg/kg bw. For Subset 3, doses in male and female rats were 0, 0.15, 0.20 or 0.25 mg/kg bw. Dose volumes were 2 mL/kg bw for both sexes in all subsets. Groups of rats were killed at 30, 60, 90, 120, 180, 240 or 360 minutes after dose administration (Subset 1); 30 minutes after dose administration (Subset 2); or 30 or 240 minutes after dose administration (Subset 3). For all subsets, blood and brain were collected at time of kill to determine AChE activity. No clinical observations and statistical evaluation were performed.

In the experiment (Subset 1) to evaluate AChE activity in RBCs and brain in pre-weanling male and female rat pups dosed at 0.1 mg/kg bw, the lowest RBC and brain AChE activities occurred at 30 minutes after dosing for male RBC and brain AChE, and female brain AChE. The greatest degree of AChE inhibition in pre-weanling male and female rat pups dosed with 0.1 mg/kg bw generally occurred at 30 minutes after dosing. Female RBC AChE activities were lowest at 90 minutes after dosing. However, due to the variability in AChE values at 30, 60, 90, 120 and 180 minutes, the biological relevance of the apparent depression in female RBC AChE at 90 minutes is questionable.

Results from the other experiment (Subset 2) showed that RBC and brain AChE activities were slightly lower than control at doses of 0.075, 0.1 and 0.125 mg/kg bw (RBC AChE was inhibited by 15–20%, while brain AChE was inhibited by 7–12%), but with no dose response. At 0.15 mg/kg bw, RBC AChE was inhibited 36–38% and brain AChE was inhibited 23% for both sexes. For a given dose and sex, inhibition of AChE activity in the brain was slightly less than that of RBCs. There were no marked sex differences in RBC and brain AChE activity.

At 30 minutes after dosing, both RBC and brain AChE activities decreased in a dose-dependent manner (Fig 3. and Fig. 4). Brain AChE activity in females at 0.15 mg/kg bw were similar to control and recovery values. In both males and females, the inhibition of brain AChE activity was less affected than the inhibition of RBC AChE activity. At 240 minutes after dosing, RBC and brain AChE activities of treated male and female rats were similar to control values. The Malley (1997a) reversibility study showed a complete reversibility of oxamyl-induced RBC and brain AChE activities after three hours post dosing, at a dose of 1.0 mg/kg bw. Therefore, any apparent changes in this study at 240 minutes after dosing were considered to represent normal variability in RBC and brain AChE measurements.

During dose–response studies, RBC and brain AChE activities were decreased in pups and adult rats (adults at higher doses only) at 30 minutes after dosing, and returned to baseline by 240 minutes

post dose for both sexes. For both adults and pups, the degree of RBC AChE inhibition was greater than brain AChE, indicating that RBC AChE activity is the more sensitive parameter. The coefficient of variation for RBC AChE activity ranged from 4–31% for pups and 17–30% for adults. For brain AChE activity, the coefficient of variation ranged from 1–18% for pups and 5–18% for adults. The variability in RBC and brain AChE activities in both pups and adults was consistent between sexes and was not affected by the dose of test substance administered.

Figure 3. Pup versus adult RBC AChE inhibition (a, males; b, females)

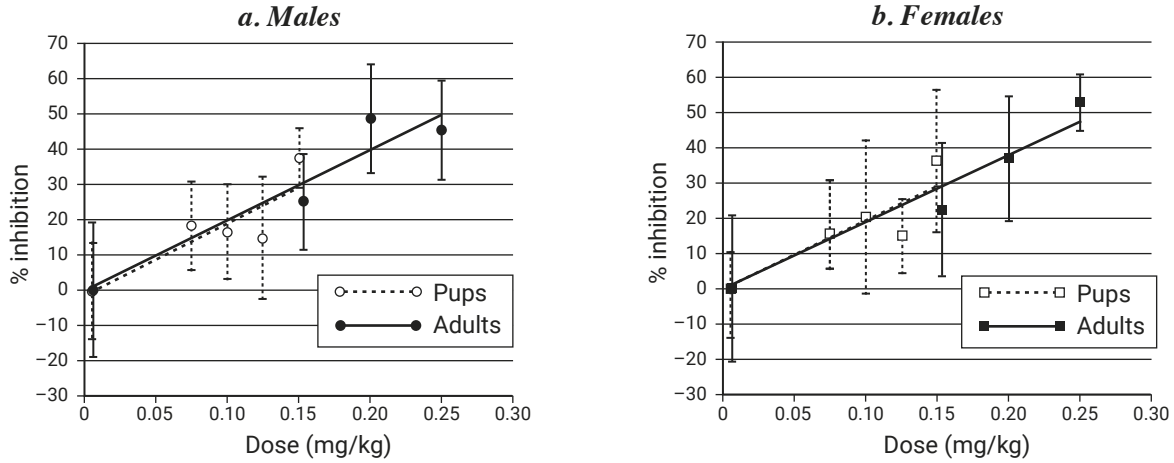
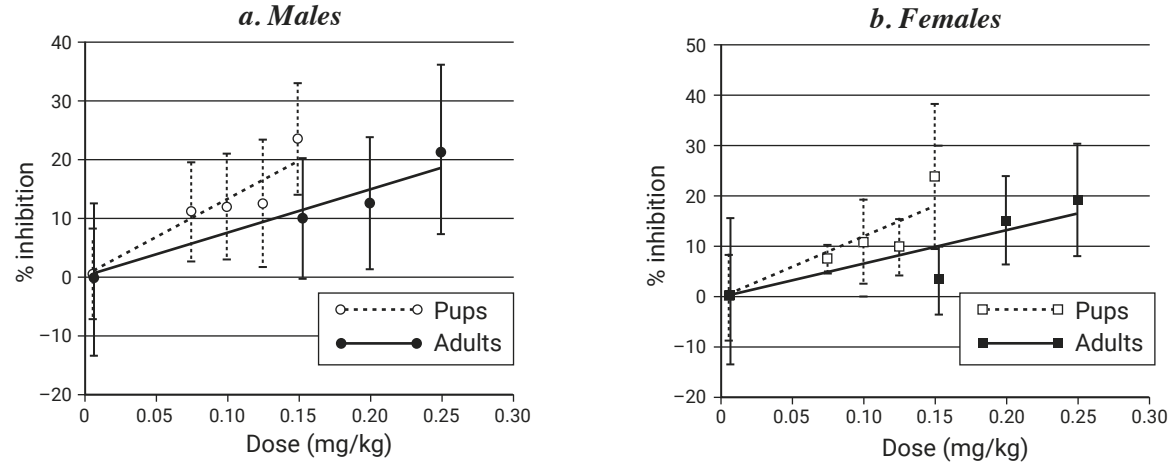


Figure 4. Pup versus adult brain AChE inhibition (a, males; b, females)



AChE: acetylcholinesterase; RBC: red blood cells

Source: Redrawn from Malley (2005)

RBC and brain AChE activity inhibition at the dose (0.15 mg/kg bw) common to both pups and adults was used to evaluate relative sensitivity. The inhibition was also calculated by comparing each individual rat's AChE activity value with the appropriate mean control value. For RBC AChE inhibition, effects were essentially the same in pre-weanling pups and adults, based on results at the dose in common (0.15 mg/kg bw) and linear trends across the range of doses tested (Fig. 3). For brain AChE, pre-weanling pups appeared to be slightly more sensitive than adults, based on results at the common dose (Fig. 4). No statistically significant differences between pup and adult rats were identified for brain and RBC AChE inhibition. At lower doses (0.075, 0.1 and 0.125 mg/kg bw), however, the range of responses for brain AChE activity in pups tended to overlap with the linear trend line in the dose response for adults. These data indicate that pups were generally not more sensitive than adults to the effect on AChE (Malley, 2005).

Neurotoxicity after repeated doses

In a study performed according to USEPA Guideline 81-8, Crl:CD rats (24/sex per group) received oxamyl (purity: 98.3%) in the diet at a concentration of 0, 10, 30 or 250 ppm (equal to 0, 0.55, 1.7 and 15 mg/kg bw per day for males and 0, 0.67, 2.0 and 20 mg/kg bw per day for females, respectively) for 90 days. Two higher concentrations of 100 and 300 ppm were reduced to 30 and 250 ppm because of the severe toxicity observed at the higher doses. Body weights and feed consumption were measured weekly. Rats were checked for clinical signs daily for the first two weeks (the first four weeks for rats at the highest doses) and weekly thereafter. FOB and motor activity tests were performed with 12 rats/sex per dose pretest and 4, 8 and 13 weeks after the treatment initiation. AChE activity in brain, plasma and erythrocytes was determined in 10 rats/sex per dose at weeks 4, 8 and 13.

Dose-related clinical signs, including tremors, abnormal gait, hunched posture, exophthalmos, ptosis, hyperreactivity, piloerection, coloured discharge from the eyes, wet chin, stained perineum or inguen and lachrymation, were observed at 100 and 300 ppm during the first week of treatment. After reducing the highest concentrations administered, treatment-related clinical signs were still observed in both sexes at 250 ppm. Although clinical signs were observed throughout the treatment, the incidences were highest during the first four weeks of treatment. It is not clear whether this higher incidence was due to a higher intake of oxamyl during the first weeks of treatment or the result of an adaptive response to prolonged oxamyl treatment, or both. Body weight and body weight gain were reduced in both sexes at 100 and 300 ppm during the first week. After reducing the two higher dietary concentrations on day 7, the body weights and body weight gain recovered completely in males at 30 ppm and females at 30 and 250 ppm, and partially in males at 250 ppm. During the first week, feed consumption and feed efficiency were decreased in animals at 100 and 300 ppm, and thereafter in males at 250 ppm.

At 250 ppm, hindlimb grip strength in the FOB was reduced in males and females at 250 ppm at week 4 and in females at weeks 8 and 13; hindlimb foot splay was decreased in females only at week 8 and in males and females at week 13 Increased incidences of ptosis, absent pupillary responses, piloerection, abnormal gait and absent defecation were seen at 250 ppm throughout treatment. Although these increases were not always significant, they were associated with AChE inhibition and were considered treatment related. Motor activity (duration and number of movements) was slightly reduced at 250 ppm, in males in weeks 4, 8 and 13, and in females at week 13. The effects on AChE activity are summarized in Table 8.

The reduction in AChE activity was constant over the measurement intervals. In general, AChE activity was more severely inhibited in females than in males, perhaps due to a slightly higher intake by females. Generally, the inhibition of AChE activity was less than 20% at 10 and 30 ppm and in only one instance reached statistical significance. No macroscopic or microscopic neuropathological changes were observed.

The NOAEL was 30 ppm (equal to 1.7 mg/kg bw per day) based on the effects on body weight and body weight gain, clinical effects, behavioural changes in the FOB and the inhibition of AChE activity in brain, erythrocytes and plasma at 250 ppm (equal to 15 mg/kg bw per day) (Malley, 1998).

Table 8. Effects of oxamyl on AChE activity in rats at 250 ppm in a 13-week neurotoxicity study

	% change in AChE activity per week of administration					
	Males			Females		
Tissue / organ	**4**	**8**	**13**	**4**	**8**	**13**
Blood						
Plasma	−34	−28	−24	−67	−56	−60
Erythrocyte	−40	−26	−48	−65	−41	−55
Brain						
Cortex	−46	−35	−40	−55	−36	−51
Hippocampus	−31	−34	−27	−60	−40	−42
Midbrain	−30	−36	−31	−40	−20	−51
Cerebellum	−31	−24	−32	−37	−32	−39
Half brain	−38	−35	−42	−56	−41	−49

AChE: acetylcholinesterase; ppm: parts per million *Source*: Malley (1998)

(b) Studies of oxamyl with other carbamates

Oxamyl was evaluated in a study along with six other carbamates (carbaryl, carbofuran, formetanate, methiocarb, methomyl and propoxur). First, in a time-course assay, pre-weanling (postnatal day 17) rats (6 males/group) were give oxamyl (purity: ≥ 99%) by gavage at a single dose of 0 or 0.5 mg/kg bw. AChE activity in RBCs and brain (whole) were determined at 15–20, 45–55, 90–95, 180–190 or 1440–1450 minutes after dosing. Second, in a dose–response study, pre-weanling (postnatal day 17) rats (10 males/group) were given oxamyl (purity: ≥ 99%) by gavage at a single dose of 0, 0.07, 0.2, 0.5 or 0.8 mg/kg bw. Motor activity was evaluated 20 minutes after dosing and immediately after the activity session (40–45 minutes after dosing). Animals were killed for tissue sampling to determine AChE activity, by radiometric assay, in RBCs and brain (whole).

Results from the time-course assay showed that the peak of RBC and brain AChE activity inhibition occurred at 45–90 minutes; recovery to control values was mostly complete after 24 hours. RBC AChE was generally inhibited to a greater degree than brain.

Results from the dose–response assay showed that RBC AChE activity was significantly inhibited at all doses in a dose-related manner. Brain AChE activity was significantly inhibited from 0.2 mg/kg bw, in a dose-related manner. Red blood cell AChE activity was generally more affected than brain. Motor activity counts were significantly decreased at 0.8 mg/kg bw, evidencing very little concordance between motor activity depression and the degree of AChE inhibition (Moser et al., 2010).

Oxamyl was evaluated in a study of acute toxicity along with six other *N*-methyl carbamate pesticides. Long Evans rats (10 males/groups) were given oxamyl (purity: ≥ 99%) by gavage at a single dose of 0, 0.05, 0.1, 0.5, 1 or 1.5 mg/kg bw. Approximately 10–12 minutes after dosing, all animals were observed for clinical signs of toxicity, including, but not limited to, lachrymation, miosis, fasciculations, smacking, tremors, polyuria and diarrhoea. Motor activity (horizontal and vertical) assessment began 15 minutes after dosing, and tissues were collected within 35–40 minutes after dosing to determine AChE activity.

No significant changes were observed at 0.05 or 0.1 mg/kg bw. At 0.5, 1 and 1.5 mg/kg bw, RBC and brain AChE activity were significantly inhibited in a dose-dependent manner with RBC AChE inhibited at a greater degree than brain AChE. Motor activity (horizontal and vertical) was significantly decreased at 0.5, 1 or 1.5 mg/kg bw. Only one rat at 1 mg/kg bw and two at 1.5 mg/kg bw showed cholinergic signs (McDaniel et al., 2007).

Oxamyl was evaluated in a study of acute toxicity along with six other *N*-methyl carbamate pesticides. Adult Long Evans rats (5 males/group) were given oxamyl (purity: 99%) in water by gavage at a single dose of 1 mg/kg bw. Brain tissue and blood were sampled from 0.5 to 24 hours after dosing for analysis of AChE activity. Two different methods were used to measure AChE activity in all *N*-methyl carbamates; a radiometric method (according to Johnson & Russel, 1975) and a spectrophotometric method (Ellman method using traditional, unmodified conditions).

AChE activity was inhibited by 80% in RBCs and 50% in brain at 30 minutes after dosing. The RBC AChE was inhibited slightly more than brain AChE, but by four hours after dosing both RBC and brain AChE had recovered to control values. Comparison of AChE activity using two different assays showed that the spectrophotometric method underestimated AChE inhibition (Padilla et al., 2007).

(c) Hormonal activity studies

In a study performed according to USEPA Guideline 890.1500 to evaluate juvenile/peripubertal development and thyroid function in male rats, juvenile/peripubertal (postnatal day 23–53 or 54) Crl:CD(SD) rats (15 males/groups) were given oxamyl (purity: 98%) in deionized water by gavage at a dose level of 0, 0.25 or 0.5 mg/kg bw per day. All animals were observed twice daily for mortality and moribundity, and clinical observations were recorded daily. Animals were also observed for signs of toxicity for approximately 30–45 minutes after dosing. Body weights were measured on the day of randomization, daily before dosing and on the day of scheduled kill. All animals were observed daily (from postnatal day 30) for balanopreputial separation. A complete necropsy was conducted on all rats

found dead or that survived to the scheduled kill (on postnatal day 53 or 54); selected organs were weighed and preserved. Hormone (thyroxine [T_4], thyroid-stimulating hormone [TSH] and testosterone) and clinical pathology evaluations (creatinine and urea nitrogen) were conducted on all surviving animals on postnatal day 53 or 54, and histopathological evaluation of thyroid, kidneys, testis and epididymis was performed.

All animals survived until scheduled kill. There were no test substance-related effects on mortality, clinical signs at daily and 30–45 minute (post dosing) observations, body weight parameters, balanopreputial separation, T_4, TSH or serum testosterone, serum chemistry (creatinine or urea nitrogen), gross or microscopic pathology or organ weights, at any level tested.

Under the conditions of the study, oxamyl did not alter the hypothalamic–pituitary–gonadal axis or the hypothalamic–pituitary–thyroid axis function at oral dosage levels of 0.25 and 0.5 mg/kg bw per day (Toot, 2012a).

In a study performed according to USEPA Guideline 890.1500 to evaluate juvenile/peripubertal development and thyroid function in female rats, juvenile/peripubertal (postnatal day 22–42 or 43) male Crl:CD(SD) rats (15/group) were given oxamyl (purity: 98%) in deionized water by gavage at a dose level of 0, 0.25 or 0.5 mg/kg bw per day. All animals were observed twice daily for mortality and morbidity, and clinical observations were recorded daily. Animals were also observed for signs of toxicity for approximately 30–45 minutes after dosing. Body weights were measured on the day of randomization, daily before dosing and on the day of scheduled kill. All animals were observed daily (from postnatal day 22) for vaginal opening, and vaginal lavages were performed for each female to determine the stage of the estrous cycle. A complete necropsy was conducted on all rats found dead or that survived to the scheduled kill (postnatal day 42 or 43), and selected organs were weighed and preserved. Hormone (T4 and TSH) and clinical pathology evaluations (creatinine and urea nitrogen) were conducted on all surviving animals on postnatal day 53 or 54. In addition, histopathological evaluations of the thyroid, kidney, ovaries and uterus were performed.

All animals survived, and there were no clinical or macroscopic findings observed at any dose tested. No test substance-related effects were noted on mean body weight parameters. The mean ages at vaginal opening at 0.25 and 0.5 mg/kg bw per day were statistically significantly (pair-wise and trend test) lower than the control group. When adjusted for body weight, the differences were significant for a trend test. Of note, the mean ages at vaginal opening at 0.25 and 0.5 mg/kg bw per day were within the laboratory historical control range, and there was no indication of dose response. According to the author, the higher control mean values were partially due to a single female (no. 33061-11) that did not attain vaginal opening prior to necropsy (postnatal day 43); adjusting the mean age at vaginal opening for this female, using the age at which partial separation was first observed (i.e. postnatal day 34), the mean day of attainment was 35.0 days. A separate statistical analysis subsequently performed showed no significant differences between control and treatment groups.

In addition, the mean age of attainment of vaginal opening and the coefficient of variation value in the control group (35.7 days and 7.76, respectively) exceeded the recommended maximum value in the performance criteria (35.62 days and 6.52, respectively). When a second analysis was performed, adjusting the age at vaginal opening with the age at which partial separation was first observed in the female with three or more days of incomplete opening, the mean age of attainment of vaginal opening and the coefficient of variation value range in the control group (35.0 days and 4.45, respectively) were within the performance criteria acceptable range.

The mean body weights at vaginal opening at 0.25 and 0.5 mg/kg bw per day were lower than the control group, achieving statistical significance (pair-wise and trend test) at 0.5 mg/kg bw per day; the values were within the laboratory historical control range. According to the author, the higher control mean values were partially due to a single female (no. 33061-11) that did not attain complete vaginal opening prior to necropsy (postnatal day 43). The body weight of this control female on postnatal day 43 was 162.4 g and at time of partial vaginal opening on postnatal day 34 was 119.2 g; adjusting for this body weight results in an adjusted control mean value of 124.1 g.

The mean age at the first occurrence of estrus, estrous cyclicity and mean estrous cycle length in these groups were unaffected by oxamyl administration.

Uterus weights (blotted and wet) at 0.25 and 0.5 mg/bw per day were significantly higher than in the control group, this was due to the estrous cycle state of the females on the day of necropsy. In the control, 0.25 and 0.5 mg/kg bw per day groups, 1, 8 and 3 females, respectively, were assumed to be in the proliferative phase and 10, 6 and 7 females, respectively, were assumed to be in the non-proliferative phase. Therefore, the increased number of females in the proliferative phase (0.25 mg/bw per day only) relative to the increase number of females in the non-proliferative phase (control group), along with the noted increase in blotted and wet uterus weights across each group, was responsible for the indicated statistical significance. Furthermore, these changes in organ weights did not fit a dose–response pattern, were not associated with any microscopic change in the uterus, and were considered to be, in part, due to biological variability.

No test substance-related effects on serum creatinine, urea nitrogen, T_4 or TSH levels were noted at either dosage level. No test substance-related microscopic findings were noted in these groups (Toot, 2012b).

In a study performed according to USEPA Guideline 890.1200, oxamyl (purity: 98%) was evaluated for its ability to inhibit the activity of human recombinant microsomal aromatase (CYP19), an enzyme responsible for the conversion of androgens to estrogens. Oxamyl, was tested at concentrations from 1.0×10^{-10} to 1.0×10^{-3} mol/L. The positive control, 4-hydroxyandrostenedione, was evaluated to verify test system performance. As expected, 4-hydroxyandrostenedione showed effects consistent with aromatase inhibition in three independent assays. The estimated log of the median inhibitory concentration ($\log_{IC50}$) for 4-hydroxyandrostenedione was approximately 7.2 log mol/L.

Under the conditions of the study, oxamyl did not inhibit aromatase activity when tested at up to a maximum concentration of 1.0×10^{-3} mol/L (Snajdr, 2012b).

In a study performed according to OECD TG 455, oxamyl (purity: 98%) was evaluated for its ability to act as an agonist of human estrogen receptor alpha (hERα) using the hERα HeLa 9903 cell line. The final concentrations in ultrapure water of oxamyl were $10^{-10.3}$, $10^{-9.3}$, $10^{-8.3}$, $10^{-7.3}$, $10^{-6.3}$, $10^{-5.3}$, $10^{-4.3}$ and $10^{-3.3}$ mol/L for the first and second runs. The duration of exposure was 24 hours. A complete concentration–response curve for each of four reference compounds (17β-estradiol, 17α-estradiol, corticosterone and 17α-methyltestosterone) was run each time the transcriptional activation assay was performed.

The maximum concentration of oxamyl selected for use in the transcriptional activation assays was $10^{-4.3}$ mol/L as no cytotoxicity (≥20% reduction in cell viability) or precipitation was observed at concentrations higher than $10^{-4.3}$ mol/L. In two independent runs of the transcriptional activation assay, oxamyl did not result in an increase in luciferase activity (RPC_{max} < 10%) at any of the viable concentrations tested.

Based on the results of this study, oxamyl is not an agonist of human estrogen receptor alpha (hERα) in the HeLa 9903 model system (Willoughby, 2012).

In a study performed according to USEPA Guideline 890.1250, oxamyl (purity: 98%) was evaluated for its ability to bind to the estrogen receptors in rat uterine cytosol. Saturation binding assays measured the affinity (K_d) of a radiolabelled estrogen ligand, 17β-estradiol ($[^3H]E_2$), for the estrogen receptor and the concentration of the estrogen receptors (B_{max}) present in the cytosol. This is determined by measuring specific binding of increasing concentrations of radioligand under conditions of equilibrium. Three independent runs were performed using hexatritiated 17β-estradiol ($[^3H]E_2$) as the radioligand to characterize the rat uterine cytosol. The K_d was approximately 0.081 nmol/L $[^3H]E_2$ and the B_{max} was approximately 32.5 fmol/100 µg protein, which is consistent with the acceptable range listed in the test guideline.

The performance criteria for radioinert 17β-estradiol and for 19-norethindrone were slightly outside acceptable ranges. According to the study author, this does not warrant performing another run since these values, except for one, were only marginally outside of the suggested range and because the range criteria in the test guideline are suggested ranges, not mandatory limits. All runs were within or very close to the suggested performance criteria, and therefore the assay was considered valid.

Under the conditions of the study, oxamyl did not competitively bind to the estrogen receptor when tested up to a maximum concentration of 1.0×10^{-3} mol/L (Snajdr, 2012c).

In a study performed according to USEPA Guideline 890.1600, oxamyl (purity: 98%) was evaluated for its potential estrogenic effects when administered by oral gavage to ovariectomized rats for three days. Young adult ovariectomized Crl:CD(SD) rats (6/group) were dosed by oral gavage with 0, 0.1, 0.25 or 0.5 mg/kg bw per day for three consecutive days and killed approximately 24 hours after the last administered dose. A separate ovariectomized positive control group was administered 0.1 mg/kg bw per day of the estrogen receptor agonist 17β-ethinyl estradiol. Body weights, feed consumption and clinical observations were recorded daily. Vaginal cytology was evaluated daily to assess the potential of oxamyl to induce cytological changes consistent with those observed with the positive control. At necropsy, uterine weights were collected.

No test substance-related deaths occurred, and no clinical observations were noted during treatment. There were no test substance-related effects on body weight. Mean body weight gain was slightly increased in the groups treated with oxamyl at 0.25 and 0.5 mg/kg bw per day, by 10.4% and 7.6% relative to control, respectively, but this change did not achieve statistical significance. At 0.25 mg/kg bw per day, feed consumption was statistically significantly increased by 7% but lacked a dose response. There were no test substance-related effects indicative of estrogenic activity. All animals remained in diestrus for the duration of the study. At necropsy, no gross observations or effects on uterine weight were noted.

The results with 17β-ethinyl estradiol were consistent with those of an estrogen receptor agonist.

Under the conditions of this study, oxamyl did not induce estrogenic effects in the uterotrophic assay when administered up to 0.5 mg/kg bw per day for three consecutive days (Snajdr, 2011a).

In a study performed according to USEPA Guideline 890.1400, oxamyl (purity:v98%) was evaluated for potential androgenic and anti-androgenic effects when administered by oral gavage to castrated rats for 10 days (Hershberger bioassay). The study consisted of two separate studies, one designed to detect androgenic activity (i.e. androgen receptor agonist-like activity) and the other to detect anti-androgenic activity (i.e. androgen receptor antagonist-like activity). In both studies, four groups of young adult castrated Crl:CD(SD) rats were given oxamyl by gavage at 0, 0.1, 0.25 or 0.5 mg/kg bw per day for 10 consecutive days and killed approximately 24 hours after the last dose. The positive control group for the androgenic study was given testosterone propionate at 0.4 mg/kg bw per day. The positive control group for the anti-androgenic study was given a flutamide at 3 mg/kg per day. In addition to the test substance or positive control treatment, all treatment groups in the antiandrogenic study also received a daily injection of 0.4 mg/kg bw of the reference androgen receptor agonist, testosterone propionate. Body weights and clinical observations were recorded daily; feed consumption was recorded once over the duration of the study (test days 9–10). At necropsy, organ weights (liver, ventral prostate, seminal vesicle plus fluids and coagulating glands, levator ani bulbocavernosus muscle, paired Cowper's glands and the glans penis) were collected.

In both the androgenic and anti-androgenic studies, no test substance-related deaths occurred and no clinical observations were noted during treatment. Nonstatistically significant reductions in body weight gain were observed over the duration of the study in all oxamyl-treated groups (6.7–9.5%). Feed consumption was similarly decreased in all oxamyl-treated groups in the antiandrogenic study (5–9.8%) and was statistically significant at 0.25 mg/kg bw per day. There were no test substance-related effects indicative of androgenic or antiandrogenic activity. At necropsy, there were no test substance-related effects on organ weights and no gross observations were noted.

In the androgenic study in rats given testosterone propionate, no deaths occurred and no clinical observations were noted during treatment. Mean final body weight was statistically significantly increased on test days 9 and 10 (~107%), and mean body-weight gain was statistically significantly increased (~25%) over the duration of the study (test days 0–10), and accompanied by an increase in mean daily feed consumption (~100%) over the duration of the study (test days 0 to 10), and increase on feed efficiency (~114%). As expected, rats administered testosterone propionate showed effects consistent with an androgen receptor agonist.

In the anti-androgenic study in rats given flutamide plus testosterone propionate, no deaths occurred and no clinical observations were noted during treatment. No effects on body weight or nutritional parameters were observed. No gross observations were noted at necropsy. Microscopic evaluation was deemed unnecessary. In both the androgenic and anti-androgenic studies, the performance criteria in the control and high-dose group (0.5 mg/bw per day) were met.

Under the conditions of this study, oxamyl did not induce androgenic or anti-androgenic activity in the Hershberger bioassay when administered to castrated male rats up to 0.5 mg/kg per day for 10 consecutive days (Snajdr, 2011b).

In a study performed according to USEPA Guideline 890.1550, oxamyl (purity: 98%) was evaluated for its potential to interact with the steroidogenic pathway for the production of testosterone and estradiol/estrone, using the human cell line H295R steroidogenesis assay. H295R cells cultured in vitro in 24-well plates were incubated with oxamyl (in 0.05% deionized water) at concentrations of 100, 10, 1, 0.1, 0.01, 0.001 and 0.000 1 µmol/L in triplicate for 48 hours. Testosterone and 17β-estradiol levels were measured by HPLC coupled with tandem mass spectrometry (HPLC-MS/MS). Three independent runs were performed. A quality control plate was included with each independent run of the test chemical with known inducer of testosterone and 17β-estradiol production, forskolin at 1 and 10 µmol/L, and inhibitor, prochloraz, at 0.1 and 1 µmol/L.

Oxamyl did not cause biologically relevant changes in 17β-estradiol synthesis relative to the vehicle control in three independent experimental runs. A slight but statistically significant decrease in testosterone levels at 100 µmol/L (the highest concentration tested) was accompanied by a slight reduction in cell viability, but the changes do not follow a dose–response curve. Performance criteria for a valid assay were met.

Under the conditions of this study, the test substance was judged to be negative for the induction or inhibition of steroid biosynthesis (Nabb, 2012).

In a study performed according to USEPA Guideline 890.1150, oxamyl (purity: 98%) was evaluated for its ability to bind to the androgen receptors in rat prostate cytosol. Saturation binding assays were conducted to measure the affinity of a radiolabelled androgen ligand, methyltrienolone (R1881) for the androgen receptor, and the androgen receptor B_{max} in the cytosol. Three independent runs, with three replicates at each concentration, were performed using hexatritiated R1881 ([^{3}H]R1881) as the radioligand. A competitive binding assays conducted to measure the binding of the radioligand to the receptors with increasing concentrations of oxamyl (eight concentrations between 1.0×10^{-10} and 1.0×10^{-3} mol/L). Three independent runs, with three replicates at each concentration, were performed. Radioinert R1881, the androgen receptor agonist reference standard, and dexamethasone, a weak androgen receptor agonist used as the positive control, were used to verify test system performance.

Results from the saturation assay showed that the K_d values were within the range provided by the test guideline, while B_{max} was lower that the suggested guideline range (from 10 to 150 fmol/100 µg protein). Nonspecific binding were slightly higher than the test guideline-suggested value (< 20%) in runs 1 and 3, at 10 nmol/L of [^{3}H]R1881. However, the reference ranges provided in the test guideline should be considered as suggested values rather that performance criteria.

The $\log_{IC50}$ values for radioinert R1881 for each of the three runs were −9.12, −9.01 and −9.01 log mol/L [^{3}H]R1881, with an average of −9.05 log mol/L [^{3}H]R1881. The $\log_{IC50}$ values for dexamethasone for each of the three runs were −4.34, −4.47 and −4.32 log mol/L [^{3}H]R1881, with an average of −4.37 log mol/L [^{3}H]R1881. The relative binding affinity of dexamethasone, compared to radioinert R1881, for each of the three runs was 0.001 7%, 0.002 8% and 0.002 0%, with an average of 0.002 2%. A $\log_{IC50}$ was not determined for oxamyl since there were no test substance-related effects on androgen receptor binding up to the concentration of 1 mmol/L, which represents the highest concentration required by the test guideline.

The performance criteria for radioinert R1881 and dexamethasone were within the acceptable ranges as specified in the test guideline. All runs were within or very close to the suggested performance criteria, and therefore the assay was considered valid.

Under the conditions of the study, oxamyl did not competitively bind to the androgen receptor when tested up to a maximum concentration of 1.0×10^{-3} mol/L. Therefore, oxamyl is not an inhibitor in the androgen receptor binding assay (Snajdr, 2012a).

(d) Studies on metabolites

Acute toxicity studies

The acute toxicity of oxamyl metabolites was studied only in male ChR-CD rats treated orally.

Rats received DMCF (purity unknown) at a single dose of 90–1000 mg/kg bw by gavage. Deaths occurred at 450 mg/kg bw and above. The clinical signs were low posture, salivation, hyper-responsiveness to noise, weakness and body weight loss. The acute oral LD_{50} of DMCF in male rats was greater than 300 mg/kg bw (Ashley, 1974).

DMOA (purity unknown) was administered to rats (5/group) by gavage at doses of 2500 or 5000 mg/kg bw. All the high-dose animals but none of the low-dose animals died. The clinical signs were irregular respiration, low posture, half-closed eyes, pallor, weakness and body weight loss. The acute oral LD_{50} of IN-D2708 in male rats was 3540 mg/kg bw (Barbo, 1972).

Methyl *N*-hydroxy-*N*′-methyl-1-thiooxamimidate (MTO; purity unknown) was administered by gavage to groups of 10 rats/group at doses of 4000–7000 mg/kg bw. At 6000 mg/kg bw and higher, dose-related mortality occurred. At 4500 and 5000 mg/kg bw, no mortality was observed; at 4000 mg/kg bw, one animal died. Clinical signs were lethargy, prostration, ruffled fur, half-closed eyes, polyuria, pallor and body weight loss (Dale, 1973).

DMTO (purity unknown) was administered orally at doses of 90–11 000 mg/kg bw. Mortality occurred at the highest dose. Clinical signs were discomfort, light-coloured faeces, half-closed eyes, salivation and weight loss (Fretz, 1968).

Short-term studies of oral toxicity

In a limited 10-day study, young adult ChR-CD rats (six males) received DMCF (purity unknown) orally at dose of 90 mg/kg bw, 5 days/week for two weeks. Three rats were killed four hours after the last dose, the other three rats were killed after a 14-day recovery period. Treatment decreased body weight and body weight gain, the absolute weights of the liver and kidney, and the absolute and relative weights of spleen and thymus. The weight of testis showed an absolute decrease, but a relative increase. All animals showed vacuolation of hepatocytes of the centrilobular area of the liver. At the end of the treatment period, slight atrophy of spleen, thymus and bone marrow were observed and one animal suffered from acute pancreatitis. After the recovery period, no atrophy was observed. The animals were limp during treatment, and mild lethargy was reported during the recovery period. The study report was very brief, with no data on individuals or groups, and the observed effects were only summarized (Dashiell, 1976).

ChR-CD rats (16/sex per group) received diets containing DMCF (purity: 100%) at a concentration of 50, 150 or 450 ppm (equal to 4.9, 11.4 and 34.3 mg/kg bw per day for males and 4.2, 12.6 and 35.7 mg/kg bw per day for females, respectively). The parameters assessed were clinical signs (daily), feed consumption and body weight (weekly), haematology, clinical chemistry and urine (at 30, 60 and 90 days). After 90 days, rats (6/sex per group) were selected for a one-generation study of reproductive toxicity. The remaining rats were killed; gross and microscopic pathological examinations were performed.

There was no deaths. Body weight gain and feed consumption were decreased in rats at 450 ppm. Exophthalmus and alopecia was observed in two females at 450 ppm, while males had decreased erythrocyte volume fraction and decreased erythrocyte counts after 30, 60 and 90 days; at 90 days, haemoglobin concentration was also decreased. Female rats in this group had lower erythrocyte counts and haemoglobin concentration at 30, 60 and 90 days and lower erythrocytes volume fraction and leukocyte counts at 60 and 90 days. At 150 ppm, males and females had lower erythrocyte counts

and erythrocytes volume fraction, and female rats had lower leukocyte counts at day 90. No gross or micropathological treatment-related effects were noted.

The female rats selected for the reproduction study were paired with three males of the same dietary group, each for a period of five days. In rats at 150 ppm the percentage of matings resulting in pregnancy was decreased. At 450 ppm, the average weight of the pups at weaning was decreased.

The NOAEL for systemic toxicity was 50 ppm (equal to 4.2 mg/kg bw per day) based on reduced white blood cells (WBC). The NOAEL for reproductive toxicity was 450 ppm (equal to 34.3 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 150 ppm (equal to 12.6 mg/kg bw per day), based on reduced pup weight at weaning (Kaplan, 1976).

ChR-CD rats (6 males/group) received DMTO dissolved in corn oil by gavage at doses of 0 or 1000 mg/kg bw per day, 5 days/week for two weeks or 2200 mg/kg bw per day for five days. At 2200 mg/kg bw per day, 4/6 animals died. Clinical signs were stained perineal area, chromodacryorrhoea, weakness, unkemptness and continued body weight loss. The pathological changes were atrophy of the spleen and thymus, hypoplasia of bone marrow, centrilobular necrosis, congestion and haemorrhage of the liver. At 1000 mg/kg bw per day, clinical signs were weakness, unkemptness and body weight loss. Atrophy of the spleen and thymus, hypoplasia of bone marrow and depletion of hepatic glycogen was observed in the three animals killed at the end of treatment; in the three remaining animals allowed to recover for 14 days, the body weight was partially retained. Gross and microscopic examination showed no pathological changes in these animals. The study report was very brief and no group or individual data are reported (Wasileski, 1971).

Genotoxicity

DMCF (purity: 100%) was tested for reverse mutation at doses of 250–10 000 µg/plate, in the absence and presence of an exogenous metabolic system, in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538. The result of the test was negative. Cytotoxicity was evident att 3000 µg/plate and above (Sipple, 1978)

3. Observations in humans

3.1 Single-dose studies in volunteers

A number of toxicologically relevant effects of oxamyl (purity: 97.6%) were determined in human volunteers (5 males/dose, 10 control males; 19–39 years old) given a capsule containing a single dose of 0.005, 0.015, 0.03, 0.06, 0.09 or 0.15 mg/kg bw. The capsules were administered five minutes after a standard breakfast. The study was conducted as a double-blind ascending dose escalation clinical trial in which the dose increased for a subsequent dose group only if the results for all lower groups indicated it was safe to do so. Blood samples for determining erythrocyte and plasma AChE activity were collected at screening, two days, 16 hours and 30 minutes before dosing; every 15 minutes for the first two hours after dosing; and at 3, 4, 6, 8, 12 and 24 hours and 7 ± 2 days after dosing. The baseline was defined as the mean of all available values before dosing. At 16 hours, and 30 minutes before dosing and 1, 2, 3, 4, 8 and 24 hours after dosing, initial pupil size, pupil reaction to and recovery from a light flash, and quantity of saliva secreted within five minutes were assessed. Haematology and clinical chemistry were performed on blood samples taken at screening, 30 minutes before dosing and 24 hours after dosing. Urine was analysed at screening and 24 hours after dosing. To ensure safety, physical examinations were included and vital signs (blood pressure, heart rate, clinical signs), oral temperature and electroencephalographic trace were monitored.

No treatment-related effects were observed in the electroencephalogram, heart rate, pulse, blood pressure, respiratory rate, body temperature, haematology parameters, clinical chemical (except ChE activity) or urine parameters or pupil size and reaction. At doses up to 0.09 mg/kg bw, no effects on salivary secretion were observed. Occasional changes in erythrocyte or plasma AChE activity at doses up to 0.06 mg/kg bw were considered incidental and not toxicologically relevant.

Significant effects on erythrocyte AChE activity (Table 9) and plasma AChE activity were observed in men given 0.09 and 0.15 mg/kg bw. At 0.09 mg/kg bw, small but statistically significant decreases in AChE activity were observed in erythrocytes (7.3% at 30 minutes) and plasma (10–12% from 75–120 minutes) were observed, but these decreases were considered not adverse as they were less than 20% and similar changes in plasma and erythrocyte AChE activities were observed in men in the control group. At 0.15 mg/kg bw, plasma AChE activity was decreased by 21–43% from 30 minutes to two hours after dosing and erythrocyte AChE activity was decreased by 23–28% from 30 to 60 minutes after dosing. These men also had a significant increase in saliva production (161%) one hour after dosing.

The NOAEL was 0.09 mg/kg bw, based on the inhibition of plasma and erythrocyte AChE activity and increased salivation at 0.15 mg/kg bw (McFarlane & Freestone, 1999).

Table 9. Erythrocyte AChE activity in male volunteers after a single oral dose of oxamyl

Time (h)	% change from baseline per dose [a]								
	0 mg/kg bw			0.09 mg/kg bw			0.15 mg/kg bw		
	Mean	Minimum	Maximum	Mean	Minimum	Maximum	Mean	Minimum	Maximum
0.25	4.9	−8	26	1.0	−12	12	−2.3	−25	12
0.5	7.8	−5	27	−7.3*	−18	5	−23.2*	−33	−6
0.75	6.5	−9	22	0.2	−17	13	−27.9*	−38	−6
1	9.4	−4	28	−0.3	−9	8	−27.1*	−43	−17
1.25	5.5	−11	28	−0.7	−9	10	−16.8*	−30	9
1.5	11.1	0	29	1.8	−5	10	−15.9*	−28	−3
1.75	4.5	−17	25	−1.1	−9	6	−9.2*	−19	1
2	7.9	−2	34	−2.1	−15	6	−8.0	−24	3
3	10.3	−4	23	−0.2	−14	15	−0.4	−7	10
4	12.4	−2	31	3.0	−16	16	3.7	−14	15
6	3.5	−20	24	21.3	3	38	10.9	4	22

AChE: acetylcholinesterase; bw: body weight; *: significantly different from baseline

[a] Baseline was defined as the mean of all available values before dosing, at screening and −2 days, −16 h and −30 min.

Source: McFarlane & Freestone (1999)

3.2 Epidemiological studies

A systematic literature search of epidemiological studies, up to May 2017, was conducted in the Medline and Embase databases. Only one study was identified. Data on two case-control study populations of 731 infants with neural tube defects (NTD) (anencephaly, spina bifida cystica, craniorrhachischisis and iniencephaly) and 940 controls delivered in California between 1987 and 1991 were pooled to investigate the relationship between potential maternal exposure to several pesticides, including oxamyl, during early gestation and the risk of NTD. Maternal exposure was evaluated by linking mothers' addresses with agricultural pesticide use reports, obtained from the California Department of Pesticide Regulation, and crop maps. Women were categorized into exposed (residential proximity within 1000 m of pesticide applicators) and not exposed. A slightly but not statistically significantly increased risk for NTD was associated with maternal exposure to oxamyl (odds ratio = 1.4; 95% confidence interval: 0.7–2.8). The main limitation of the study was the assessment of the pesticide exposure, which could lead to misclassification of the potential maternal exposure (Rull, Ritz & Shawn, 2006).

Comments

Biochemical aspects

Absorption of oxamyl was rapid and nearly complete after oral administration to rats. Elimination was rapid, mainly in urine (80% within 24 hours and 95% within 168 hours). The tissue concentrations were low at 168 hours. Studies of biotransformation in vitro and in vivo indicated that oxamyl is metabolized in rats and mice via two major pathways: non-enzymatic hydrolysis to the oxime, DMTO and enzymatic conversion to DMOA via DMCF. These and other metabolites were present as polar conjugates in urine of rats. No marked sex difference was observed in the excretion pattern, tissue distribution or metabolite profile in rats (Harvey & Han, 1978; Belasco, 1979; Chang & Knowles, 1979; Hawkins et al., 1990).

Toxicological data

The oral LD_{50} in rats was 2.5 mg/kg bw; the inhalation median lethal concentration (LC_{50}; four hours, nose-only) in rats was 0.05 mg/L; and the dermal LD_{50} in rabbits was greater than 2000 mg/kg bw. Signs of acute intoxication with oxamyl were consistent with inhibition of AChE activity. Oxamyl was not irritating to eyes or skin of rabbits; however, ocular treatment induced signs of acute intoxication consistent with inhibition of AChE activity (Ladics, 2001b). Oxamyl did not sensitize the skin of guinea pigs and was not phototoxic in vitro (Tayfun, 1969a,b; Hinckle, 1980; Brock, 1988a; Kelly, 2001; Carpenter, 2009; Markell, 2015).

Oxamyl was tested in a number of repeated-dose toxicity studies in mice, rats and dogs. The effect most relevant to humans was inhibition of AChE activity, often accompanied by clinical signs at the same or higher doses. The effect of oxamyl on AChE activity was rapid and completely reversible. The NOAELs after dietary administration were higher than those after treatment by gavage. Other observed effects were on body weight and body weight gain and, to a lesser degree, on feed consumption and feed efficiency, sometimes accompanied by effects on organ weights.

In a 90-day dietary study in dogs fed oxamyl at a concentration of 0, 50, 100 or 150 ppm (equal to 0, 1.5, 2.8 and 5.0 mg/kg bw per day for males and 0, 1.3, 2.6 and 4.2 mg/kg bw per day for females, respectively), the NOAEL was 150 ppm (equal to 4.2 mg/kg bw per day), the highest dose tested. AChE activity was not measured.

In a one-year dietary study in dogs fed oxamyl at a concentration of 0, 50, 150 or 250 ppm (equal to 0, 1.6, 4.6 and 8.0 mg/kg bw per day for males and 0, 1.5, 4.5 and 7.8 mg/kg bw per day for females, respectively), no NOAEL could be identified because of the occurrence of tremors in females and decreased body weights and decreased brain AChE activity in males at 50 ppm (equal to 1.5 mg/kg bw per day), the lowest dose tested (Mebus, 1990).

On the basis of this study, a second one-year dietary study in male dogs was performed to determine the NOAEL for AChE inhibition in dogs. Animals were fed oxamyl at a concentration of 0, 12.5, 20, 35 or 50 ppm (equal to 0, 0.37, 0.58, 0.93 and 1.36 mg/kg bw per day). The NOAEL was 35 ppm (equal to 0.93 mg/kg bw per day) based on equivocal AChE inhibition at 50 ppm (equal to 1.36 mg/kg bw per day) (Dickrell, 1991). The results of these two 1-year studies were combined to establish an overall NOAEL of 35 ppm (equal to 0.93 mg/kg bw per day).

In a two-year dietary study in mice fed oxamyl at a concentration of 0, 25, 50 or 75 ppm (equal to 0, 4.2, 8.7 and 13.5 mg/kg bw per day for males and 0, 5.2, 10.8 and 16.8 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 25 ppm (equal to 5.2 mg/kg bw per day) based on early mortality in females at 50 ppm (equal to 10.8 mg/kg bw per day). No treatment-related increases in tumour incidence were observed. AChE activity was not examined (Adamik, 1981; Tompkins, 1990).

In a two-year dietary study in rats fed oxamyl at a concentration of 0, 25, 50, 100 or 150 ppm (equal to 0, 0.99, 2.0, 4.2 and 7.0 mg/kg bw per day for males and 0, 1.3, 2.7, 6.7 and 11 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 50 ppm (equal to 2.0 mg/kg bw per day) based on effects on body weights and clinical signs at 100 ppm (equal to 4.2 mg/kg bw per day). AChE activity was inadequately measured. No treatment-related increases in tumour incidence were observed (Malley, 1991).

The Meeting concluded that oxamyl is not carcinogenic in mice or rats.

Oxamyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Galloway, 1982; Rickard, 1987; Vincent, 1987; Gladnick, 1999; Gudi & Schadly, 2000; San & Clarke, 2000; Donner, 2002).

The Meeting concluded that oxamyl 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 oxamyl is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive dietary toxicity study in rats fed oxamyl at a concentration of 0, 25, 75 or 150 ppm (equal to 0, 1.4, 4.2 and 8.7 mg/kg bw per day for males and 0, 1.7, 5.4 and 12.2 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 25 ppm (equal to 1.4 mg/kg bw per day) based on decreases in body weight and body weight gain, feed consumption and feed efficiency and increase in relative testis weight (Hurtt, 1990). The NOAEL for reproductive toxicity was 75 ppm (equal to 5.4 mg/kg bw per day) based on reduced pup numbers per litter. The NOAEL for offspring toxicity was 25 ppm (equal to 1.7 mg/kg bw per day) based on reduced pup weights. Similar effects were observed at similar dose levels in an older, three-generation study of reproductive toxicity (Sherman, 1972). Neither study measured brain or erythrocyte AChE activities.

In a developmental toxicity study in rats given oxamyl by gavage at 0, 0.2, 0.5, 0.8 or 1.5 mg/kg bw per day, the NOAEL for maternal toxicity was 0.5 mg/kg bw per day based on decreases in feed consumption and body weight gain and tremors at 0.8 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 0.5 mg/kg bw per day based on decreased fetal body weights at 0.8 mg/kg bw per day (Rickard, 1988; Munley, 1998). AChE activity was not measured.

In a developmental toxicity study in rabbits given oxamyl by gavage at 0, 1, 2 and 4 mg/kg bw per day, the NOAEL for maternal toxicity was 1 mg/kg bw per day based on decreased body weight gain at 2 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 2 mg/kg bw per day based on increased incidence of resorptions at 4 mg/kg bw per day (Hoberman, 1980). AChE activity was not measured.

The Meeting concluded that oxamyl is not teratogenic.

In an acute neurotoxicity study in rats given oxamyl by gavage at 0, 0.1, 1 or 2 mg/kg bw (males) and 0, 0.1, 0.75 or 1.5 mg/kg bw (females), the NOAEL was 0.1 mg/kg bw based on inhibition of brain and erythrocyte AChE activity, clinical signs and effects on FOB parameters at 0.75 mg/kg bw (Malley, 1997b).

In a 90-day neurotoxicity study, rats fed oxamyl at a dietary concentration of 0, 10, 30 or 250 ppm (equal to 0, 0.55, 1.7 and 15 mg/kg bw per day for males and 0, 0.67, 2.0 and 20 mg/kg bw per day for females, respectively), the NOAEL was 30 ppm (equal to 1.7 mg/kg bw per day) based on effects on body weights and body weight gain, clinical effects, the behavioural changes in FOB and the inhibition of AChE activity in brain and erythrocytes (Malley, 1998).

An open literature article on non-guideline-compliant, acute neurotoxicity studies evaluated differences in sensitivities between pups and adult rats in terms of oxamyl inhibiting AChE activity. The data indicated that pups are generally not more sensitive than adults (Malley, 2005).

There was no evidence that a single 20 mg or 40 mg dose of oxamyl to hens induced delayed polyneuropathy (Lee, 1970).

The Meeting concluded that oxamyl is neurotoxic through inhibition of AChE activity.

Oxamyl was tested in a range of guideline- and GLP-compliant in vivo and in vitro assays for its potential to interact with the endocrine system. No significant effects were observed (Snajdr, 2011a,b; 2012a,b,c; Nabb, 2012; Toot, 2012a,b; Willoughby, 2012).

Toxicological data on metabolites and/or degradates

A number of non-guideline, non-GLP toxicity tests were carried out with oxamyl metabolites IN-A2213, IN-D2708 and IN-N0079. Studies were submitted in the form of one-page data sheets.

Metabolite IN-A2213 (also referred to as DMTO or oxamyl oxime)

Metabolite IN-A2213 is a plant, rat and mouse metabolite (44% of radioactivity in murine toxicokinetic studies). The acute oral LD_{50} of IN-A2213 in rats was greater than 7500 mg/kg bw (Fretz, 1968).

Metabolite IN-D2708 (also referred to as DMOA)

Metabolite IN-D2708 is a plant and rat metabolite. No quantitative data on metabolism were available. The acute oral LD_{50} of IN-D2708 in male rats was 3540 mg/kg bw (Barbo, 1972).

Metabolite IN-N0079 (also referred to as DMCF)

Metabolite IN-N0079 is a rat (no quantitative data available) and plant metabolite. Acute oral, short-term toxicity studies and a genotoxicity Ames test were performed with this metabolite.

The acute oral LD_{50} in male rats was greater than 300 mg/kg bw (Ashley, 1974).

In a 90-day dietary toxicity study, which incorporated a modified one-generation reproductive toxicity phase, rats were fed IN-N0079 at a concentration of 0, 50, 150 or 450 ppm (equal to 0, 4.9, 11.4 and 34.3 mg/kg bw per day for males and 0, 4.2, 12.6 and 35.7 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 50 ppm (equal to 4.2 mg/kg bw per day) based on reduced white blood cell counts. The NOAEL for reproductive toxicity was 450 ppm (equal to 34.3 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 150 ppm (equal to 12.6 mg/kg bw per day) based on reduced pup weight at weanling (Kaplan, 1976).

IN-N0079 did not induce reverse mutations in bacteria (Sipple, 1978).

Metabolite IN-QKT34 (IN-A2213 glucoside or metabolite A)

Metabolite IN-QKT34 ([(hexopyranosyloxy)imino]-*N*,*N*-dimethyl-2-(methylthio)acetamide; also referred to as IN-A2213 glucoside or DMTO glucoside)) is a rat (70–80% of radioactivity) and plant metabolite. No specific toxicological data were available, but the Meeting concluded that metabolite IN-QKT34 would be covered by toxicological studies on the parent.

The Meeting noted that the studies provided for metabolites IN-A2213, IN-D2708 and IN-N0079 suggest that these plant and animal metabolites are less toxic than oxamyl. The Meeting also noted that none of the metabolites contain the carbamate moiety responsible for AChE inhibition.

Human data

In a human study male volunteers received a single gelatine capsule containing oxamyl at a dose of 0, 0.005, 0.015, 0.03, 0.06, 0.09 or 0.15 mg/kg bw. The NOAEL was 0.09 mg/kg bw based on erythrocyte AChE activity inhibition (> 20% inhibition within 0.5–1 hour) and increased salivation at 0.15 mg/kg bw (McFarlane & Freestone, 1999).

No information was provided on the health of workers involved in the manufacture or use of oxamyl. No information on accidental or intentional poisoning in humans was made available to the Meeting.

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

Toxicological evaluation

The Meeting reaffirmed the ADI for oxamyl of 0.009 mg/kg bw, established by the 2002 JMPR, on the basis of the NOAEL of 0.09 mg/kg bw per day in a single-dose male volunteer study, in whom increased salivation and decreased erythrocyte AChE activity were observed at higher doses of 0.15 mg/kg bw. A safety factor of 10 was applied.

The toxicological profile of oxamyl showed rapid and complete restoration of AChE activity after inhibition; repeated administration did not change the recovery characteristics. Moreover, no sex differences were found with respect to the effects of oxamyl in experimental animals. The Meeting considered it appropriate to establish both the ADI and the ARfD from a single-dose human volunteer study conducted in males.

The Meeting reaffirmed the ARfD for oxamyl of 0.009 mg/kg bw, established by the 2002 JMPR, on the basis of the NOAEL of 0.09 mg/kg bw per day in a single-dose male volunteer study. A safety factor of 10 was applied. Both the ADI and the ARfD are supported by the NOAEL of 0.1 mg/kg bw in the acute neurotoxicity study in rats.

Levels relevant to risk assessment of oxamyl

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a,b]	Toxicity	25 ppm, equivalent to 5.2 mg/kg bw per day	50 ppm, equivalent to 10.8 mg/kg bw per day
		Carcinogenicity [c]	75 ppm, equal to 13.5 mg/kg bw per day	–
Rat	Two-year study of toxicity and carcinogenicity [a,b]	Toxicity	50 ppm, equal to 2 mg/kg bw per day	100 ppm, equal to 4.2 mg/kg bw per day
		Carcinogenicity [c]	150 ppm, equal to 7 mg/kg bw per day	–
	Two-generation study of reproductive toxicity [a,b]	Reproductive toxicity	75 ppm, equal to 5.4 mg/kg bw per day	150 ppm, equal to 12.2 mg/kg bw per day
		Parental toxicity	25 ppm, equal to 1.4 mg/kg bw per day	75 ppm, equal to 4.2 mg/kg bw per day
		Offspring toxicity	25 ppm, equal to 1.7 mg/kg bw per day	75 ppm, equal to 5.4 mg/kg bw per day
	Developmental toxicity [b,d]	Maternal toxicity	0.5 mg/kg bw per day	0.8 mg/kg bw per day
		Embryo/fetal toxicity	0.5 mg/kg bw per day	0.8 mg/kg bw per day
	Acute neurotoxicity [d]	Neurotoxicity	0.1 mg/kg bw	0.75 mg/kg bw per day
	Ninety-day neurotoxicity [a]	Neurotoxicity	30 ppm, equal to 1.7 mg/kg bw per day	250 ppm, equal to 15 mg/kg bw per day
Rabbit	Developmental toxicity [b,d]	Maternal toxicity	1 mg/kg bw per day	2 mg/kg bw per day
		Embryo/fetal toxicity	2 mg/kg bw per day	4 mg/kg bw per day
Dog	One-year studies of toxicity [a,e]	Toxicity	35 ppm, equal to 0.93 mg/kg bw per day	50 ppm, equal to 1.36 mg/kg bw per day
Human	Study in volunteers with single doses [f]	AChE inhibition, salivation	0.09 mg/kg bw per day	0.15 mg/kg bw per day

[a] Dietary administration. [b] (Adequate) measurement of AChE activity not included. [c] Highest dose tested.
[d] Gavage administration. [e] Two studies combined. [f] Capsule administration.

Estimate of acceptable daily intake for oxamyl, IN-A2213, IN-QKT34, IN-D2708 and IN-N0079

0–0.009 mg/kg bw

Estimate of acute reference dose for oxamyl, IN-A2213, IN-QKT34, IN-D2708 and IN-N0079

0.009 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 oxamyl

Absorption, distribution, excretion and metabolism in animals	
Rate and extent of absorption	Rapid and extensive: 81% within 24 h
Dermal absorption	No data
Distribution	Throughout the body; highest concentration in blood, heart, liver, kidney, lung, spleen and gastrointestinal tract
Potential for accumulation	Low
Rate and extent of excretion	Relatively rapid (mouse: 76% after 6 h, 89% after 24 h; rat: 81% after 24 h); mainly in urine
Metabolism in animals	Extensively metabolized; no parent compound in urine
Toxicologically significant compounds in animals and plants	Oxamyl
Acute toxicity	
Rat, LD_{50}, oral	2.5 mg/kg bw
Rabbit, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	0.05 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating (signs of acute toxicity apparent)
Dermal sensitization	Non-sensitizing (Buehler)
Short-term studies of toxicity	
Target/critical effect	Inhibition of AChE activity in brain and erythrocytes, clinical and behavioural effects associated with AChE inhibition, reduction in body weight and weight gain
Lowest relevant oral NOAEL	0.93 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	2.5 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduction in body weight and body weight gain (AChE activity not assessed)
Lowest relevant NOAEL	2 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mouse or rat
Genotoxicity	Not genotoxic in vitro and in vivo
Reproductive toxicity	
Target/critical effect	Reduction in number of pups per litter (in the presence of parental toxicity)
Lowest relevant parental NOAEL	1.4 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	1.7 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	5.4 mg/kg bw per day (rat)

(*Continued on next page*)

Developmental toxicity	
Target/critical effect	Reduction in body weight (in the presence of maternal toxicity)
Lowest relevant maternal NOAEL	0.5 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	0.5 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	0.1 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	1.7 mg/kg bw per day (rat)
Delayed neurotoxicity	No concern
Developmental neurotoxicity NOAEL	No data
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
IN-A2213	LD_{50} > 7500 mg/kg bw (rat)
IN-D2708	LD_{50} = 3540 mg/kg bw (male rat)
IN-N0079	LD_{50} > 300 mg/kg bw (male rat)
	90-day rat, with a one-generation reproductive toxicity phase: Systemic/parental toxicity NOAEL: 4.2 mg/kg bw per day Offspring toxicity NOAEL: 12.6 mg/kg bw per day Reproductive toxicity NOAEL: 34.3 mg/kg bw per day, the highest dose tested
	Not genotoxic in vitro (Ames test)
Human data	
Single-dose volunteer study	Inhibition of AChE activity in erythrocytes increased saliva production
Lowest relevant oral NOAEL	0.09 mg/kg bw
	No information provided on the health of workers involved in the manufacture or use of oxamyl

Summary

	Value	Study	Safety factor
ADI [a]	0–0.009 mg/kg bw	Acute toxicity (human)	10
ARfD [a]	0.009 mg/kg bw	Acute toxicity (human)	10

[a] Applies to oxamyl, IN-A2213, IN-QKT34, IN-D2708 and IN-N0079.

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Propylene oxide

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Explanation

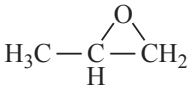
Propylene oxide is the ISO-approved name for methyloxirane (International Union of Pure and Applied Chemistry [IUPAC] name), with the Chemical Abstract Services (CAS) number 75-56-9. Propylene oxide is a highly reactive, volatile compound (boiling-point 34ºC) that is used as a gas or pressurized liquid for fumigation and sterilization to control insect infestations and microbial spoilage in a range of food commodities (e.g. herbs, spices and nuts). The primary residues detected after Propylene oxide use are propylene oxide, propylene chlorohydrin (chloropropanol), propylene bromohydrin (bromopropanol) and propylene glycol.

Propylene oxide was reviewed for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2011 (Annex 1, ref. 122) when an acceptable daily intake (ADI) of 0–0.04 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.04 mg/kg bw were established. The 2011 Meeting was unable to establish ADIs or ARfDs for propylene chlorohydrin or propylene bromohydrin due to shortcomings in the databases.

Propylene oxide was re-reviewed at the 2017 Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). Additional data were made available on Propylene oxide, propylene chlorohydrin and propylene bromohydrin.

The original database for propylene oxide and propylene chlorohydrin consists mainly of published papers, often with limited levels of detail and no statements of compliance with good laboratory practice (GLP). The new studies contain GLP compliance statements and meet the requirements of national or international test guidelines. The structure of propylene oxide is shown in Fig. 1.

Figure 1. Structure of propylene oxide



Evaluation for acceptable daily intake

1 Biochemical aspects

No new data were submitted.

2. Toxicological studies

2.1 Acute toxicity

No new data were submitted.

2.2 Short-term studies of toxicity

No new data were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

No new data were submitted.

2.4 Genotoxicity

No new data were submitted.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

No new data were submitted.

(b) Developmental toxicity

Rat

No new data were submitted.

Rabbit

Groups of 25 inseminated New Zealand White rabbits received propylene oxide (purity 99.99%; batch GATX26473M15) by gavage in water on days 7–28 of gestation. Dose levels were 0, 75, 150 or 300 mg/kg bw per day. On day 29, does were killed and the uterine contents examined. Fetuses were investigated for viability, weight and sex and examined for malformations and variations.

At the dose level of 300 mg/kg bw per day, 10 does died or were euthanized. Maternal body weight gain was significantly lower than controls at 300 mg/kg bw per day. Fetal weights were significantly lower in the 300 mg/kg bw per day group. There were no treatment-related changes in malformations, variations or ossification. An apparent reduction in viable fetuses per litter in treated groups was associated with reduced corpora lutea number, which is defined before dosing is started and is considered to be unrelated to treatment (Table 1).

Table 1. Findings in the developmental toxicity study in rabbits with Propylene oxide

	Measure per dose level			
	0 mg/kg bw per day	**75 mg/kg bw per day**	**150 mg/kg bw per day**	**300 mg/kg bw per day**
Maternal data				
Doe weight gain days 7–10 (g)	65	98	96	51
Doe weight gain days 7–29 (g)	400	391	373	93**
Mean number of corpora lutea/dam	10.6	9.6	10.0	9.1

(*Continued on next page*)

	Measure per dose level			
	0 mg/kg bw per day	75 mg/kg bw per day	150 mg/kg bw per day	300 mg/kg bw per day
Fetal data				
Mean no. of viable fetuses/litter	9.2	8.9	8.4	8.3
Mean fetal weight (g)	41.7	41.8	40.8	33.7**
Number of fetuses with malformations	11	3	6	4

**: $P < 0.01$ *Source*: Edwards (2016)

The no-observed-adverse-effect level (NOAEL) for developmental toxicity was 150 mg/kg bw per day based on reduced fetal weights at 300 mg/kg bw per day.

The NOAEL for maternal toxicity was 150 mg/kg bw per day based on reduced body weights and body weight gain, and mortality at 300 mg/kg bw per day (Edwards, 2016).

2.6 Special studies

(a) Neurotoxicity

No new data were submitted.

(b) Immunotoxicity

No new data were submitted.

(c) Studies on metabolites

Propylene chlorohydrin

New studies of acute oral toxicity, developmental toxicity in rats and rabbits, and acute neurotoxicity in rats were submitted. Studies of repeated-dose toxicity, genotoxicity, chronic toxicity and reproductive toxicity were available in 2011.

Acute oral toxicity – propylene chlorohydrin

Female SD rats were administered propylene chlorohydrin (batch FQLSC; purity 98.8%) in water, in an up-and-down procedure (according to Organisation for Economic Co-operation and Development [OECD] Guideline 425). The acute oral median lethal dose (LD_{50}) was calculated to be 532 (95% confidence limits: 64–2000) mg/kg bw (Kuhn, 2013a). This value is consistent with the studies reviewed in 2011 (Table 2).

Table 2. Summary of acute toxicity studies with propylene chlorohydrin

Species	Strain	Sex	Route	LD_{50} / LC_{50}	Purity (%), batch	Vehicle	Reference
Rat	Sprague Dawley	F	Oral	532 mg/kg bw	98.8 FQLSC	Water	Kuhn (2013a)
Rat	Caworth Wistar	–	Oral	200–250 mg/kg bw	–	Not stated	Cited by NTP (1998)
Guinea pig	–	–	Oral	720 mg/kg bw	–	Not stated	Cited by NTP (1998)
Dog	–	–	Oral	~200 mg/kg bw	–	Not stated	Cited by NTP (1998)
Rabbit	New Zealand White	–	Dermal	500 mg/kg bw	–	Not stated	Cited by NTP (1998)
Rat	–	–	Inhalation 6 h	>3.8 mg/L (1000 ppm)	–	None	NTP (1998)

bw: body weight; F: female; LC_{50}; median lethal concentration; LD_{50}; median lethal dose; ppm: parts per million

Developmental toxicity in rats – propylene chlorohydrin

Groups of 25 inseminated SD rats received propylene chlorohydrin (purity 100%; batch USEWL) by gavage in water on days 6–19 of gestation. Dose levels were 0, 10, 30 or 100 mg/kg bw per day. On day 20, dams were killed and the uterine contents examined. Fetuses were investigated for viability, weight and sex and examined for malformations, anomalies and variations.

Reduced defecation and nasal discharge were reported in dams receiving 100 mg/kg bw per day. At 100 mg/kg bw per day, maternal body weight gain was significantly lower than controls from the start of dosing. Fetal weights were significantly lower in the 100 mg/kg bw per day group. There were no treatment-related changes in malformations, only single incidences with no pattern or dose relationship, or visceral variations. Reduced ossification was noted in some bones in fetuses from the 100 mg/kg bw per day group, consistent with the lower body weight and likely related to retarded development (Table 3).

The NOAEL for developmental toxicity was 30 mg/kg bw per day based on reduced fetal weights and reduced ossification at 100 mg/kg bw per day.

The NOAEL for maternal toxicity was 30 mg/kg bw per day based on reduced body weights and transient body weight loss at 100 mg/kg bw per day (Edwards, 2015a).

Table 3. Findings in the developmental toxicity study in rats with propylene chlorohydrin

	Measure per dose level			
	0 mg/kg bw per day	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**
Maternal data				
Dam weight gain, days 6–9 (g)	12	11	9	−12**
Dam weight gain, days 6–19 (g)	122	120	117	75**
Fetal data				
No. of fetuses/litter	14.7	15.0	15.3	15.3
Mean fetal weight (g)	3.9	3.7	3.7	3.3**
No. of fetuses with malformations	0	2	2	2

bw: body weight; no.: number; **: $P < 0.01$

Source: Edwards (2015a)

Developmental toxicity in rabbits – propylene chlorohydrin

Groups of 25 inseminated New Zealand White rabbits received propylene chlorohydrin (purity 100%; batch USEWL) by gavage in water on days 7–29 of gestation. Dose levels were 0, 10, 50 or 175 mg/kg bw per day. On day 30, does were killed and the uterine contents examined. Fetuses were investigated for viability, weight and sex and examined for malformations and variations.

At 175 mg/kg bw per day, two does exhibited signs of toxicity, aborted and died. Maternal body weight gain was significantly lower than controls at 175 mg/kg bw per day from the beginning of dosing. Fetal weights were significantly lower in the 175 mg/kg bw per day group. There were no treatment-related changes in malformation, variations or ossification. An apparent increase in malformations (primarily lung lobe agenesis) at the low and mid dose levels was not reproduced at the top dose level and is considered to be unrelated to treatment (Table 4).

Table 4. Findings in the developmental toxicity study in rabbits with propylene chlorohydrin

	Measure per dose level			
	0 mg/kg bw per day	10 mg/kg bw per day	50 mg/kg bw per day	175 mg/kg bw per day
Maternal data				
Doe weight gain, days 8–9 (g)	22	31	22	4
Doe weight gain, days 7–29 (g)	424	412	366	189**
Fetal data				
Fetal numbers	169	179	213	163
Mean fetal weight (g)	43.2	42.1	40.7	34.6*
No. with malformations	5	11	12	4
No. with lung lobe agenesis	4	8	7	0

bw: body weight; no.: number; *: $P < 0.05$; **: $P < 0.01$

Source: Edwards (2015b)

The NOAEL for maternal toxicity was 50 mg/kg bw per day based on reduced body weights and body weight gain, abortion and mortality at 175 mg/kg bw per day.

The NOAEL for developmental toxicity was 50 mg/kg bw per day based on reduced fetal weights at 175 mg/kg bw per day (Edwards, 2015b).

Acute neurotoxicity – propylene chlorohydrin

SD rats (10/sex per group) received one dose of propylene chlorohydrin (purity 100%; batch USEWL) by gavage in water. The dose levels of 0, 25, 75 or 200 mg/kg bw were based on effects seen in a range-finding study. An extensive functional observational battery (FOB) was performed pretest, at eight hours post dosing (day 0), the approximate time-to-peak effect in the range-finding study, and on days 7 and 14. Rats were perfused and killed on day 15. Brain weights and dimensions were recorded and microscopic examinations of a range of tissues from the peripheral and central nervous systems from five control and five rats from the highest dose group per sex were performed.

There were no unscheduled deaths or changes in clinical signs and no notable changes in FOB parameters other than motor activity counts. There were no effects on brain weight or morphology nor on the findings of the examination of nervous system tissues. Transient nasal and oral discharges were seen in the 75 and 200 mg/kg bw groups shortly after dosing but had resolved within six hours. These findings are considered to be secondary to gavage dosing with an irritant compound and not relevant to human dietary exposures. Rats in the 200 mg/kg bw groups exhibited body weight loss on the day after dosing, but body weight gains were similar in all groups subsequently. Total and ambulatory activity counts were reduced (by 40–50%) in the first two measurement periods, and overall on day 0 in both sexes receiving the 200 mg/kg bw dose (Table 5). A reduction was also seen in males from the 75 mg/kg bw group, and while the total counts were not statistically significantly lower, the counts for every individual 10-minute segment were markedly below controls. Motor activity counts typically show a large inter-animal and inter-group variation pretest (Table 5). However, the pattern of the activity counts over the different evaluation segments in males at 75 mg/kg bw is considered indicative of a treatment-related adverse effect.

The NOAEL was 25 mg/kg bw based on reduced activity counts in males at 75 mg/kg bw (Herberth, 2015).

Table 5. Motor activity counts in rats in the acute neurotoxicity test with propylene chlorohydrin

	Day	Measure per dose level [a]			
		0 mg/kg bw	25 mg/kg bw	75 mg/kg bw	200 mg/kg bw
Males					
Total activity counts	Pretest	1802 ± 623	1571 ± 619	1650 ± 464	1718 ± 740
	Day 0 [b]	1699 ± 885	1451 ± 805	1275 ± 344	1109 ± 327*
Ambulatory activity counts	Pretest	423 ± 218	387 ± 188	409 ± 130	400 ± 219
	Day 0 [b]	356 ± 207	336 ± 222	294 ± 68	221 ± 50
Females					
Total activity counts	Pretest	2091 ± 721	2168 ± 839	1847 ± 415	2100 ± 1001
	Day 0 [b]	1715 ± 687	1915 ± 593	1810 ± 610	900 ± 347**
Ambulatory activity counts	Pretest	564 ± 160	610 ± 219	477 ± 142	606 ± 286
	Day 0 [b]	482 ± 179	481 ± 169	512 ± 163	211 ± 89 **

bw: body weight; SD: standard deviation; *: $P < 0.04$, **: $P < 0.002$ (linear trend test)

[a] Results expressed as group mean ± SD.

[b] Day of dosing.

Propylene bromohydrin

New studies of acute oral toxicity, 28-day repeated-dose toxicity and genotoxicity have been submitted. The only data available in 2011 were published genotoxicity studies.

Acute oral toxicity – propylene bromohydrin

Female SD rats were administered propylene bromohydrin (purity 98.7%; batch V@SMN)) in water, in an up-and-down procedure (OECD Test Guideline 425). The acute oral LD_{50} was calculated to be 175 (95% confidence limits 29–714) mg/kg bw (Kuhn, 2013b).

Subacute toxicity in rats – propylene bromohydrin

Groups of F344 rats (5/sex per group) received propylene bromohydrin (purity 99.9%; batch 1791061) in water by gavage for 28 days. Dose levels were 0, 10, 30 or 80 mg/kg bw per day. Investigations of haematology, clinical chemistry and gross and microscopic pathology were performed on day 28. Microscopic pathology investigations were restricted to the liver for the 10 and 30 mg/kg bw per day groups, but covered a full range of tissues for control and highest dose groups. An extensive FOB was performed on all rats between days 23 and 25.

There were no treatment-related clinical signs, changes in body weight, haematology, clinical chemistry or alterations in FOB parameters. Motor activity was lower in all treated female groups, but there was no consistent pattern, and inter-group variation was large. Inflammation of the liver was reported in two females in the 80 mg/kg bw per day group and one male in the 30 mg/kg bw per day group. The finding in the females is considered to be a potentially adverse effect of treatment, even though there were no changes in clinical chemistry normally associated with liver damage. There were no other treatment-related changes. A reduction in thymus weight of approximately 16% in females receiving the highest dose had no related changes in histopathology or haematology and is not considered to be adverse.

The NOAEL was 30 mg/kg bw per day based on the finding of liver inflammation in females at 80 mg/kg bw per day (Randazzo, 2016a).

Genotoxicity – propylene bromohydrin

Two in vitro and one in vivo genotoxicity studies were submitted on propylene bromohydrin. These confirmed the genotoxic potential of propylene bromohydrin in vitro and are summarized in Table 6.

Table 6. Results of studies of genotoxicity with propylene bromohydrin

End-point	Test object	Concentration	Purity (%) / batch no.	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 *Escherichia coli* (WP2*uvrA*, CM891, CM871)	25–5000 µg/plate (± S9)	99.9%, batch no. 1791061	Positive with TA100 and TA1535 (± S9) Negative other strains	Hurtado (2016)
Chromosomal aberration	Human lymphocytes (2 male donors)	350, 700, 1400 µg/mL (± S9, 3 h) 115, 224, 448 µg/mL (−S9, 22 h)	99.9%, batch no. 1791061	Positive (+S9, 3 h; –S9, 22 h)	Wells (2016)
In vivo					
Micronucleus assay	CD rat bone marrow (gavage)	0, 20, 40, 80 mg/kg bw per day (3 d)	99.9%, batch no. 1791061	Negative	Randazzo (2016b)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Gene mutation in bacteria. Propylene bromohydrin was positive in an Ames test in strains TA100 and TA1535 with and without metabolic activation, from 100 µg/plate and greater concentrations (Hurtado, 2016).

Chromosomal aberrations in vitro. Propylene bromohydrin was positive in a chromosomal aberration test with human lymphocytes, with and without metabolic activation at 1400 µg/mL after a 3-hour incubation and 448 µg/mL after a 22-hour incubation. There were no effects on polyploidy or endoreduplication (Wells, 2016).

In vivo micronucleus. Propylene bromohydrin was negative in a micronucleus assay with rat peripheral blood and bone marrow cells. CD rats were dosed with propylene bromohydrin in water by gavage. Doses were 0, 20, 40 or 80 mg/kg bw per day for three days. There were no signs of toxicity other than nasal and oral discharge (Randazzo, 2016b).

Read-across – propylene chlorohydrin and bromohydrin

A comparison of propylene bromohydrin and propylene chlorohydrin using (Q)SAR (quantitative structure–activity relationships) methodology identified the same structural alerts for both molecules.

The results on the limited studies with propylene bromohydrin have been compared with equivalent data on propylene oxide and propylene chlorohydrin (Table 7). The data show a consistent pattern of genotoxicity with all three compounds: positive in TA100 and TA1535; positive in chromosomal aberration assays in vitro; and negative in oral in vivo micronucleus assays. All three compounds are of moderate acute oral toxicity. In acute and repeated-dose toxicity studies, the toxicity of propylene oxide is related to site of contact irritant effects, whereas propylene chlorohydrin and propylene bromohydrin have systemic effects including a common target of the liver. Taking account of dose spacing and the limited database, propylene bromohydrin appears to be approximately two to three times more potent than propylene chlorohydrin. Direct comparisons of potency cannot be made as there are no studies where both propylene bromohydrin and propylene chlorohydrin have been tested using identical protocols.

Although the database on propylene bromohydrin is limited, there is a degree of similarity in the findings with propylene chlorohydrin: the chemical structures differ only by the substitution of one halide atom with another. Reading across from the data on propylene chlorohydrin to address the toxicity of propylene bromohydrin can be performed with appropriate consideration of relative potency and uncertainties associated with read-across.

Table 7. Comparison of findings in studies with Propylene oxide, propylene chlorohydrin and propylene bromohydrin

End-point	Propylene oxide	Propylene chlorohydrin	Propylene bromohydrin
Acute oral LD_{50} (mg/kg bw)	300–1000	200–532	175
28-day NOAEL (mg/kg bw per day)	200	35 (90 d)	30
28-day LOAEL (mg/kg bw per day)	300	100 (90 d)	80
28-day target tissue	Gastric irritation	Blood, liver, pancreas	Liver
Rat developmental NOAEL (mg/kg bw per day)	No oral data	30	No data
Rabbit developmental NOAEL (mg/kg bw per day)	150	50	No data
Ames test	Positive; TA100 and TA1535 (± S9)	Positive; TA100 and TA1535 (± S9)	Positive; TA100 and TA1535 (± S9)
Cytogenetics	Positive (± S9)	Positive (± S9)	Positive (± S9)
In vivo genotoxicity	Negative oral micronucleus Positive i.p. micronucleus	Negative micronucleus (14 weeks)	Negative micronucleus (three doses)
Chronic toxicity NOAEL (mg/kg bw per day)	40	> 34 (HDT)	No data
Carcinogenicity (mg/kg bw per day)	40	> 34 (HDT)	No data

bw: body weight; i.p.: intraperitoneal; LD_{50}: median lethal dose;
NOAEL: no-observed-adverse-effect level; LOAEL: lowest-observed-adverse-effect level

3. Observations in humans

No new data were submitted.

Comments

Biochemical aspects

There were no reliable in vivo data on the kinetics or biotransformation of propylene oxide. By analogy with ethylene oxide, it is likely that propylene oxide is rapidly and extensively absorbed via the inhalation route. Oral exposure to propylene oxide is likely to result in hydrolysis to propylene glycol in the stomach. In vitro work has shown that propylene oxide hydrolyses significantly more rapidly in human synthetic gastric juice (pH 1.48; $t_{½} \sim 2$ minutes) than in rat synthetic gastric juice (pH 4.8; $t_{½} > 2$ hours). Absorbed propylene oxide is likely to be hydrolysed to propylene glycol by epoxide hydrolase or bound to non-protein sulfhydryl groups such as glutathione. There were no data that permit comparison of systemic exposures to propylene oxide by the inhalation and oral routes. It is expected that inhalation exposures to propylene oxide will result in greater systemic levels than equivalent oral exposures when taking into account the likely hydrolysis rates in the human stomach combined with kinetic data on propylene oxide levels in blood following inhalation exposure and a physiologically based pharmacokinetic model for inhalation exposures to propylene oxide.

For the purposes of this assessment, a simplistic conversion between inhalation exposures to propylene oxide and oral dosing has been performed. This conversion assumed standard breathing rates and volumes, a rat body weight of 250 g and 100 % absorption via each exposure route. The conversion resulted in an atmospheric concentration of 100 ppm (240 mg/m^3) inhaled for six hours/day, five days/week, being approximately equivalent to an oral dose of 40 mg/kg bw per day in rats and 80 mg/kg bw per day in mice. This is likely to be a conservative estimate for systemic Propylene oxide exposures via the oral route.

Toxicological data

The acute toxicity of propylene oxide has been investigated orally (LD_{50} values, 300–1000 mg/kg bw), dermally (LD_{50} = 950–1250 mg/kg bw) and by inhalation (LC_{50} = 1–9.5 mg/L). propylene oxide is an irritant to skin, respiratory tract and eyes. There are no data on its sensitizing potential.

Short-term studies of toxicity with propylene oxide have been performed in mice and rats, mainly via the inhalation route, in which no systemic effects other than body weight deficits were evident. No effects on the nasal cavity were reported in rats or mice exposed for 14 weeks (six hours/day, five days/week) at up to 500 ppm. In a gavage study in rats dosed 18 times in 24 days, reduced body weight gain, gastric irritation and hepatotoxicity were reported at 300 mg/kg bw per day, with a NOAEL of 200 mg/kg bw per day.

In a chronic toxicity and carcinogenicity study in mice exposed via inhalation at 200 or 400 ppm for six hours/day, five days/week, survival was reduced at both concentrations. Body weights were significantly lower in the 400 ppm groups during the second half of the study. Inflammation of the nasal epithelia was seen in all treated groups. Low incidences of squamous cell carcinoma and adenocarcinoma of the nasal epithelia were present in high-dose animals. There was also an increase in haemangiosarcoma and haemangioma of the vascular plexus below the nasal epithelium. An increase in mammary gland adenocarcinoma seen in females was statistically significant in the high-dose group when corrected for survival; the incidences were within the historical control range and considered to be not clearly treatment related. A no-observed-adverse-effect concentration (NOAEC) for site of contact toxicity could not be derived for this study due to the inflammation of the nasal epithelia seen at both concentrations. The NOAEC for carcinogenicity was 200 ppm (~160 mg/kg bw per day orally) based on the nasal tumours seen at 400 ppm (~320 mg/kg bw per day orally). The NOAEC for systemic toxicity was 200 ppm (~160 mg/kg bw per day orally) based on reduced body weight gain at 400 ppm (320 mg/kg bw per day orally).

In a published 150-week study, female rats were exposed to propylene oxide by gavage twice a week at 15 or 60 mg/kg bw per administration, in a volume of 1 mL (equal to 4.3 or 17 mg/kg bw per day). The extent of the tissues examined and level of reporting were less than those carried out in a normal regulatory study, with minimal or no reporting of body weights, clinical signs or non-neoplastic lesions. Within the limitations of the investigative procedure, the only organ with an increased incidence of non-neoplastic lesions (hyperkeratosis) or tumours was the stomach/forestomach (data not presented separately). The incidence of squamous cell carcinoma in the stomach/forestomach showed a clear dose–response relationship. The lowest dose level gave a slight increase in squamous cell carcinoma of the stomach/forestomach. The NOAEL for carcinogenicity was less than 4.3 mg/kg bw per day. The study did not demonstrate a NOAEL for chronic toxicity because of the presence of hyperkeratosis at 4.3 mg/kg bw per day, the lowest dose tested.

In a 28-month inhalation study in rats, survival was reduced in the 300 ppm groups and in 100 ppm females at the end of the study (after week 115). Body weights were reduced in the 300 ppm groups. Increases in relative liver weights (10–15%) were statistically significant at 300 ppm in males sacrificed at 24 and 28 months and in females sacrificed at 24 months. Local effects on the basal mucosa, nasal turbinates and olfactory epithelium were seen at 300 ppm and occasionally at 100 ppm from 12 months onwards. Non-neoplastic findings were seen in the heart, liver, lung and kidneys at 300 ppm; the effects at 100 and 30 ppm are unclear due to the limited number of tissues examined. There were no increases in tumour incidence in the nose or respiratory tract. Increased incidences of mammary gland fibroadenomas and thyroid tumours (follicular cell adenoma and parafollicular cell adenoma) were recorded in the 300 ppm groups. The incidences of multiple mammary gland tumours were increased in all treated female groups but were reported to be within the historical control range. The NOAEC for systemic effects was 100 ppm (~40 mg/kg bw per day orally) based on body weight gain reductions at 300 ppm (~120 mg/kg bw per day orally). The increased mortality at 100 ppm at week 115 was not considered relevant as this occurred beyond the normal lifespan of laboratory rats.

In a second chronic inhalation study, rats were exposed to propylene oxide for six hours/day, five days/week, for two years. Body weights were slightly lower (< 10%) in the 400 ppm groups than in controls. Inflammation of the nasal cavity was increased at 400 ppm and in males at 200 ppm. Tumours of the nasal cavity (papillary adenoma) were increased in both sexes at 400 ppm, outside the historical control range. Other tumours showing increased incidences were mammary gland, uterus and thyroid

tumours in females. The uterine stromal sarcoma incidences were above the historical control range at both concentrations of propylene oxide but did not exhibit a dose–response relationship. The thyroid gland C-cell tumours were at the upper end of the historical control range, and as there was no related increase in hyperplasia, the relationship to propylene oxide was considered equivocal. The mammary gland tumours were not increased statistically significantly and were within the historical control range, but are consistent with results in other studies, and their relationship to propylene oxide is equivocal. The NOAEC for tumours was 200 ppm (~80 mg/kg bw per day orally) based on the increase in papillary adenomas of the nasal cavity at 400 ppm (~160 mg/kg bw per day orally). The NOAEC for chronic site of contact toxicity was less than 200 ppm (~80 mg/kg bw per day orally) based on nasal cavity inflammation. For systemic toxicity, the NOAEC was 200 ppm (~80 mg/kg bw per day orally) based on reduced body weight gain at 400 ppm (~160 mg/kg bw per day orally).

Evidence of carcinogenicity was seen in long-term studies of toxicity and carcinogenicity with propylene oxide in rats via both oral (stomach/forestomach) and inhalation routes (nasal cavity and mammary tumours) and in mice via inhalation (nasal cavity and mammary tumours). The relevance of these tumours to human exposures to relatively low levels of propylene oxide via the diet is equivocal. In vitro work has shown that propylene oxide hydrolyses significantly more rapidly in human synthetic gastric juice than in rat synthetic gastric juice. This indicates that the stomach tumours seen in the rat gavage study might be associated with a much more prolonged exposure to propylene oxide than would occur in humans.

Similarly, the nasal cavity tumours seen in the inhalation studies with rats and mice could be associated with chronic irritation of the epithelial cells and depletion of sulfhydryl groups and not relevant to oral exposures. However, there have been no specific mechanistic investigations to demonstrate that site of contact mutagenic effects do not occur. A threshold concentration for nasal tumours in chronic studies appears to be 300 ppm (720 mg/m^3), which is consistent with data on non-protein sulfhydryl group depletion in nasal mucosa.

In mice and rats exposed to propylene oxide by inhalation, increases in mammary tumours were noted, but these were reported to be inside the historical control ranges.

The Meeting concluded that there was no convincing evidence that propylene oxide caused systemic tumorigenicity in mice and rats.

The potential genotoxicity of propylene oxide has been investigated in an adequate battery of tests in vitro and in vivo. Positive results were seen in a range of in vitro assays. In vivo assays (for micronuclei and dominant lethal mutations) using oral administration were negative; positive results were seen following high-dose intraperitoneal administration in mice and a high-concentration inhalation study in fruit flies. There were no in vivo data from tissues directly exposed to propylene oxide rather than its metabolites. propylene oxide produces deoxyribonucleic acid (DNA) adducts (primarily N7G, plus N3A, N3C and N1A) in respiratory mucosa and liver of exposed rats, and 1-hydroxypropyl-adenine was reported in the leukocytes of a group of propylene oxide production plant workers.

The Meeting concluded that propylene oxide is genotoxic in vitro but is unlikely to be genotoxic via the oral route due to hydrolysis to propylene glycol in the stomach.

The Meeting concluded that propylene oxide is carcinogenic to experimental animals at the site of initial contact, but because of the likely rapid hydrolysis to propylene glycol in the human stomach and negative genotoxicity in vivo via oral administration, it is unlikely to be carcinogenic to humans following exposure via the oral route to propylene oxide residues in the diet.

In a rat reproductive toxicity study using inhalation exposure, no effects were reported on mating performance, fertility, litter size, pup survival or development at the highest concentration tested (300 ppm, six hours/day, five days/week). Reduced body weight gain was seen in parental animals and pups at 300 ppm. The NOAEC for reproductive toxicity was 300 ppm (~120 mg/kg bw per day orally), the highest dose tested. The NOAEC for parental and pup toxicity was 100 ppm (~40 mg/kg bw per day orally) based on reduced body weight gain at 300 ppm (~120 mg/kg bw per day orally).

The Meeting concluded that propylene oxide does not adversely affect reproduction via the inhalation route at exposure concentrations producing parental toxicity.

In a well-reported developmental toxicity study, rats were exposed to propylene oxide at 0, 100, 300 or 500 ppm for six hours/day on gestation days 6–15. Maternal body weight gain was reduced at 500 ppm. There was no increase in malformations, and the NOAEC for teratogenicity was 500 ppm (~260 mg/kg bw per day orally)[1]. There were no effects on litter size, postimplantation losses, fetal viability or litter size. The only significant developmental finding was an increase in accessory cervical ribs at 500 ppm. The NOAECs for maternal and developmental effects were both 300 ppm (~160 mg/kg bw per day orally).

In a limited developmental toxicity study, rats were exposed by inhalation to a single concentration of propylene oxide (500 ppm) for seven hours/day during various phases of gestation. Body weight gain was reduced in treated animals, whereas kidney, liver, lung and spleen weights were increased. Decreases were reported in numbers of corpora lutea and implantation sites and in live fetus weights, lengths and numbers. The only visceral, skeletal or external alterations were increased incidences of wavy ribs and reduced ossification of the ribs and vertebrae in the exposed groups. The single air concentration tested (500 ppm; ~200 mg/kg bw per day orally) was a NOAEC for teratogenicity and a lowest-observed-adverse-effect concentration (LOAEC) for maternal and developmental toxicity.

In an almost identical study in rabbits, there were reductions in maternal body weight gain, histopathological changes to a number of organs and increases in resorptions and minor skeletal abnormalities. There were no reported increases in malformations. The single concentration tested (500 ppm; ~75 mg/kg bw per day orally) was reported to be a NOAEC for teratogenicity and a LOAEC for maternal toxicity and developmental toxicity in rabbits.

In an oral developmental toxicity study, rabbits were exposed to propylene oxide at 0, 75, 150 or 300 mg/kg bw per day by gavage on gestation days 7–28. Maternal deaths and reductions in maternal and fetal body weights were reported at 300 mg/kg bw per day. There were no reported increases in malformations or fetal anomalies/variations. The NOAELs for maternal toxicity and embryo/fetal effects were 150 mg/kg bw per day (Edwards, 2016).

The Meeting concluded that propylene oxide is not teratogenic.

Toxicological data on metabolites and/or degradates

Propylene chlorohydrin

Propylene chlorohydrin (1-chloro-2-propanol, 2-chloro-1-propanol) is a plant metabolite formed following the use of propylene oxide. Data have been generated on a 3:1 mixture of 1-chloro-2-propanol and 2-chloro-1-propanol.

Limited, qualitative data indicate that propylene chlorohydrin is absorbed following oral administration, conjugated to glucuronic acid or glutathione and excreted in the urine.

The acute toxicity of propylene chlorohydrin has been investigated via the oral route (rat $LD_{50} > 200$ mg/kg bw), the dermal route (rabbit $LD_{50} = 500$ mg/kg bw) and by inhalation ($LC_{50} > 3.8$ mg/L). Propylene chlorohydrin was not irritating to rabbit skin but was a severe eye irritant. There were no data on the skin-sensitizing potential of propylene chlorohydrin.

In a 14-day drinking-water study in mice, reductions in body weight were seen at the top dose level (10 000 mg/L). Alterations in pancreatic acinar cells and pancreatic degeneration and hepatocyte vacuolation were reported at 3300 mg/L and above. The NOAEL was 330 mg/L (equivalent to 33 mg/kg bw per day) based on hepatocyte vacuolation at 1000 mg/L (equivalent to 100 mg/kg bw per day). In a subsequent 14-week study, findings were similar (including pancreatic acinar cell degeneration and fatty change of the pancreas), but it was not possible to identify a NOAEL due to hepatocyte vacuolation at the lowest dose tested, 33 mg/L (equal to 7 mg/kg bw per day).

In a 14-day drinking-water study in rats, reduced body weight was seen at high dose levels. Indications of red cell effects (splenic haematopoiesis, bone marrow atrophy) and pancreatic degeneration/acinar cell changes were seen at 1000 mg/L (equal to 100 mg/kg bw per day). A NOAEL could not be determined due to the limited investigations at dose levels below 1000 mg/L (equal to 100 mg/kg bw per day). In an equivalent 14-week study, body weight, erythrocyte, pancreas and liver effects were seen at 1000 mg/L, with a NOAEL of 330 mg/L (equal to 35 mg/kg bw per day).

[1] A different conversion rate was used, as exposures occurred every day as opposed to five days/week.

Chronic toxicity and carcinogenicity studies have been performed in mice and rats exposed to propylene chlorohydrin in the drinking-water for two years. In both of the studies, there were no indications of carcinogenicity or general toxicity, including of the pancreas and liver. Haematological and clinical chemistry examinations were not performed. The NOAELs were the highest concentrations tested, 1000 mg/L (equal to 100 mg/kg bw per day) in mice and 650 mg/L (equal to 34 mg/kg bw per day) in rats.

The potential genotoxicity of propylene chlorohydrin has been investigated in an adequate battery of tests in vitro and in vivo. Positive results were seen in a range of in vitro assays. Negative results were seen in vivo with oral administration, although a mutation assay in *Drosophila* using injection administration was positive.

The Meeting concluded that propylene chlorohydrin is genotoxic in vitro but recognized that there was a negative in vivo assay.

Taking note of the absence of genotoxicity in vivo in mammals and the absence of carcinogenicity in rats and mice, the Meeting concluded that propylene chlorohydrin is unlikely to be carcinogenic to humans.

In a continuous breeding reproductive toxicity study, rats were exposed to propylene chlorohydrin in drinking-water over two generations. Reduced body weight gain was seen in dams and pups at 650 mg/L. There were no adverse effects on reproduction or pup viability at any dose level. An increase in numbers of abnormal sperm and slightly extended estrous were reported in parental animals at 1300 mg/L, but these were without any reproductive consequence and are considered not to be adverse. The reproductive NOAEL was 1300 mg/L (equal to 130 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 300 mg/L (equal to 30 mg/kg bw per day) based on reduced body weights at 650 mg/L (equal to 65 mg/kg bw per day). The NOAEL for offspring toxicity was 300 mg/L (equal to 30 mg/kg bw per day) based on reduced body weight gain at 650 mg/L (equal to 65 mg/kg bw per day).

The Meeting concluded that propylene chlorohydrin is not toxic to reproduction.

In a limited developmental toxicity study, propylene chlorohydrin was administered to five pregnant rats per group. Fetuses were examined only for gross external abnormalities. Maternal body weight gain was reduced at the top dose level of 125 mg/kg bw per day. There were no treatment-related increases in external findings and no effects on viable fetal numbers. This study is inadequate, with respect to group size and extent of investigations, to permit identification of a NOAEL for developmental toxicity.

In a study of developmental toxicity, propylene chlorohydrin was administered to pregnant rats by gavage at 0, 10, 30 or 100 mg/kg bw per day on gestation days 6–19. Maternal body weight gain was reduced at 100 mg/kg bw per day throughout the dosing period, with body weight loss at the start of the dosing period. Fetal weights in the 100 mg/kg bw per day group were lower than in controls. There were no reported increases in malformations or fetal anomalies. A reduced level of ossification was reported in the 100 mg/kg bw per day group. The NOAELs for maternal toxicity and embryo/fetal toxicity were 30 mg/kg bw per day (Edwards, 2015a).

In a developmental toxicity study, propylene chlorohydrin was administered to pregnant rabbits by gavage at 0, 10, 50 or 175 mg/kg bw per day on gestation days 7–29. Maternal body weight gain was reduced at 175 mg/kg bw per day throughout the dosing period. Fetal weights in the 175 mg/kg bw per day group were lower than controls. There were no treatment-related increases in malformations or fetal anomalies or changes in ossification. The NOAELs for maternal toxicity and embryo/fetal toxicity were 50 mg/kg bw per day (Edwards, 2015b).

The Meeting concluded that propylene chlorohydrin is not teratogenic.

In an acute neurotoxicity study, rats were administered a single dose of propylene chlorohydrin by gavage at 0, 25, 75 or 200 mg/kg bw. There were no deaths or changes in clinical signs and no notable changes in FOB parameters other than motor activity counts. There were no effects on brain weight or morphology or on nervous system tissues. Transient body weight loss and reductions in motor activity counts were reported in the 200 mg/kg bw groups. Reduced motor activity counts were seen in males at 75 mg/kg bw. The NOAEL was 25 mg/kg bw (Herberth, 2015b).

The Meeting concluded that propylene chlorohydrin is not neurotoxic.

Propylene bromohydrin

Propylene bromohydrin (1-bromo-2-propanol; 2-bromo-1-propanol) is a plant metabolite formed following the use of propylene oxide.

The acute toxicity of propylene bromohydrin has been investigated via the oral route (LD_{50} = 175 mg/kg bw) (Kuhn, 2013b).

In a 28-day study of toxicity, rats were administered propylene bromohydrin by gavage at 0, 10, 30 or 80 mg/kg bw per day. The NOAEL was 30 mg/kg bw per day based on liver inflammation in females at 80 mg/kg bw per day (Randazzo, 2016a).

Genotoxicity data show that propylene bromohydrin is genotoxic in vitro (Hurtado, 2016; Wells, 2016), but negative results were obtained in an in vivo micronucleus assay (Randazzo, 2016b). Comparative data in the published literature indicate that in some bacterial mutagenicity tests, bromopropanol derivatives were more potent mutagens than the equivalent chloro-compounds.

The Meeting concluded that propylene bromohydrin is genotoxic in vitro but recognized that there was a negative in vivo assay.

A comparison of the toxicity of propylene oxide, propylene chlorohydrin and propylene bromohydrin identified a consistent pattern of genotoxicity. The limited in vivo toxicity data on propylene bromohydrin indicate it is qualitatively similar but more potent than propylene chlorohydrin by a factor of about three.

Propylene glycol

Propylene glycol (1,2-propanediol) is a plant metabolite formed following the use of propylene oxide. It is also an approved food additive (e.g. E1520). It was reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 (FAO/WHO, 2002), when an ADI of 0–25 mg/kg bw was derived.

Hydroxypropylvaline adducts of haemoglobin have been detected in workers in industrial facilities using or producing propylene oxide. 1-Hydroxypropyladenine was reported in the leukocytes of a group of propylene oxide production plant workers. Epidemiological studies of workers exposed to propylene oxide as well as other chemicals have been inconclusive.

Epidemiological studies of workers in plants producing propylene chlorohydrin and other chlorinated hydrocarbons identified an excess of mortality due to pancreatic cancer, leukaemia and all lymphatic and haematopoietic cancers. The involvement, if any, of propylene chlorohydrin in these effects is unclear.

The Meeting concluded that the existing database on propylene oxide was adequate to characterize the potential hazards to the general population, fetuses, infants and children by the inhalation route.

Most of the database on propylene oxide consists of studies by the inhalation route. Taking account of the likely hydrolysis to propylene glycol following oral exposure, the Meeting concluded that the overall database of inhalation studies, supplemented by an oral developmental toxicity study in rabbits, was adequate to provide sufficient information to assess the risks to the general population fetuses, infants and children via the oral route.

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

The Meeting concluded that the existing database on propylene bromohydrin, in combination with the database on the structurally very similar compound propylene chlorohydrin, was adequate to provide sufficient information to assess the risks to the general population, fetuses, infants and children.

Toxicological evaluation

Propylene oxide

The Meeting re-affirmed the ADI of 0–0.04 mg/kg bw derived from the NOAEC for systemic effects (reduced body weight gain) in the chronic inhalation studies in rats of 100 ppm (equivalent to approximately 40 mg/kg bw per day orally), supported by the NOAEC of 100 ppm (equivalent to approximately 40 mg/kg bw per day orally) for offspring and parental toxicity (reduced body weight gain) in the reproductive toxicity study in rats. Kinetic and metabolic data indicate that there is likely to be greater systemic exposure to propylene oxide following inhalation exposures relative to equivalent oral exposures; thus, the extrapolation is likely to be conservative. A safety factor of 1000 was applied. An additional factor of 10 was applied to the default safety factor of 100 to maintain a margin of 100 to the lowest-observed-adverse-effect level (LOAEL) of 4.3 mg/kg bw per day for findings at the site of contact in the 150 week gavage carcinogenicity study for which a mode of action had not been conclusively determined. The 150-week oral study in rats was not used in the establishment of the ADI, as there was limited investigation of non-neoplastic systemic effects and the critical findings reported were local effects in the rat stomach that are considered not relevant to human exposures to propylene oxide residues in the diet.

The Meeting re-affirmed the ARfD of 0.04 mg/kg bw on the same basis as the ADI.

The Meeting concluded that there was inadequate information to support the derivation of a value based on specific acute effects from dietary exposure.

Propylene chlorohydrin

The Meeting established an ADI of 0–0.3 mg/kg bw based on the NOAEL of 25 mg/kg bw per day in the acute neurotoxicity study in rats based on reduced motor activity in males at 75 mg/kg bw per day, and using a 100-fold safety factor. This is supported by NOAELs of 30 mg/kg bw per day for maternal toxicity and embryo/fetal toxicity in the developmental toxicity study in rats and for parental and offspring toxicity in the multigeneration reproductive toxicity study in rats.

The Meeting established an ARfD of 0.3 mg/kg bw based on the NOAEL of 25 mg/kg bw per day in the acute neurotoxicity study in rats based on reduced motor activity at 75 mg/kg bw per day, and using a 100-fold safety factor. This is supported by the NOAEL in the rat developmental toxicity study based on body weight loss at the start of the dosing period.

Propylene bromohydrin

The Meeting established an ADI of 0–0.03 mg/kg bw based on read across from the ADI for propylene chlorohydrin with an additional factor of 10. The Meeting concluded that based on the closely related chemical structure and a comparison of the available toxicity data, propylene bromohydrin was likely to have similar toxicity to propylene chlorohydrin. However, there were uncertainties in the read-across as not all end-points had been tested, there were no single studies which used both compounds and there was evidence propylene bromohydrin was of greater potency than propylene chlorohydrin. The Meeting concluded that these uncertainties could be addressed by the application of an extra 10-fold factor.

The Meeting established an ARfD of 0.03 mg/kg bw on the same basis as the ADI, as the ADI was based on some effects seen after a single dose.

Levels relevant to risk assessment of propylene oxide

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a]	Toxicity	200 ppm (~160 mg/kg bw per day orally) [b]	400 ppm (~320 mg/kg bw per day orally) [b]
		Carcinogenicity	200 ppm (~160 mg/kg bw per day orally) [b]	400 ppm (~320 mg/kg bw per day orally) [b]
Rat	Two-year study of toxicity and carcinogenicity [c]	Toxicity	–	4.3 mg/kg bw per day [d]
		Carcinogenicity	–	4.3 mg/kg bw per day [d]
	Twenty-eight month study of toxicity and carcinogenicity [a]	Toxicity	100 ppm [e] (~40 mg/kg bw per day orally) [b]	300 ppm (~120 mg/kg bw per day orally) [b]
		Carcinogenicity	300 ppm [f] (~120 mg/kg bw per day orally) [b]	–
	Multigeneration study of reproductive toxicity [a]	Reproductive toxicity	300 ppm [f] (~120 mg/kg bw per day orally) [b]	–
		Parental toxicity	100 ppm (~40 mg/kg bw per day orally) [b]	300 ppm (~120 mg/kg bw per day orally) [b]
		Offspring toxicity	100 ppm (~40 mg/kg bw per day orally) [b]	300 ppm (~120 mg/kg bw per day orally) [b]
	Developmental toxicity study [a]	Maternal toxicity	300 ppm (~160 mg/kg bw per day orally) [g]	500 ppm (~260 mg/kg bw per day orally) [g]
		Embryo and fetal toxicity	300 ppm (~160 mg/kg bw per day orally) [g]	500 ppm (~260 mg/kg bw per day orally) [g]
Rabbit	Developmental toxicity study [c]	Maternal toxicity	150 mg/kg bw per day	300 mg/kg bw per day
		Embryo and fetal toxicity	150 mg/kg bw per day	300 mg/kg bw per day

[a] Inhalation exposure.

[b] Assuming 100 ppm = 240 mg/m^3, 100% absorption; 250 g body weight; standard breathing rates and volumes; exposures for six hours/day, five days/week.

[c] Gavage dosing.

[d] Lowest concentration/dose tested.

[e] Limited examination.

[f] Highest concentration tested.

[g] Assuming 100 ppm = 240 mg/m^3, 100% absorption; 250 g body weight; standard breathing rates and volumes; exposures for six hours/day on gestation days 6–15.

Estimate of acceptable daily intake (ADI)

0–0.04 mg/kg bw

Estimate of acute reference dose (ARfD)

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; further information to establish the mode of action underlying the stomach tumours seen following gavage administration of propylene oxide

Levels relevant to risk assessment of propylene chlorohydrin

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity [a]	Toxicity	100 mg/kg bw per day [b]	–
		Carcinogenicity	100 mg/kg bw per day [b]	–
Rat	Study of acute neurotoxicity [c]	Toxicity	25 mg/kg bw	75 mg/kg bw
	Two-year study of toxicity and carcinogenicity [a]	Toxicity	34 mg/kg bw per day [b]	–
		Carcinogenicity	34 mg/kg bw per day [b]	–
	Multigeneration study of reproductive toxicity [a]	Reproductive toxicity	130 mg/kg bw per day [b]	–
		Parental toxicity	30 mg/kg bw per day	65 mg/kg bw per day
		Offspring toxicity	30 mg/kg bw per day	65 mg/kg bw per day
	Developmental toxicity study [c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo/fetal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
Rabbit	Developmental toxicity study [c]	Maternal toxicity	50 mg/kg bw per day	175 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	175 mg/kg bw per day

[a] Drinking-water administration.

[b] Highest dose tested.

[c] Gavage administration.

Estimate of acceptable daily intake (ADI)

0–0.3 mg/kg bw

Estimate of acute reference dose (ARfD)

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

Levels relevant to risk assessment of propylene bromohydrin

Species	Study	Effect	NOAEL	LOAEL
Rat	Twenty-eight day study of toxicity [a]	Toxicity	30 mg/kg bw	80 mg/kg bw

[a] Gavage administration.

Estimate of acceptable daily intake (ADI)

0–0.03 mg/kg bw

Estimate of acute reference dose (ARfD)

0.03 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; additional information on the toxicity of propylene bromohydrin to permit a more robust comparison with the toxicity of propylene chlorohydrin

Critical end-points for setting guidance values for exposure to propylene oxide

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	No data
Dermal absorption	No data
Distribution	No data
Potential for accumulation	Unlikely
Rate and extent of excretion	No data
Metabolism in animals	Hydrolysed to propylene glycol or conjugated
Toxicologically significant compounds in animals and plants	Propylene oxide, propylene chlorohydrin, propylene bromohydrin
Acute toxicity	
Rat, LD_{50}, oral	300–1000 mg/kg bw
Rat, LD_{50}, dermal	950 mg/kg bw
Rat, LC_{50}, inhalation	3.2–3.4 mg/L (4 h, nose only)
Rabbit, dermal irritation	Severe
Rabbit, ocular irritation	Moderate to severe
Guinea pig, dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Body weight gain
Lowest relevant oral NOAEL	200 mg/kg bw per day (rats)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	250 ppm (600 mg/m^3) (mice and rats)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Site of contact irritation (nasal cavity inflammation; stomach hyperkeratosis); systemic toxicity – reduced body weight gain
Lowest relevant LOAEL	4.3 mg/kg bw per day (lowest dose tested) (rat)
Lowest relevant NOAEC	100 ppm (rat) (~40 mg/kg bw per day oral)
Carcinogenicity	Site of contact tumours (nasal cavity; stomach) [a]
Genotoxicity	Genotoxic in vitro; unlikely to be genotoxic in humans at dietary exposure levels [a]
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEC	100 ppm (rat) (~40 mg/kg bw per day oral)
Lowest relevant offspring NOAEC	100 ppm (rat) (~40 mg/kg bw per day oral)
Lowest relevant reproductive NOAEC	300 ppm (rat) (~120 mg/kg bw per day oral)
Developmental toxicity	
Target/critical effect	Reduced fetal weight
Lowest relevant maternal NOAEL	150 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	150 mg/kg bw per day (rat)

Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Genotoxicity	DNA and haemoglobin adduct formation in rats and humans; depletion of non-protein sulfhydryl groups
Human data	Epidemiological studies of production plant workers inconclusive

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Critical end-points for setting guidance values for exposure to propylene chlorohydrin

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	> 11% (limited information)
Dermal absorption	No data
Distribution	No data
Potential for accumulation	Unlikely
Rate and extent of excretion	> 11% (urine, rabbit)
Metabolism in animals	Glucuronide and glutathione conjugates
Toxicologically significant compounds in animals and plants	Propylene chlorohydrin
Acute toxicity	
Rat, LD_{50}, oral	532 mg/kg bw
Rat, LD_{50}, dermal	500 mg/kg bw
Rat, LC_{50}, inhalation	> 3.8 mg/L (6 hours)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Severe
Guinea-pig, dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Liver (hepatocyte vacuolation); pancreas (acinar cell alterations)
Lowest relevant oral NOAEL	35 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	None
Lowest relevant NOAEL	34 mg/kg bw per day (highest dose tested) (rat) 100 mg/kg bw per day (highest dose tested) (mouse)
Carcinogenicity	Not carcinogenic [a]
Genotoxicity	Genotoxic in vitro; negative in an in vivo assay [a]
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	30 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	30 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	130 mg/kg bw per day (rat)

Developmental toxicity	
Target/critical effect	Reduced fetal weight and reduced ossification
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	30 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	25 mg/kg bw per day (rat)
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
	See below
Human data	Epidemiological studies of production plant workers – inconclusive

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Critical end-points for setting guidance values for exposure to propylene bromohydrin to support the read-across to propylene chlorohydrin

Acute toxicity	
Rat, LD_{50}, oral	175 mg/kg bw
Short-term studies of toxicity	
Target/critical effect	Liver inflammation
Lowest relevant oral NOAEL	30 mg/kg bw per day (rat)
Genotoxicity	Genotoxic in vitro; negative in an in vivo assay[a]

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
Propylene oxide			
ADI	0–0.04 mg/kg bw	Rat chronic inhalation	1000
ARfD	0.04 mg/kg bw	Rat chronic inhalation	1000
Propylene chlorohydrin			
ADI	0–0.3 mg/kg bw	Rat acute neurotoxicity; rat developmental toxicity; rat multigeneration	100
ARfD	0.3 mg/kg bw	Rat acute neurotoxicity; rat developmental toxicity	100
Propylene bromohydrin			
ADI	0–0.03 mg/kg bw	Rat acute neurotoxicity; rat developmental toxicity; rat multigeneration with propylene chlorohydrin	1000
ARfD	0.03 mg/kg bw	Rat acute neurotoxicity; rat developmental toxicity with propylene chlorohydrin	1000

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THIOPHANATE-METHYL

First draft prepared by
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Explanation

Thiophanate-methyl is the International Organization for Standardization (ISO)–approved common name for dimethyl 4,4′-(o-phenylene)bis(3-thioallophanate) (International Union of Pure and Applied Chemistry [IUPAC]), which has the Chemical Abstracts Service number 23564-05-8. Thiophanate-methyl is a systemically active benzimidazole fungicide that inhibits the synthesis of β-tubulin. Thiophanate-methyl was previously evaluated by the Joint Meeting on Pesticide Residues (JMPR) in 1973, 1975, 1977, 1995, 1998 and 2006. In 1998, an acceptable daily intake (ADI) of 0–0.08 mg/kg body weight (bw) was established based on the no-observed-adverse-effect level (NOAEL) of 8 mg/kg bw per day in a three-generation study of reproductive toxicity in rats and in a one-year study in dogs (both of these studies have been evaluated at earlier meetings) and a safety factor of 100. In 2006, the Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for thiophanate-methyl.

Thiophanate-methyl was re-evaluated by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Both new toxicity studies with thiophanate-methyl and previously submitted studies were considered by the present Meeting. No toxicological data were submitted on carbendazim.

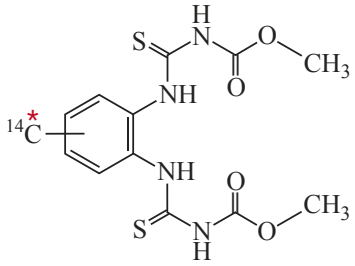
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with the relevant Organisation for Economic Co-operation and Development (OECD) test guidelines (TGs) or similar guidelines of the European Union or United States Environmental Protection Agency, unless otherwise indicated.

Evaluation for acceptable intake

1 Biochemical aspects

Absorption, distribution, metabolism and excretion (ADME) studies were conducted in rats using thiophanate-methyl radiolabelled with ^{14}C in the phenyl ring (Fig. 1).

Figure 1. Position of ^{14}C label in thiophanate-methyl used in rat ADME studies



1.1 Absorption, distribution and excretion

(a) Oral route

Mouse

In a study with reporting and study design deficiencies, male dd-Y mice received the thiophanate-methyl (batch and purity not specified; ^{14}C and ^{35}S radiolabels were used) at 1, 1.1, 2 or 3 mg/mouse. Excreta and blood samples were collected after 3, 6, 12, 24, 48, 60, 72, 84 and 96 hours. Tissue samples (brain, thyroid, thymus, heart, lung, liver, spleen, kidney, testicle, fat, muscle, gastrointestinal tract, thigh-bone) were taken at 3, 6, 12, 24, 48 and 72 hours after administration.

The radioactivity was excreted within 24 hours post administration. The excretion rate was 82–89% via urine and 19–29% via faeces. The highest radioactivity was found in liver and kidney followed by kidneys and blood (Table 1). Low levels of thiophanate-methyl were also detected in thyroid and testis. Tissue radioactivity peak levels were generally found at three hours post dosing and remained fairly high up to six hours post administration. Thereafter, the radioactivity levels rapidly declined (Noguchi, 1970; Noguchi & Kosaka, 1971).

Table 1. Radiolabel content in mouse tissues and organs after administration of a single dose [^{14}C]thiophanate-methyl

Matrix	^{14}C content in tissue (10^3 dpm/g)		
	3 hours	6 hours	12 hours
Blood	80	76	43
Liver	141	94	37
Thyroid	24	27	19
Kidney	122	96	24
Testis	23	23	23

Sources: Noguchi (1970); Noguchi & Kosaka (1971)

Rat

The kinetics and metabolism of [^{14}C]thiophanate-methyl (radiochemical purity >97.3–98.5%, batch numbers C-109-2, C-109-3, C-109-3-1, EC-01-06; unlabelled batch TIF-1016, purity 96.6%), was studied in male and female Fischer 344 rats.

In a preliminary study, the excretion of volatile [^{14}C] products after the administration of a low dose [^{14}C]thiophanate-methyl was investigated. The kinetics and metabolism were studied in groups of five male and five female Fischer 344 rats (except for group B2) dosed as follows:

- Group B: a single low dose (13 mg/kg bw) of [^{14}C]thiophanate-methyl;
- Since the metabolic pattern of group B slightly differed from that of group C an additional group B2 was added in which three male rats were given a single low dose (13.3 mg/kg bw) of [^{14}C]thiophanate-methyl;
- Group C: one dose (14 mg/kg bw) of unlabelled thiophanate-methyl daily for 14 days and thereafter one dose (10 mg/kg bw) of [^{14}C]thiophanate-methyl;
- Group D: a single high dose (140 mg/kg bw) of [^{14}C]thiophanate-methyl;
- Group D2: a single high dose (173 mg/kg bw) of [^{14}C]thiophanate-methyl.

Thiophanate-methyl was dissolved in acetone and formulated in a 1% aqueous carboxymethylcellulose (CMC) suspension and administered by gavage. The rats were placed in metabolism cages and their excreta were collected separately every 24 hours for up to 96 hours. Blood was collected at 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 24 and 48 hours. Tissue samples (liver, kidney, fat, spleen, heart, femoral bone, lung, brain, femoral muscle, thyroid, testis, and ovary) were gathered at day 4 after administration. The identification of thiophanate-methyl and its metabolites in tissues and excreta is described in section 1.2 (Tanoue, 1992a,b).

No detectable levels of volatile [^{14}C] products were found in the preliminary study. Blood peak levels were reached 1–3 hours after the administration of the low dose (groups B and C). At this time the blood radioactivity concentration was 1.7–4.2 µg equivalents/g blood. After administration of thiophanate-methyl at high-dose, peak levels were reached at 4–7 hours (D) and 2–4 hours (D2) post dosing. The blood radioactivity concentration was then 14–22 (D) and 17–27 (D2) µg equiv./g blood. The initial elimination half-lives ($t_{½}$) in blood were calculated to be between 1.6 and 2.8 hours for the low-dose groups (B, C) and between 2.4 and 7.8 hours for the high-dose groups (D, D2).

Over the period of 96 hours after the administration, approximately 100% of the administered radioactivity was excreted via urine and faeces (Table 2). The administered radioactivity in the low-dose groups was mainly excreted via urine, while there was a shift to faecal excretion in the high-dose groups. This indicates that the relative absorption from the gastrointestinal tract decreased after the administration of high doses, although it may also be an indication of increased excretion via bile at high doses. Nearly the same faecal and urinary excretion rates were found in the pretreated group (C) as in the single dose group (B).

Table 2. Excretion balance in dose groups 96 hours after administration of [^{14}C]thiophanate-methyl

	Radioactivity (% of administered dose)				
	B (single low dose) [a]	**B2 (single low dose)** [b]	**C (repeated low dose)** [c]	**D (single high dose)** [d]	**D2 (single high dose)** [e]
Urine	71.0	60.0	51.5	31.0	NA
Faeces	28.6	39.7	48.2	68.8	NA

bw: body weight; NA: not applicable *Source*: Tanoue (1992a,b)

[a] Group B: Single low dose (13 mg/kg bw) of [^{14}C]thiophanate-methyl.

[b] Group B2: Single low dose (13.3 mg/kg bw) of [^{14}C]thiophanate-methyl to males only.

[c] Group C: Low doses (14 mg/kg bw) of unlabelled thiophanate-methyl daily for 14 days followed by one dose (10 mg/kg bw) of [^{14}C]thiophanate-methyl.

[d] Group D: Single high dose (140 mg/kg bw) of [^{14}C]thiophanate-methyl.

[e] Group D2: a single high dose (173 mg/kg bw) of [^{14}C]thiophanate-methyl.

Tissue residue levels are presented in Table 3. Less than 0.5% of the administered dose was retained in the rat's tissues and carcass after 96[14]hours post dosing. The highest residue levels were detected in the liver, thyroid and kidney (Tanoue, 1992a,b).

Table 3. Residue levels in rat tissues and organs 96 hours after administration of [^{14}C]thiophanate-methyl

Matrix	Residue levels (µg eq/g)					
	B (single low dose) [a]		C (repeated low dose) [b]		D (single high dose) [c]	
	Males	Females	Males	Females	Males	Females
Blood	0.05	0.05	0.03	0.02	0.21	0.14
Liver	0.29	0.37	0.17	0.32	1.72	2.15
Thyroid	0.36	0.37	0.04	0.22	2.49	1.81
Kidney	0.08	0.11	0.04	0.07	0.51	0.30
Testis	0.02	–	0.00	–	0.05	–
Ovary	–	0.02	–	0.01	–	0.17

bw: body weight; eq: equivalents *Source*: Tanoue (1992a,b)

[a] Group B: Single low dose (13 mg/kg bw) of [^{14}C]thiophanate-methyl.

[b] Group C: Low doses (14 mg/kg bw) of unlabelled thiophanate-methyl daily for 14 days followed by one dose (10 mg/kg bw) of [^{14}C]thiophanate-methyl.

[c] Group D: Single high dose (140 mg/kg bw) of [^{14}C]thiophanate-methyl.

The metabolism and excretion of a gavage dose of 171 mg/kg bw of [^{14}C]thiophanate-methyl (labelled batch no. CFQ 4519, radiochemical purity 98.0%; unlabelled batch no. batch TIF-1016, purity 96.6%) dissolved in 1% CMC were studied in CD-1 mice (5 males/group). Levels of radioactivity were measured in urine and faeces collected daily over a 96-hour post-administration sampling period. The identification of thiophanate-methyl and its metabolites in tissues and excreta is described in section 1.2 (Nabetani & Mori, 1993).

Most radioactivity (98.9%) was rapidly excreted, mainly via faeces (73.3%) and urine (25.6%) within 1 day of dosing. Less than 0.1% of radioactivity was found in the tissues after 96 hours (Nabetani & Mori, 1993).

The blood and plasma kinetics of [^{14}C]thiophanate-methyl (labelled batch no. XIX/17, radiochemical purity 96.9%; unlabelled batch no. KAS6107, purity 99.8%) dissolved in 1% CMC were studied over a 48-hour post-administration sampling period following a single gavage dose of 14 mg/kg bw to Fischer (F344/NCrHsd) rats (9/sex per group). Blood samples were collected from three males and three females at 0.25, 0.5, 1, 2, 3, 4, 8, 24 and 48 hours post dosing. Blood and plasma kinetics (area under the concentration–time curve [AUC], half-life, maximum concentration, time to reach maximum concentration) of the test substance was assessed by measuring the concentration of radioactivity in blood and plasma by liquid scintillation counting.

The toxicokinetic parameters are presented in Table 4.

Table 4. Kinetic parameters of thiophanate-methyl after single gavage administration of 14 mg/kg bw to rats

Matrix / sex	C_{max} (µg equiv./g)	T_{max} (h)	$t_{½}$ (h)		AUC (µg × h/g)		
			Initial	Terminal	0–24 [a]	Last [b]	Infinity [c]
Blood							
Males	4.8	2	3.4	12.4	37.9	41.4	43.0
Females	4.7	2	4.6	10.5	42.4	46.3	47.7

(*Continued on next page*)

Matrix / sex	C_{max} (µg equiv./g)	T_{max} (h)	$t_{½}$ (h)		AUC (µg × h/g)		
			Initial	Terminal	0–24 [a]	Last [b]	Infinity [c]
Plasma							
Males	5.9	2	3.5	8.7	48.7	51.4	52.0
Females	5.4	2	5.1	8.9	51.4	55.0	56.0

AUC: area under the plasma concentration–time curve; equiv.: equivalent; *Source*: Bernard (2011a)
C_{max}: maximum concentration; T_{max}: time to reach maximum concentration (C_{max}) t½: elimination half-life;
[a] AUC up to sampling time at 24 hours.
[b] AUC up to last sampling time
[c] AUC extrapolated to infinity.

From 0.25 to eight hours, mean blood to plasma ratios averaged 0.79 (range: 0.75–0.83) in males and 0.84 (range: 0.80–0.87) in females. This ratio increased to about one at 24 hours, and two at 48 hours post dose in males and to about one at 24 hours, and to 1.3 at 48 hours in females (Bernard, 2011a).

The absorption, distribution and excretion of [^{14}C]thiophanate-methyl (labelled batch no. XIX/17, radiochemical purity 96.9%; unlabelled batch no. KAS6107, purity 99.8%) dissolved in 1% CMC were studied over a 48-hour post-administration sampling period following a single gavage dose of 14 mg/kg bw to bile duct–cannulated Fischer F344/NCrHsd rats (6/sex per group). Bile was collected at intervals of 0–1, 1–2, 2–4, 4–8, 8–12, 12–24, 24–32, 32–48 hours. Urine and faeces were collected at intervals of 0–24 and 24–48 hours. Blood samples were collected at 48 hours post dosing. The concentration of radioactivity was determined in bile, urine, faeces and residues (blood, plasma, gastrointestinal tract with contents and carcass) using liquid scintillation counting.

Oral absorption of thiophanate-methyl was rapid and almost complete (88–89% of the administered dose, based on excretion in urine and bile and residual radioactivity in blood, plasma, gastrointestinal tract and carcass). Radioactivity was predominantly excreted with urine (47% of the administered dose) and secreted in bile (40% of the administered dose) within 48 hours post dosing. Approximately 7% of the administered dose was recovered in faeces. Up to 0.1% of the administered dose was recovered from the gastrointestinal tract (including contents) and less than 0.3% of the administered dose was recovered from the remaining carcass 48 hours after administration. Detailed results are presented in Table 5 (Bernard, 2011b).

Table 5. Recovery of radioactivity after single gavage administration of 14 mg/kg bw [^{14}C]thiophanate-methyl

Matrix	Radioactivity (% of administered dose) [a]	
	Males	Females
Bile (0–48 hours)	40.2 ± 5.7	39.6 ± 2.4
Urine (0–48 hours)	47.9 ± 7.6	47.0 ± 4.9
Faeces (0–48 hours)	6.9 ± 4.5	7.0 ± 4.0
Cage wash	0.7 ± 0.4	1.0 ± 0.8
Excreted radioactivity	95.8 ± 1.6	94.6 ± 1.9
Blood	0.02 ± < 0.01	0.02 ± < 0.01
Plasma	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Gastrointestinal tract (including contents)	0.06 ± 0.03	0.04 ± 0.03
Carcass	0.31 ± 0.04	0.32 ± 0.07
Total recovery	96.1 ± 1.6	95.0 ± 1.9
Systemic absorption	89.2 ± 3.4	88.0 ± 3.4

bw: body weight; SD: standard deviation
[a] Results expressed as group mean % of the administered dose ± standard deviation.
Source: Bernard (2011b)

In a study with reporting and study design deficiencies, 15 female Wistar rats were treated with [^{14}C] thiophanate-methyl (batch and purity not specified) via the diet at 45 ppm (~2.25 mg/kg bw per day) for 20 consecutive days and then observed for 7 days. Urine and faeces were collected at regular intervals and tissue samples were obtained from three rats each at three hours and 1, 3 and 7 days after the last day of exposure.

Every day during the administration period nearly 90% of the ingested radioactivity was excreted, approximately 54% via the urine and approximately 35% via the faeces. Radioactivity was mainly found in the gastrointestinal tract as well as in the thyroid, adrenals and liver. After cessation of treatment, radioactivity declined rapidly in all tissues. No potential for accumulation was identified (Kosaka et al., 1975).

In another study with reporting and study design deficiencies, male Wistar rats were orally dosed with [^{14}C]thiophanate-methyl (batch and purity not specified) at 24.2 mg per rat (approximately 65 mg/kg bw). Excreta were collected for up to 96 hours.

About 60% of the administered radioactivity was excreted via the faeces, while 30% were found in the urine. The majority of the radioactivity was excreted within 24 hours of dosing (Fujino et al., 1973).

Dog

In a study with reporting and study design deficiencies, thiophanate-methyl (batch and purity not specified) was orally administered via capsule to male beagle dogs at 9.85 mg/dog. Faeces, urine and blood were separately collected at 8, 15 and 30 minutes and 1, 2, 3, 6, 12, 24, 36, 48, 60, 72 and 96 hours post dosing.

At the end of the collection period, 74% of the administered radioactivity was excreted via the urine, while 14% was retrieved in the faeces. The majority of the radioactivity was excreted within 24 hours after dosing (Noguchi & Kosaka, 1971).

(b) Dermal route

Rat

A single dermal administration of [^{14}C]-labelled thiophanate-methyl (labelled batch no. not specified, radiochemical purity 98.1%; unlabelled batch no. not specified, purity 99.0%) dissolved in 1% CMC was applied to approximately 10% of the total skin surface of groups of male CD rats (4/dose per exposure time) at doses of 0.30 or 31.93 mg/rat (0.162 MBq [4.37 µCi] per rat). The rats were exposed for 0.5, 1.5, 10 or 24 hours, during which time, urine and faeces were collected. At the end of the exposure, radioactivity was determined in these urine and faeces samples and in skin wash, carcass, blood, liver, kidneys and muscle. The metabolites in the urine were analysed by thin layer chromatography directly and after arylsulfatase treatment. It was noted that tape stripping was not performed.

The percentage of the administered dose obtained in skin (washing), tissues, carcass, urine and faeces at different time points after administration are presented in Table 6. The percentage absorption at the high dose was lower than at the low dose. The significant excretion in faeces indicates that the bile is an important excretion route. A relatively small amount of the applied radioactivity was found in the tissues (blood, liver, kidneys and muscle) at the low and high doses. The data indicate that at least about 53% and 23% of the applied thiophanate-methyl is absorbed at a low and high dose level, respectively. As it is not clear what percentage of the radioactivity in the skin might be absorbed, no conclusion on the total dermal absorption of thiophanate-methyl in rats can be drawn (Anonymous, 1981).

Table 6. Recovery of radioactivity after percutaneous application of [^{14}C]thiophanate-methyl rats

Dose	Exposure time (h)	Recovery of radioactivity (% of applied dose)					Total absorbed
		Washing + skin	Tissues	Carcass	Urine	Faeces	
Low (0.30 mg/rat)	0.5	95.42	0.09	4.49	0.00	0.00	4.58
	1.5	95.35	0.20	4.15	0.30	0.00	4.65
	10	70.81	0.69	15.41	11.34	1.75	29.19
	24	47.09	0.64	16.12	25.18	10.97	52.91
High (31.93 mg/rat)	0.5	97.32	0.06	2.54	0.08	0.00	2.68
	1.5	97.83	0.02	1.83	0.15	0.17	2.17
	10	91.29	0.11	5.69	2.64	0.27	8.71
	24	76.35	0.14	5.24	9.84	8.44	23.66

Source: Anonymous (1981)

1.2 Biotransformation

Mouse

The metabolism and excretion of a gavage dose of 171 mg/kg bw of [^{14}C]thiophanate-methyl (labelled batch no. CFQ 4519, radiochemical purity 98.0%; unlabelled batch no. TIF-1016, purity 96.6%) dissolved in 1% CMC were studied in CD-1 mice (5 males/group). Levels of radioactivity were measured in urine and faeces collected daily over a 96-hour post-administration sampling period. The excretion of thiophanate-methyl is described in section 1.1.

One day after dosing, a high level of thiophanate-methyl (47.9 %) was detected in faeces. The major metabolite in urine was 5-hydroxycarbendazim sulfate (5-OH-MBC-S; 8.7%) and in faeces was carbendazim (MBC; 6.3%). In addition to the faecal metabolites in rats, 4-hydroxy-dimethyl [(1,2-phenylene)bis(iminocarbonyl)]bis(carbamate)-sulfate (4-OH-FH 432-S) was found in the mouse faeces. The metabolites detected in male mouse urine and faeces are detailed in Table 7. The proposed metabolic pathway in mice is depicted in Fig. 2 (Nabetani & Mori, 1993).

Table 7. Metabolites in mouse excreta after a single gavage administration of thiophanate-methyl

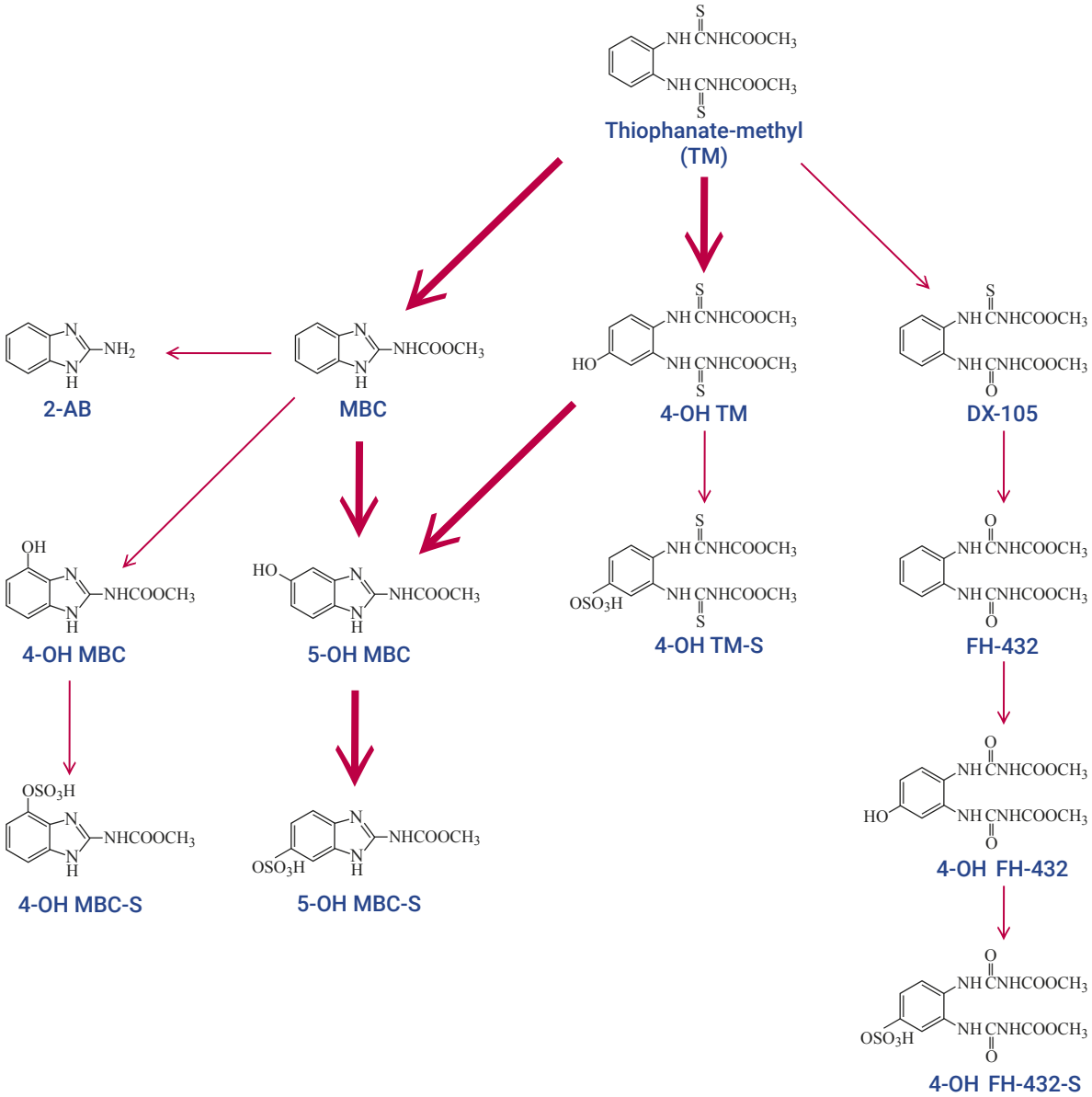
Parent compound and metabolites (for structures see Fig. 2)	% of administered dose[a]	
	Urine	Faeces
Thiophanate-methyl (TM) parent compound	0.1	47.9
DX-105	Not detected	1.0
4-OH-TM	0.4	1.5
Carbendazim (MBC)	0.5	6.3
5-OH-MBC	0.9	1.0
2-AB	0.1	0.5
5-OH-MBC-S	8.7	1.2
4-OH-FH 432-S	Not determined	0.3
4-OH-MBC + 4-OH-FH 432	0.1	1.9
4-OH-TM-S + 4-OH-MBC-S	1.4	0.5
UK-3 (unknown metabolite)	3.8	0.4
UK-4 (unknown metabolite)	2.9	0.1

2-AB: 2-aminobenzimidazole; *Source*: Nabetani & Mori (1993)
5-OH-MBC-S: 5-hydroxycarbendazim sulfate;
DX-105: (methyl N-[2-(N'-methoxycarbonylthioureido)phenylaminocarbonyl]carbamate);
FH 432: dimethyl [(1,2-phenylene)bis(iminocarbonyl)]bis(carbamate);
[a] One day after a single gavage administration of 171 mg/kg bw of thiophanate-methyl.

The metabolism of low or high doses of [^{14}C]thiophanate-methyl (radiochemical purity >97.3–98.5%; batch nos C-109-2, C-109-3, C-109-3-1, EC-01-06; unlabelled batch TIF-1016, purity 96.6%) was studied to identify unchanged compound and rat urinary and faecal metabolites using thin layer chromatography, high-performance liquid chromatography, proton nuclear magnetic resonance analysis and mass spectrometry. The study design and toxicokinetics are described in section 1.1 (Tanoue, 1992a,b).

The identified metabolites in urine and faeces accounted for 71.8–87.5% of the administered radioactivity. The major urinary and faecal metabolite detected was 5-hydroxycarbendazim sulfate (5-OH-MBC-S) and 4-OH-thiophanate-methyl, respectively. There was a high level of unchanged thiophanate-methyl in the faeces from rats which were administered the high dose of thiophanate-methyl or which were pretreated with unlabelled thiophanate-methyl for 14 days. The metabolites are presented in Table 8 (urinary) and Table 9 (faecal). The proposed metabolic pathway in the rat is presented in Fig. 3.

Figure 2. Proposed metabolic pathway of thiophanate-methyl in the mouse



TM: thiophanate-methyl, or dimethyl [(1,2-phenylene)bis(iminocarbonothioyl)]bis(carbamate);

2-AB: 2-aminobenzimidazole; DX-105: methyl *N*-[2-(*N*'-methoxycarbonylthioureido)phenylaminocarbonyl]carbamate;

FH-432: dimethyl [(1,2-phenylene)bis(iminocarbonyl)]bis(carbamate);

MBC: carbendazim, or methyl 2-benzimidazolylcarbamate; UK: unknown metabolite, chemical structure could not be identified

Source: Redrawn from Nabetani & Mori (1993)

No significant difference in the identified metabolites between sexes and/or dose groups was observed (Tanoue, 1992a,b).

Table 8. Urinary metabolites after administration of [^{14}C]thiophanate-methyl in rat

Parent compound and metabolites, *(for chemical names and structures see Fig. 3 legend)*	% of administered radioactivity						
	B (single low dose)[a]		B2 (single low dose)[b]	C (repeated low dose)[c]		D (single high dose)[d]	
	Males	Females	Males	Males	Females	Males	Females
Thiophanate-methyl (TM)	0.2	0.7	0.2	0.2	0.4	0.2	0.4
4-OH-TM	2.4	2.1	1.4	1.5	1.2	1.1	0.7
4-OH-TM-S	0.7	0.7	0.8	0.8	1.1	0.4	0.3
DX-105	<0.1	0.1	0.1	<0.1	<0.1	<0.1	0.1
Carbendazim (MBC)	0.6	1.1	0.9	0.4	0.1	0.7	0.9
5-OH-MBC	1.8	1.5	2.1	1.7	2.8	1.0	0.9
4-OH-MBC+UK-2	0.4	0.5	0.4	0.3	0.6	0.2	0.2
5-OH-MBC-S	42.0	34.4	36.0	27.3	20.6	18.9	13.9
4-OH-MBC-S	1.0	1.9	1.0	1.3	2.0	0.6	0.8
2-AB	0.3	0.2	1.1	0.6	0.7	0.2	0.3
FH-432	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
4-OH-FH 432	0.2	0.2	0.2	0.1	0.1	0.1	0.1
4-OH-FH 432-S+UK-3	3.9	6.2	2.5	2.9	5.1	1.4	2.8
UK-1	2.2	1.8	1.2	1.0	0.6	0.7	0.2
UK-4	2.6	5.8	2.0	2.3	3.3	1.0	2.4

[a] Group B: Single low dose (13 mg/kg bw) of [^{14}C]thiophanate-methyl. *Source*: Tanoue (1992a,b)

[b] Group B2: Single low dose (13.3 mg/kg bw) of [^{14}C]thiophanate-methyl to males only.

[c] Group C: Low doses (14 mg/kg bw) of unlabelled thiophanate-methyl daily for 14 days followed by one dose (10 mg/kg bw) of [^{14}C]thiophanate-methyl.

[d] Group D: Single high dose (140 mg/kg bw) of [^{14}C]thiophanate-methyl.

Table 9. Faecal metabolites after administration of [^{14}C]thiophanate-methyl in rat

Parent compound and metabolites, *(for chemical names and structures see Fig. 3 legend)*	% of administered radioactivity						
	B (single low dose)[a]		B2 (single low dose)[b]	C (repeated low dose)[c]		D (single high dose)[d]	
	Males	Females	Males	Males	Females	Males	Females
Thiophanate-methyl (TM)	1.1	1.1	9.8	24.0	21.4	52.2	55.7
DX-105	0.1	0.1	0.3	0.2	0.8	0.3	0.6
Carbendazim (MBC)	0.5	0.5	2.0	1.9	2.7	0.9	2.0
5-OH-MBC	3.1	2.9	4.9	1.6	1.4	1.0	0.9
4-OH-TM	10.5	8.4	9.2	6.5	5.7	4.0	3.5
FH-432	<0.1	<0.1	0.1	0.1	0.2	0.1	0.1
4-OH-FH 432	0.3	0.2	0.2	0.2	0.3	<0.1	0.2
4-OH-MBC+UK-2	0.7	0.6	0.6	0.5	0.5	0.2	0.2
UK-1	0.7	0.8	0.5	0.3	0.5	0.3	0.3

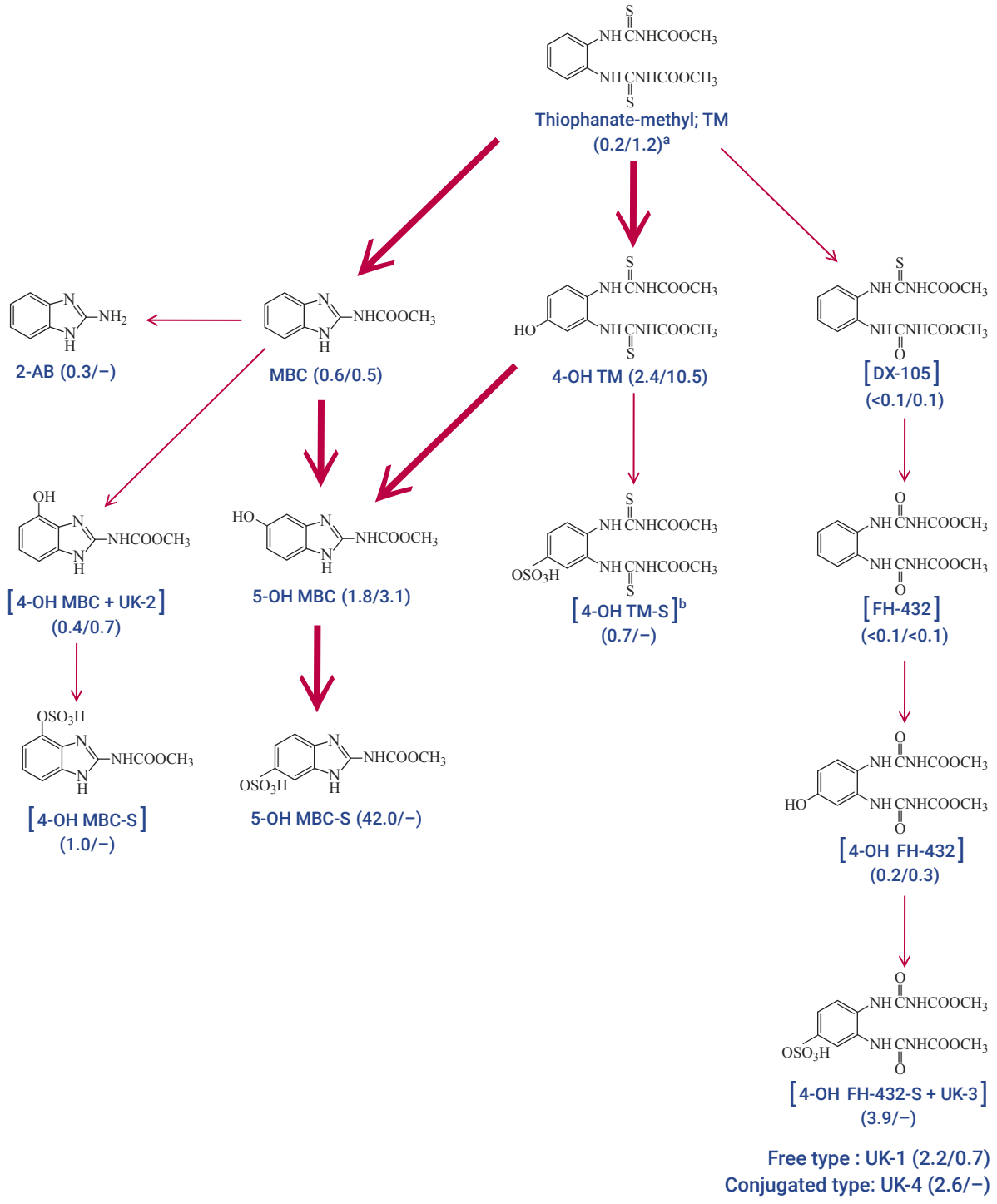
[a] Group B: Single low dose (13 mg/kg bw) of [^{14}C]thiophanate-methyl. *Source*: Tanoue (1992a,b)

[b] Group B2: Single low dose (13.3 mg/kg bw) of [^{14}C]thiophanate-methyl to males only.

[c] Group C: Low doses (14 mg/kg bw) of unlabelled thiophanate-methyl daily for 14 days followed by one dose (10 mg/kg bw) of [^{14}C]thiophanate-methyl.

[d] Group D: Single high dose (140 mg/kg bw) of [^{14}C]thiophanate-methyl.

Figure 3. Proposed metabolic pathway of thiophanate-methyl in the rat



[a] Percent of radioactivity found in the urine/faeces of male rats in Group B

[b] Metabolites in [square brackets] were not identified by spectrometry

2-AB: 2-aminobenzimidazole;

MBC: carbendazim, or methyl 2-benzimidazolylcarbamate;

DX-105: methyl *N*-[2-(*N*'-methoxycarbonylthioureido)phenylaminocarbonyl]carbamate;

FH-432: dimethyl [(1,2-phenylene)bis(iminocarbonyl)]bis(carbamate);

TM: thiophanate-methyl, or dimethyl [(1,2-phenylene)bis(iminocarbonothioyl)]bis(carbamate);

UK: unknown metabolite, chemical structure could not be identified

Source: Redrawn from Tanoue (1992a)

Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with thiophanate-methyl are summarized in Table 10. In an old, non-GLP study by Noguchi & Hashimoto (1970a) with deficiencies in study design and reporting, the following oral LD_{50} values were reported:

- Mouse: 3514 and 3400 mg/kg bw (male/female)
- Rat: 7500 and 6640 mg/kg bw (male/female)
- Guinea pig: 3640 and 6700 mg/kg bw (male/female) and
- Rabbit: 2270 and 2500 mg/kg bw (male/female);

Dermal LD_{50} values for mouse, rat, guinea-pig and rabbit were greater than 10 000 mg/kg bw (male/female).

This study is considered as supporting evidence only in the present risk assessment (Noguchi & Hashimoto, 1970a).

Table 10. Results of studies of acute toxicity with thiophanate-methyl

Species	Strain	Sex	Route	Vehicle	Purity (%), batch no.	LD_{50} / LC_{50}	Reference
Rat	Crj:CD(SD)	M/F	Oral	Distilled water	96.6 TIF-1016	> 5000 mg/kg bw (M/F)	Nishibe (1990b) [a]
Rabbit	Kbs:JW	M/F	Dermal	Moistened with distilled water	96.6 TIF-1016.	> 2000 mg/kg bw (M/F)	Nishibe (1990c) [b]
Rat	Crj:CD(SD)	M/F	Inhalation	–	95.3 TP-544.	1.7 mg/L (M) 1.9 mg/L (F)	Nishibe (1987) [c]

bw: body weight; F: female; M: male; LC_{50}: median lethal concentration; PEG: polyethylene glycol; LD_{50}: median lethal dose; OECD TG: Organisation for Economic Co-operation and Development Test Guideline

[a] Study design resembles OECD TG 423. Rats (5/sex per group) received thiophanate-methyl by gavage at 5000 mg/kg bw. No mortality, clinical signs or gross pathological findings were observed.

[b] Study design resembles OECD TG 402. Rats (5/sex per group) were dermally exposed to thiophanate-methyl at 2000 mg/kg bw. No mortality, clinical signs of systemic toxicity or gross pathological findings were observed. Erythema was noted on the thiophanate-methyl treated area for two days in seven rats.

[c] Study design resembles OECD TG 403. Rats (5/sex per group) were exposed whole body for four hours to actual thiophanate-methyl concentrations of 0, 1.0, 1.5, 1.6 and 1.9 mg/L. During the study period, 5/5 males and 3/5 females at 1.9 mg/L and 1/5 females at 1.0 mg/L died. Clinical signs of toxicity observed in rats exposed to thiophanate-methyl were decreased motor activity, low sensitivity, ataxia, ptosis, incontinence of urine, tremor, convulsion, hypotonia and ventral position. Clinical signs of toxicity were observed in female rats at 1 mg/L or higher and in male rats at 51.5 mg/L or higher. Body weight decrease and growth depression were observed for 1–3 days after the exposure in many rats of the dosed groups; however, their body weight increased thereafter. At necropsy one rat that died showed a dark reddish lung.
Mass median aerodynamic diameter was 3.7–4.5 ± 2.3 µm at 0.895 mg/L and 7.5 ± 2.1 µm at 4.873 mg/L.

(b) Dermal irritation

In a primary dermal irritation study with a study design resembling OECD TG 404, the intact skin of six male New Zealand White rabbits was exposed for four hours under occlusion to 0.5 g thiophanate-methyl (purity 96.2%; batch no. TM-948) moistened with water. Dermal irritation was scored at 0, 0.5, 1, 24, 48 and 72 hours after patch removal.

No skin irritation was observed at any time point (Nishibe, 1986b).

(c) Ocular irritation

In an acute eye irritation study with a study design resembling OECD TG 405, 100 mg of thiophanate-methyl (purity 96.2%; batch no. TM-948) was instilled into the conjunctival sac of the right eye of each of nine male New Zealand White rabbits. The treated eyes of three rabbits were washed two minutes after the treatment for 30 seconds. The untreated eye served as a control. The eyes were examined macroscopically for signs of irritation at 1, 24, 48 and 72 hours post instillation.

In four rabbits of the unwashed group and three of the washed group, conjunctival changes (redness, grade 1–2) were observed at one hour after the applications; after 24 h redness (grade 1) was observed in only one rabbit of the unwashed group.

It was concluded that thiophanate-methyl is not an eye irritant (Nishibe, 1986a).

(d) Dermal sensitization

In a dermal sensitization study using the Magnusson and Kligman maximization test, with a study design resembling OECD TG 406, thiophanate-methyl (purity 96.2%; batch no. TM-948) formulated in corn oil was tested in 20 female Hartley guinea pigs. A positive control group of 20 females was treated with 2,4-dinitrochlorobenzene. No vehicle control group was included. In the induction phase, the guinea pigs received 3.5% thiophanate-methyl intradermally, followed by epidermal treatment with 42% thiophanate-methyl in Vaseline on day 8. The challenge on day 21 was performed with epidermal application of 42% thiophanate-methyl in Vaseline. Skin reactions were assessed 24, 48 and 72 hours after the challenge.

After challenge application, skin effects (grade 1 to 3) were observed in all guinea pigs in the treatment group after 24–48 hours and in 19/20 guinea pigs after 72 hours. It is concluded that thiophanate-methyl is a skin sensitizer under the conditions of the study (Nishibe, 1989).

In a dermal sensitization study using the Buehler test, performed in accordance with OECD TG 406, thiophanate-methyl (purity 96.6%; batch no. TIF-1016) suspended in saline, was tested in 10 female Hartley guinea pigs. No vehicle control group was included. In the induction phase, the guinea pigs were exposed to 30% thiophanate-methyl in saline for six hours under occlusive dermal conditions on experimental day 0, 7 and 14. After 14 days the challenge was performed (30% thiophanate-methyl, six hours occlusive patch exposure). Skin reactions were assessed 24 and 48 hours after the challenge. A group of 10 guinea pigs served as controls. As a positive control group, 10 females were treated with 2,4-dinitrochlorobenzene.

After challenge application, no skin effects were observed. 2,4-dinitrochlorobenzene induced the expected dermal response upon challenge. It is concluded that thiophanate-methyl is not a skin sensitizer under the conditions of the study (Nishibe & Mochizuki, 1993).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a limited non-GLP six-month dietary toxicity study conducted prior to OECD guideline implementation, thiophanate-methyl (batch and purity not specified) was administered to ICR mice (12/sex per group) at 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 2, 10, 50, 250 and 1240 mg/kg bw per day for males and 0, 2, 11, 52, 231 and 1630 mg/kg bw per day for females, respectively). Mice were checked daily for mortality and clinical signs of toxicity. Body weight and feed consumption were measured weekly. At study termination blood was sampled for haematological investigations. All mice were macroscopically examined, selected organs were weighed and a wide range of organs and tissues were histopathologically examined.

No treatment-related mortality or clinical signs were reported. Body weight gain was decreased at 8000 ppm in males (11%) and females (17%). A slight reduction in body weight gain (7%) was observed in females at 1600 ppm. At 8000 ppm a slight reduction in haematocrit (13% in males and 8% in females) and red blood cell (RBC) counts (7% in males and 12% in females) was found. At 8000 ppm,

increased absolute (18% and 17% in males and females, respectively) and relative (31% and 43% in males and females) liver weights were observed. Histopathological examination revealed hepatocyte hypertrophy in high-dose males and females. No other changes in organ weight or histopathology were noted.

The NOAEL was 1600 ppm (equal to 231 mg/kg bw per day) based on decreased body weight gain and slight reductions in haematocrit and RBC counts in males and females at 8000 ppm, equal to 1240 mg/kg bw per day (Noguchi & Hashimoto, 1970b).

Rat

In a 13-week dietary toxicity study, with a study design resembling OECD guideline 408, thiophanate-methyl (purity 96.6%; batch no. TIF-1016) was administered to groups of Fischer 344 rats (10/sex per group) at 0, 200, 2200, 4200, 6200 or 8200 ppm (equal to 0, 13.9, 155, 293, 427 and 565 mg/kg bw per day for males and 0, 15.7, 173, 323, 479 and 647 mg/kg bw per day for females, respectively). The rats were checked daily for mortality and clinical signs. Ophthalmological examinations were conducted on the control and high-dose rats at the beginning and during week 12 of the treatment period. Body weights and feed consumption were measured weekly. Haematology, clinical biochemistry (including thyroid hormone measurements in dose groups 0, 200, 2200 and 8200 ppm) were performed at week 13 and urine analysis was performed at week 12. All rats were necropsied, and weights of brain, heart, testes, liver, lung, spleen, adrenal, kidney, ovary, thyroid, pituitary and thymus were recorded. A wide range of tissues of all rats was examined microscopically.

No treatment-related mortality, clinical signs or effects on body weight or ophthalmology were observed. Feed consumption was reduced by 9–17% during week 1 and 2 of treatment in males at 6200 ppm and higher, and in females at 4200 ppm and higher. Haematology showed effects of slight anaemia, that is, decreased haemoglobin, haematocrit, mean cell volume (MCV), mean cell haemoglobin and mean cell haemoglobin concentration at 2200 ppm and higher (Table 11).

Table 11. Results of haematology investigations in male rats after 90 days exposure to thiophanate-methyl

	Measure per dose level					
	0 ppm	**200 ppm**	**2200 ppm**	**4200 ppm**	**6200 ppm**	**8200 ppm**
Males						
PCV (%)	47.5	47.3	46.3	46.2	46.0*	44.9** (–6%)
Hb (g/dL)	15.2	15.0	14.6* (−4%)	14.5**	14.3**	13.9** (−9%)
MCV (μm^3)	58.6	58.5	57.5**	57.4**	57.8	57.6* (−2%)
MCH (pg)	18.7	18.6	18.2**	18.0**	18.0**	17.8** (−5%)
MCHC (%)	31.9	31.8	31.6	31.3**	31.2**	30.9** (−3%)
Females						
PCV (%)	47.7	47.2	46.4	45.2**	45.0**	45.1** (−5%)
Hb (g/dL)	15.3	15.0	14.7*	14.2**	14.2**	14.1** (−8%)
MCV (μm^3)	62.5	62.6	61.4	61.1**	60.6**	60.6** (−3%)
MCH (pg)	20.1	20.0	19.5**	19.2**	19.1**	19.0** (−5%)
MCHC (%)	32.2	31.9	31.8*	31.5**	31.6**	31.4** (−2%)

Hb: haemoglobin; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; MCV: mean cell volume; PCV: packed cell volume; *: $P < 0.05$; **: $P < 0.01$

Source: Nishibe & Takaori (1990)

Clinical chemistry revealed a dose-related increase in both sexes in total cholesterol (41–129%), total protein (8–19%) and albumin (8–15%) and in males in calcium (4–6%) at 2200 ppm or higher. Triiodothyronine (T_3) was statistically significantly increased (56%) in females at 8200 ppm ($P < 0.001$). The 37% increase in T_3 in males was not statistically significant. Tetraiodothyronine (T_4, or thyroxine) was not affected by treatment. Plasma cholinesterase was dose-dependently increased in males at 4200 ppm and above, and dose-dependently decreased at 2200 ppm and above in females. However, as

only total plasma cholinesterase was measured and levels increased in males and decreased in females, the toxicological relevance of this finding is equivocal. Urine analysis showed an increase of urinary protein in males at 6200 and 8200 ppm an increase of ketone bodies in males at 8200 ppm.

Absolute and relative thyroid weights were increased in both sexes at 2200 ppm and above (Table 12). In females at 200 ppm there was a 31% increase in absolute thyroid weight compared to controls ($P < 0.05$), but with no accompanying histopathological changes. At 2200 ppm and higher, increases in absolute and relative liver weights (~30–60% in males; ~22–65% in females) and kidney weights (~9–19% in males) were found. Kidney weights were increased by 9% in high-dose females. Decreased absolute and relative thymus weights were found in males at 6200 ppm and higher (26–29%) and in females at 4200 ppm and higher (~15–28%). Slight (6–10%) non dose-dependent increases in absolute and relative left testes weights were seen at 4200 ppm and higher; right testes weights were not affected.

Table 12. Absolute and relative thyroid weights in male and female rats after 90 days exposure to thiophanate-methyl

	Measure per dose level					
	0 ppm	**200 ppm**	**2200 ppm**	**4200 ppm**	**6200 ppm**	**8200 ppm**
Males						
Absolute weight (g)	0.023	0.024	0.032**	0.044**	0.055**	0.066**
% increase in absolute weight	–	4	39	91	139	186
Relative weight (%)	7	7	10	14	17	21
% increase in relative weight	–	0	43	100	143	200
Females						
Absolute weight (g)	0.016	0.021*	0.024**	0.027**	0.037**	0.039**
% increase in absolute weight	–	31	50	69	131	143
Relative weight (%)	9	12	14	16	21	22
% increase in relative weight	–	33	56	78	133	144

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Nishibe & Takaori (1990)

Dose-related histopathological changes, accompanied by weight changes of the affected organs, were observed in the thyroid, liver, adrenal and kidney. Follicular hyperplasia and hypertrophy were noted in the thyroid in both sexes at doses of 2200 ppm and above. Despite the increased thyroid weight in females at 200 ppm, no histopathological lesions were noted in this dose group. Therefore the increase in thyroid weight at 200 ppm is not considered adverse. Hepatocellular swelling and increased lipofuscin pigment were noted in both sexes at 2200 ppm and above. Fatty degeneration of adrenal cortex was noted in females at 4200 ppm and in both sexes in the two higher dose groups. The severity of glomerulonephrosis, characterized by tubular lesions and hyaline droplets in the glomerular epithelium, was increased in males at 2200 ppm and above and in females at 8200 ppm.

The NOAEL was 200 ppm (equal to 13.9 mg/kg bw per day) based on haematological changes indicative of slight anaemia, increased thyroid and liver weights, follicular hyperplasia and hypertrophy of the thyroid and hepatocellular swelling and increased lipofuscin pigment in both sexes and increased severity of glomerulonephrosis, characterized by tubular lesions and hyaline droplets in the glomerular epithelium, in males observed at 2200 ppm (equal to 155 mg/kg bw per day) (Nishibe & Takaori, 1990).

In a pre-guideline six-month dietary toxicity study with limited investigations, thiophanate-methyl (batch and purity not specified) was administered to Sprague Dawley rats (12/sex per group) at 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 1, 4, 20, 95 and 500 mg/kg bw per day for males and 0, 1, 5, 22, 110 and 660 mg/kg bw per day for females, respectively). Rats were checked daily for mortality and clinical signs of toxicity. Body weight and feed consumption were measured weekly. At scheduled

kill, blood and urine were sampled for haematological and clinical examinations and urine analysis. All rats were macroscopically examined, a selection of organs was weighed and a wide range of organs and tissues was histopathologically examined.

No treatment-related mortality and clinical signs were reported. Body weight gain was decreased at 8000 ppm in males (28%) and females (30%). At 8000 ppm a slight reduction in haematocrit (8% in males and 4% in females) and RBC counts (13% in males and 10% in females) was found. At 8000 ppm decreases in glucose (27–28%), aspartate aminotransferase (ASAT) and alkaline phosphatase (ALP) and a slight increase in cholesterol (58–71%) were observed. These changes may be secondary to general toxicity at this dose. The toxicological significance of reduced ASAT and ALP levels is not clear. At 8000 ppm increased relative liver weights (45% and 62% in males and females, respectively) were observed, while absolute liver weights were slightly increased (4–15%). At the high dose, absolute and relative thyroid weights were increased (absolute: 12% in males and 18% in females; relative: 51% in males and 62% in females). No effects were noted in other organs. Histopathological examination of the thyroid revealed small follicles, cubic epithelium cells and a decrease of colloidal substance in several rats (incidences not reported) at 8000 ppm. No other changes in organ weight or histopathology were noted.

The NOAEL was 1600 ppm (equal to 95 mg/kg bw per day) based on decreased body weight gain, slight reductions in haematocrit and RBC counts, decreased glucose levels and increased cholesterol levels in males and females at 8000 ppm (equal to 500 mg/kg bw per day) (Noguchi & Hashimoto, 1970c).

Dog

In a three-month toxicity study, beagle dogs (4/sex per dose group) received thiophanate-methyl (purity 96.6%; batch no. TIF-1016) via gelatine capsule at doses of 0, 50, 200 or 800/400 mg/kg bw per day. As severe toxicity was noted at 800 mg/kg bw per day, at study week 8 the high dose level was reduced to 400 mg/kg bw per day (from day 50 until study end). The dogs were checked daily for mortality and clinical signs. A detailed physical examination and ophthalmoscopy were performed weekly. Feed consumption and body weights were measured weekly. Neurological, ophthalmological, blood pressure and electrocardiogram examinations were carried out before dosing and in week 7 and 13. Haematology, clinical chemistry and urine analysis were performed pretrial and after about 1.5 and 3 months of treatment. The clinical chemistry investigations at study termination also included the determination of the thyroid hormones T_3 and T_4 as well as thyroid-stimulating hormone (TSH). All dogs were subjected to a complete gross pathological evaluation. Selected organs were weighed and histopathology was carried out on a wide range of organs and tissues.

Three males and three females high-dose dogs looked thin and appeared dehydrated from week 2 (males) or 6 (females) onward. One male and two female mid-dose dogs also appeared thin at the end of the study. In addition, in high-dose dogs and occasionally in mid-dose dogs, decreased feed consumption, tarry stools (both dose levels) and lethargy (high dose only) were observed. One high-dose male was euthanized in a moribund condition on day 41. One low-dose male dog died unexpectedly on day 36. No cause of death could be established. A reduction in body weight was observed already during the first week of treatment in high-dose males (−400 g) and females (−300 g). After lowering the dose from 800 to 400 mg/kg bw per day at the end of week 7, body weights remained stable or slightly recovered in the high-dose dogs. In the mid-dose group body weights gradually decreased up to about week 7 and then remained stable during the rest of treatment. In the control and low-dose group the dogs gained weights throughout the duration of the study. Feed consumption was markedly reduced in high-dose males (> 70% during weeks 3 and 4) and females (> 50% during weeks 3–5) and in mid-dose males (up to 30% during week 4) and females (up to 44% in week 5). Feed consumption remained lower in high-dose dogs throughout the study duration, although the reduction was less severe after lowering the dose from 800 to 400 mg/kg bw per day on day 50. Changes in body weights are presented in Table 13.

Table 13. Group mean body weights in male and female dogs

Dose group	Sex	Body weight (+ weight change compared to week 0) in kg			
		Week 0	Week 1	Week 7	Week 13
Control	Male	9.0	9.0 (0.0)	9.8 (0.8)	10.5 (1.5)
	Female	7.1	7.4 (0.3)	7.7 (0.6)	8.0 (0.9)
50 mg/kg bw per day	Male	9.0	9.0 (0.0)	9.4 (0.4)	9.8 (0.8)
	Female	7.2	7.3 (0.1)	7.8 (0.6)	8.2 (1.0)
200 mg/kg bw per day	Male	9.0	9.0 (0.0)	8.6 (−0.4)	8.9 (−0.1)
	Female	7.1	7.0 (−0.1)	6.1 (−1.0)	6.1 (−1.0)
800/400 mg/kg bw per day	Male	9.0	8.6 (−0.4)	7.0 (−2.0) [a]	7.6 (−1.4)
	Female	7.1	6.8 (−0.3)	5.7 (−1.4) [a]	5.9 (−1.2)

bw: body weight *Source*: Auletta (1991)

[a] At the end of week 7, dose was lowered from 800 mg/kg bw per day to 400 mg/kg bw per day.

Haematological examination at termination showed the following changes in high-dose dogs: slight decreases in haemoglobin (also found in mid-dose females), haematocrit and/or RBC counts and increases in platelet counts (Table 14). Similar trends were observed at 1.5 months; however, at that time differences were not statistically significant. T_3 levels of high-dose males and T_3 and T_4 levels in mid- and high-dose females were slightly decreased. TSH values demonstrated wide variations although no dose-related trends were observed (Table 15). In mid- and high-dose dogs reductions in alanine aminotransferase (ALAT) (up to 70%), serum albumin (up to 18%) and calcium (up to 9%) and increases in cholesterol (up to 70%) and chloride (up to 5%) were observed.

Table 14. Results of haematology investigations in male and female dogs after 90 days exposure to thiophanate-methyl

	Measure per dose level							
	0 mg/kg bw per day		50 mg/kg bw per day		200 mg/kg bw per day		400 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
HCT (%)	51	49	47	49	45	44	39* (−24%)	44* (−10%)
Hb (g/dL)	18.1	17.4	16.7	17.4	15.9	15.5* (−11%)	13.6* (−25%)	15.3* (−12%)
Platelets (100 T/µL)	3.52	4.04	4.67	4.22	4.30	5.33	6.12* (74%)	6.56** (62%)

HCT: haematocrit; Hb: haemoglobin; *: $P < 0.05$; **: $P < 0.01$ *Source*: Auletta (1991)

Table 15. Results of thyroid hormone investigations in male and female dogs after 90 days exposure to thiophanate-methyl

	Measure per dose level							
	0 mg/kg bw per day		50 mg/kg bw per day		200 mg/kg bw per day		400 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
T_3	1.38	1.80	1.51	1.55	1.31	1.34**	1.08	1.35**
T_4 (ng/dL)	2.30	3.61	3.25*	3.57	2.47	1.92*	2.13	2.48
TSH	1.68	5.97 [a]	6.57	1.94	5.07	0.00 [b]	1.58	1.21 [c]

M: male; F: female; *: $P < 0.05$; **: $P < 0.01$; *Source*: Auletta (1991)

T3: triiodothyronine; T4: tetraiodothyronine (thyroxine); TSH: thyroid-stimulating hormone;

[a] TSH level was 0.00 in three dogs and 23.86 in one dog.

[b] TSH result was 0.00 in three dogs and 7.75 in one dog, but was given as 0.00 for the mean value in the study report.

[c] TSH result was 0.00 in three dogs and 4.85 in one dog.

Urine analysis and ophthalmoscopy revealed no effects of treatment. At the mid- and high-dose, absolute and relative weights of liver (both sexes) and thyroid (males only) were increased (Table 16). The weights of these organs relative to brain weight were not changed.

Table 16. Thyroid and liver weights compared to controls in male and female dogs after 90 days exposure to thiophanate-methyl

		% change relative to control value					
		50 mg/kg bw per day		200 mg/kg bw per day		400 mg/kg bw per day	
Organ / measure		Males	Females	Males	Females	Males	Females
Liver	Absolute weight	−5	+8	+6	+11	+3	+6
	Relative to body weight	+5	+0	+26**	+32*	+42**	+34**
	Relative to brain weight	−7	+3	+2	+7	+8	+13
Thyroid	Absolute (%)	−1	−4	+31	−4	+49	−10
	Relative to body weight	+9	−11	+53	+18	+107	+15
	Relative to brain weight	+0	−9	+27	−9	+60	−5

bw: body weight; *: $P \leq 0.05$; **: $P \leq 0.01$; *Source*: Auletta (1991)

Macroscopic examination showed slightly enlarged livers in a few dogs. Histopathological examination of the liver revealed minimal to moderate vacuolation/vesiculation of the hepatocellular cytoplasm. This was inversely related to the dose, and was considered to reflect the decreased feed intake in the higher dose groups resulting in a decrease of intracytoplasmic hepatocellular glycogen content. Hypertrophy of the follicular epithelial cells of the thyroid was seen in each treated group but not the control group (0/8, 2/8, 5/8, 8/8, respectively). Incidence and severity were dose related (minimal at the low dose). Minimal to marked hyperplasia of the follicular epithelium was also seen (0/8, 0/8, 1/8, 7/8, respectively). The incidence and severity of hypertrophy and hyperplasia were slightly more pronounced in males than in females. In two high-dose males with marked severity of these findings a marked decrease in the quantity of intrafollicular colloid was also evident. Other histopathological changes observed in the mid- and high-dose dogs were considered to be secondary to the decreased feed intake and reductions in body weight or body weight gains. The changes included a slight increase in the severity of intracytoplasmic vacuoles in the epithelial cells lining the gall bladder, lymphoid cell depletion/atrophy of the spleen and an increase in the incidence and severity of thymic involution/atrophy, atrophy of acinar cells in the pancreas (manifested as a decrease in the quantity of intracytoplasmic zymogen granules), hypoplasia/atrophy of the prostate gland in all mid- and high-dose males and the absence of normal cyclic activity in the ovaries and uterus of high-dose females.

The lowest-observed-adverse-effect level (LOAEL) was 50 mg/kg bw per day, based on the hypertrophy of the follicular epithelial cells of the thyroid at this dose (Auletta, 1991).

In a one-year toxicity study, beagle dogs (4/sex per dose group) received thiophanate-methyl (purity 96.6; batch no. TIF-1016) by capsule at concentrations of 0, 8, 40 or 200 mg/kg bw per day. The dogs were checked daily for mortality and clinical signs. Detailed physical examinations and ophthalmoscopy took place weekly. Body weight and feed consumption were measured weekly. Haematology, clinical chemistry including determination of the thyroid hormones T_3 and T_4 and TSH, and urine analysis were carried out after 3, 6 and 12 months. At scheduled kill all dogs were macroscopically examined, organ weights were determined and histopathology was carried out on a wide range of organs.

No mortalities were observed. Tremors were noted in all high-dose dogs during the first three weeks of treatment, first seen on day 1 of treatment, and in one mid-dose female on day 13 of treatment. As these initial, transient clinical signs (tremors) were not observed in a three-month study in dogs given thiophanate-methyl at doses of up to 800 mg/kg bw per day the Meeting considered that the tremors were not attributable to a toxicological effect of the test substance.

In high-dose dogs slight body weight loss (0.1–0.5 kg) was observed during the first week and a decreased body weight gain was observed thereafter. At the end of the study, body weights of high-dose males and females were 18% and 20% lower than that of control males and females. In mid-dose dogs body weights at scheduled kill were 6–7% lower than that of controls. Feed consumption in high-dose dogs was reduced up to about 30% from week 1 to 10 of treatment and although differences were smaller thereafter, feed consumption remained lower than controls up to the end of the study, particularly in males. Ophthalmological investigations revealed no treatment-related findings. Haematological effects in high-dose male dogs consisted of slight decreases in total erythrocyte counts (−13%), haemoglobin (−14%) and haematocrit (−14%) values at 12 months. Similar but smaller reductions in these parameters, not reaching statistical significance, were seen at three and six months. Clinical chemistry showed elevated serum cholesterol levels in mid- (30–47%) and high-dose (42–51%) males and in high-dose females (76–93%) at 6 and 12 months. Serum ALP levels were increased in high-dose males (101–200%) and females (47–82%, not statistically significant). ALAT levels were slightly decreased in high-dose males and females. The latter observation is not considered indicative of an adverse effect. Decreases in the albumin to globulin ratio resulting from decreased albumin levels (up to 19%) and increased globulin levels (up to 38%) were seen in mid- and high-dose males at 6 and 12 months. The slight decrease in blood urea nitrogen values (19–33%) was not considered indicative of toxicity. In high-dose males decreased levels of calcium (9–11%), potassium (9–18%) and phosphorous (28–42%) were observed and in mid-dose males levels of calcium were decreased (6–7%, months 6 and 12). Statistically significant decreases in T_4 levels were seen in mid- (46%) and high-dose males (79%) at 6 months and in high-dose males at 12 months (57%). No effects on T_3 or TSH levels were found. T_3, T_4 and TSH were not affected in females.

In high-dose dogs increased relative liver weights (34–45%) were found. Absolute and relative thyroid weights were increased in males and females, reaching statistical significance in females only (Table 17).

Table 17. Thyroid weights compared to controls in male and female dogs after one year exposure to thiophanate-methyl

	Weight (% of control value)[a]			
	0 mg/kg bw per day	**8 mg/kg bw per day**	**40 mg/kg bw per day**	**200 mg/kg bw per day**
Males				
Absolute weight (g) (% of control)	0.927	1.026 (111)	1.240 (134)	1.310 (141)
Weight relative to body weight (%) (% of control)	7.36	7.63 (104)	10.43 (141)	12.67 (172)
Weight relative to brain weight (% of control)	1.20	1.24 (103)	1.49 (124)	1.57 (131)
Females				
Absolute weight (g) (% of control)	0.716	0.922 (129)	1.04* (145)	0.998 (139)
Weight relative to body weight (% of control)	6.79	7.49 (110)	10.46** (154)	11.91** (175)
Weight relative to brain weight (% of control)	0.93	1.19 (128)	1.36* (146)	1.35* (145)

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

[a] Expressed in g for absolute weights and % for relative weights, with % change from control in parentheses.

Source: Auletta (1992)

Microscopic examination of the thyroid showed minimal to moderate hypertrophy of the follicular epithelium of the thyroid gland in males (0/0, 0/0, 0/0, 4/4) and females (0/0, 0/0, 2/4, 3/4) and slight hyperplasia (high-dose group, one male and one female).

The NOAEL was 8 mg/kg bw per day, based on effects on thyroid weight in both sexes and minimal to moderate hypertrophy of the follicular epithelium of the thyroid gland of females observed at 40 mg/kg bw per day (Auletta, 1992).

In a pre-guideline two-year toxicity study, with limited reporting, beagle dogs (5/sex per dose group except 4/sex at the highest dose) received thiophanate-methyl (purity and batch no. not reported) by capsule at concentrations of 0, 2, 10, 50 or 250 mg/kg bw per day. The dogs were checked for mortality and clinical signs, and findings were reported on weekly case sheets. Body weight and feed consumption were measured weekly. Ophthalmology, electrocardiography, haematology, clinical chemistry and urine analysis were performed before the start of treatment and at 6, 12, 18 and 24 months. After 12 months one dog per sex and per dose was killed, the remaining dogs were killed after 24 months. All dogs were necropsied, organs were weighed and histopathological examination was performed on a wide range of organs.

No mortalities or clinical signs were observed. At the end of the study, body weights of high-dose males and females were 14% and 5% lower than that of control males and females. Weight gained during treatment was 4.5 and 4.4 kg in high-dose males and females, respectively, versus 6.4 and 5.4 kg in control males and females, respectively. Feed consumption was not affected. Ophthalmology, haematology, clinical chemistry, urine analysis and necropsy revealed no treatment-related findings. Increased thyroid weight was observed in both sexes at mid-dose (34–50%) and high dose (45–77%). The incidence of decreased colloid tended to be increased at 50 and 250 mg/kg bw per day.

The NOAEL was 10 mg/kg bw per day, based on effects on thyroid weight and histopathology of the thyroid in both sexes at 50 mg/kg bw per day (Hashimoto et al., 1972).

(b) Dermal application

A repeated-dose dermal toxicity study in rabbits and guinea-pigs was available (Noguchi & Hashimoto, 1970a). However, as several reporting deficiencies were noted, this study was considered unsuitable for setting a NOAEL for dermal toxicity.

In a subacute dermal toxicity study with a study design resembling OECD TG 410, the shaved intact skin of New Zealand White rabbits (5/sex per dose group) was exposed to thiophanate-methyl (batch TIF-1016, purity 96.6%) in deionized water at 0, 100, 300 or 1000 mg/kg bw per day for a total of 15 topical applications per rabbit over a period of 21 days (6 hours/day). Corrected for five days exposure per week, doses were 0, 71, 214 and 714 mg/kg bw per day. The rabbits were observed daily for mortality and clinical signs including dermal irritation. Body weight was measured weekly. Weekly feed consumption was reported. Blood was sampled prior to scheduled kill for haematology and clinical chemistry. At scheduled kill the rabbits were macroscopically examined, weights of brain, kidneys, liver, ovaries and testes were recorded and liver, kidneys, skin and gross lesions were histopathologically examined.

At 1000 mg/kg bw per day a reduction in feed consumption of about 25% was observed in both sexes. Body weight at the end of treatment was slightly lower (6–7%) than controls. Clinical chemistry and haematology showed no effects of treatment. In all treatment groups sporadic occurrences of very slight erythema and desquamation with corresponding microscopic tissue changes of the application site skin (hyperkeratosis, inflammation) were observed. Hyperkeratosis of the treated skin was observed in one female in each of the treated groups. Nonsuppurative subdermal inflammation was also present for the 300 mg/kg bw per day female that had hyperkeratosis.

The NOAEL was 714 mg/kg bw per day, the highest dose tested (Naas, 1991).

2.3 Long-term studies of toxicity and carcinogenicity

Mouse

In a pre-guideline two-year dietary carcinogenicity study with limited investigations, thiophanate-methyl (purity ~ 94%; batch no. not reported) was administered to ICR-JCL mice (50/sex per group) at 0, 10, 40, 160 or 640 ppm (equal to 0, 1.2, 4.4, 20 and 82 mg/kg bw per day for males and 0, 1.3, 5.0, 19 and 82 mg/kg bw per day for females, respectively). Mice were housed four per cage. The mice were checked daily for mortality and clinical signs. Body weight was recorded monthly. Feed consumption per four mice was recorded biweekly. All mice were necropsied and a selection of organs was histopathologically examined.

There were no effects of treatment on mortality, clinical signs or feed consumption. At 640 ppm, body weights of male mice were about 10% lower than those of controls after about 1 year of treatment. No effect of treatment on terminal body weights was observed. In the testes at 640 ppm a slight reduction in spermatogenesis was observed. There was no increase in the incidence of neoplasia at any dose level.

The NOAEL was 160 ppm (equal to 20 mg/kg bw per day) based on reduced body weight gain and histopathological changes in testes of males at 640 ppm (equal to 82 mg/kg bw per day) (Nishibe et al., 1973).

In view of the limitations in study design and reporting, this study is considered supporting only in the present risk assessment.

In an 18-month dietary carcinogenicity study, with a study design resembling OECD TG 451, thiophanate-methyl (purity 96.6 and 95.9%; batches no. TIF-1016 and TIF-01016) was administered to Crl:CD-1(ICR)BR mice (60/sex per group) at 0, 150, 640, 3000 or 7000 ppm (equal to 0, 24, 99, 468 and 1079 mg/kg bw per day for males and 0, 29, 123, 558 and 1329 mg/kg bw per day for females, respectively). A group of 10/sex per dose were killed and necropsied following 39 weeks of treatment. The mice were checked daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and feed consumption were recorded weekly during the first 14 weeks and every 4 weeks thereafter. Blood was sampled for haematology and assessment of thyroid function just before the interim kill and at week 77. Ophthalmoscopy was performed before the start of treatment and at week 77. Mice found dead, killed prematurely or killed at the end of the treatment period of at least 79 weeks were necropsied. Organ weights (adrenals, brain, heart, liver, kidneys, spleen, ovaries, testes and thyroid gland) were recorded. A wide range of tissues was examined microscopically.

The cumulative mortalities were 10, 11, 14, 16 and 24 for males and 12, 13, 15, 17 and 23 for females in the 0, 150, 640, 3000 and 7000 ppm groups, respectively. Mortality was significantly higher in high-dose males and females and slightly increased at 3000 ppm. Amyloidosis in the kidney was considered to be the cause of death in 1/10, 1/11, 4/14, 5/16 and 14/24 males and 3/12, 3/13, 4/15, 11/17 and 11/23 females of the 0, 150, 640, 3000 and 7000 ppm groups, respectively. The increased death rate was to some extent attributed to atrial thrombosis. No treatment-related clinical signs or ophthalmoscopic changes were noted. Compared to controls, final body weights in both sexes were 5–6% lower at 3000 ppm and 7–8% lower at 7000 ppm. Feed consumption was occasionally slightly decreased in high-dose males and in females at all doses. The decreases were not considered toxicologically relevant. Haematology revealed a 15% lower RBC count in high-dose males. At week 39 TSH values in males at 3000 and 7000 ppm were significantly increased whereas T_4 levels were slightly and nonsignificantly decreased. Similar, but smaller effects on TSH and T_4 levels were observed in females at 3000 and 7000 ppm at 39 weeks. No effect of treatment was observed at 18 months. Necropsy showed no treatment-related changes.

Effects on organ weights are presented in Table 18. Mean absolute and relative heart weights for the 7000 ppm males and females were increased at nine months and absolute and relative heart weights for the females were increased at 18 months. The incidence of atrial thrombosis was increased grossly and microscopically in the 7000 ppm males (2 versus 0 in control) and the 3000 and 7000 ppm females (2 and 5, respectively, versus 0 in control). At nine months increased liver weights and hepatocellular centrilobular hypertrophy was observed in males and females at 3000 and 7000 ppm. No hepatocellular adenomas were observed at nine months. At study termination, increased absolute and relative liver

weights were observed in males at 7000 ppm. At the nine-month interim kill, but not at study termination, increased absolute and relative thyroid weights were observed in males at 3000 and 7000 ppm.

Table 18. Absolute and relative organ weights[a] in 18-month dietary carcinogenicity study in mice

		Organ weights per dose level[b]				
		0 ppm	**150 ppm**	**640 ppm**	**3000 ppm**	**7000 ppm**
Males						
Liver (week 39)	Absolute	2.09	2.01	2.19	2.50 (20)	2.79 (34)**
	Relative	5.05	4.97	5.27	6.33 (25)***	7.08 (40)***
Liver (week 79)	Absolute	2.27	2.21	2.06	2.81 (24)	4.13 (82)**
	Relative	5.68	5.79	5.21	6.89 (21)	10.56 (86)***
Thyroid glands (week 39)	Absolute	0.004 8	0.006 0	0.005 9	0.007 2*	0.010 1 (110)**
	Relative	0.011	0.015	0.014	0.018 (64)**	0.026 (136)**
Females						
Liver (week 39)	Absolute	1.65	1.59	1.81	2.04 (24)**	2.59 (57)**
	Relative	5.08	4.83	5.59	6.40 (26)**	8.00 (57) **
Heart (week 39)	Absolute	0.17	0.16	0.17	0.18	0.21 (23)**
	Relative	0.53	0.50	0.53	0.56	0.66 (25)**
Heart (week 79)	Absolute	0.18	0.20	0.19	0.19	0.24 (39)**
	Relative	0.55	0.59	0.58	0.59	0.77 (40)**

ppm: parts per million; *: $P < 0.05$ (Dunnett test); **: $P < 0.01$ (Dunnett test); *Source*: Tompkins (1992)

[a] Only organs where changes were significant are shown in the table.

[b] Expressed in g for absolute weights and % for relative weights, with % change from control in parentheses.

At nine months, hepatocellular centrilobular hypertrophy was noted at 640, 3000 and 7000 ppm in females and at 3000 and 7000 ppm in males. At the interim kill, hepatocellular centrilobular hypertrophy was observed in males at 3000 and 7000 ppm. The incidences of hepatocellular adenomas were increased in males and females at 3000 and 7000 ppm (Table 19).

Table 19. Incidence of mouse hepatocellular adenomas in an 18-month dietary carcinogenicity study in mice

Time of necropsy	**Incidence of hepatocellular adenomas (%)[a]**				
	0 ppm	**150 ppm**	**640 ppm**	**3000 ppm**	**7000 ppm**
Males					
Unscheduled necropsies	0/10 (0)	0/11 (0)	0/14 (0)	2/16 (12.5)	6/24 (25)
Terminal necropsies	4/40 (10)	8/39 (20.5)	7/36 (19.4)	17/34 (50)**	18/26 (69.2)**
Combined	4/50 (8)	8/50 (16)	7/50 (14)	19/50 (38)**	24/50 (48)**
Females					
Unscheduled necropsies	0/12 (0)	0/13 (0)	0/15 (0)	0/17 (0)	2/23 (8)
Terminal necropsies	0/38 (0)	0/37 (0)	3/35 (8.6)	8/33 (24.2)**	16/27 (59.3)**
Combined	0/50 (0)	0/50 (0)	3/50 (6)	8/50 (16)**	18/50 (36)**

ppm: parts per million; **: $P < 0.01$; *Source*: Tompkins (1992)

[a] Results expressed as no. of animals with the finding /no. of animals examined and, in parentheses, as a percentage.

The incidences for hepatocellular adenomas in the 150 and 640 ppm groups were not significantly above the control group incidence and the incidence for males was within the historical control range (0–16.3%). The incidence for females at 640 ppm was just outside the historical control range (0–2.7%). The study authors considered the hepatocellular centrilobular hypertrophy and the hepatocellular adenomas a consequence of induction of the cytochrome P450 drug metabolizing system.

Two hepatocellular carcinomas were seen at terminal necropsy: one in a 640 ppm male and one in a 7000 ppm male. The incidences of 2% were within the range of historical control incidence (mean 1.4%, range 0–6%). One hepatoblastoma was observed in a male at 7000 ppm. Even though this is a relatively rare tumour (0.001% historical control), the study authors considered this single occurrence not a treatment-related effect.

Atrial thrombosis was present at an increased incidence both grossly and microscopically in the 7000 ppm group for males and the 3000 and 7000 ppm group for females. The incidence was 1, 2, 0, 1 and 8 in males and 0, 2, 1, 6 and 14 in females of the 0, 150, 640, 3000 and 7000 ppm dose groups, respectively. The increased incidence in females at 3000 and 7000 ppm and in males at 7000 ppm was considered to be treatment related. The study authors considered it to be the cause of death in one male at 3000 ppm, three males at 7000 ppm and one female each at 150 and 7000 ppm.

The NOAEL was 150 ppm (equal to 29 mg/kg bw per day) based on the hepatocellular centrilobular hypertrophy at nine months and hepatocellular adenomas in females observed at 640 ppm (equal to 123 mg/kg bw per day).

The thiophanate-methyl-related increase in hepatocellular adenomas was considered a consequence of an induction of the cytochrome P450 drug metabolizing system (Tompkins, 1992).

Rat

In a two-year combined chronic toxicity and carcinogenicity study performed according to OECD TG 453, thiophanate-methyl (purity 96.6%; batch no. TIF-1016) was administered through the diet to Fischer-344 rats (60/sex per group) at 0, 75, 200, 1200 or 6000 ppm (equal to 0, 3.3, 8.8, 54 and 281 mg/kg bw per day for males and 0, 3.8, 10.2, 64 and 335 mg/kg bw per day for females, respectively). The rats were checked daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and feed consumption were recorded weekly during the first 14 weeks, at week 16 and every four weeks thereafter. Ophthalmoscopy was performed on all rats before treatment and after 12 and 24 months of treatment, and in addition at 6 and 18 months in the control and high-dose rats. Blood and urine of 10 rats per sex and per dose were sampled for haematology, clinical chemistry including cholinesterase activity, and urine analysis at 3 (haematology only), 6, 12, 18 and 24 months. TSH and thyroid hormones T_4 and T_3 were determined in eight nonfasting rats per sex per dose. Due to high mortality at 24 months, haematology and clinical chemistry was performed only on one high-dose male. The data of this one male at 24 months are not described below. Rats found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (adrenals, brain, pituitary, heart, liver, lung, kidneys, spleen, thyroid/parathyroid, ovaries and testes) were recorded. A wide range of tissues was examined microscopically.

In the same study mechanistic investigations were performed on Fischer 344 rats or ICR mice. Thiophanate-methyl (6000 ppm), or the reference compounds phenobarbital (500 ppm) or propylthiouracil (1000 ppm) substances were given to male and female rats (six weeks old) for two or eight days (males) and eight days (females). Thiophanate-methyl and phenobarbital were given by diet; propylthiouracil was given in distilled water. Measurement of T_3, T_4 and TSH in serum, and determination of drug intake, liver and thyroid weights and total cholesterol in serum were performed. Thyroid weight was measured after a recovery period of eight days. Additional administration of thiophanate-methyl to male rats (eight weeks old) was performed for eight days with daily T_4 supplementation (30 µg/kg bw subcutaneously injected). The microsome fraction of each liver was isolated. Measurements of enzyme induction of the following enzymes was performed: cytochrome P450, cytochrome b5, NADPH-cytochrome c reductase, uridine-glucuronosyltransferase (UDPGT) and microsomal protein. Isolation of microsome fraction of porcine thyroids was performed, purchased from a butchery. Measurement of peroxidase activity in the presence of thiophanate-methyl (10^{-3} to 10^{-4} mol/L) or propylthiouracil (10^{-4} to 10^{-6} mol/L) was performed.

Due to technical reasons with feeders, eight high-dose males were killed in extremis at weeks 11 and 12. These rats suffered from a fracture of the nasal bone and subsequent dyspnoea (rhinorrhagia). After week 80, the mortality in the 6000 ppm group increased extremely and only two males survived the course of the study (Table 20). The main causes of death noted in males of the 6000 ppm group were nephropathy (22 rats), thyroid follicular cell tumours (10 rats) and leukaemia (6 rats). The severe nephropathy was associated with hyperplasia of the parathyroid, demineralization of the bone and metastatic calcification in various organs.

Table 20. Mortality rates in rats in two-year dietary study with thiophanate-methyl

Dose (ppm)	Number of deaths per sex and time					
	Males			Females		
	Week 52	Week 80	Week 104	Week 52	Week 80	Week 104
0	0/60 (0)	2/50 (4)	13/50 (26)	0/60 (0)	3/50 (6)	13/50 (26)
75	0/60 (0)	2/50 (4)	15/50 (30)	0/60 (0)	1/50 (2)	12/50 (24)
200	0/60 (0)	8/50 (16)	24/50 (48)*	0/60 (0)	1/50 (2)	8/50 (16)
1200	0/60 (0)	3/50 (6)	21/50 (42)	0/60 (0)	0/50 (0)	12/50 (24)
6000	8/60 (13)*	18/55 (33)***	53/55 (96)***	1/60 (2)	3/50 (6)	11/50 (22)

ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$ (chi square test)

[a] Results expressed as no. of animals with the finding / no. of animals examined and, in parentheses, as a percentage

Source: Takaori (1993)

No treatment-related clinical signs were noted in any dose groups during the first 52 weeks. After week 52, dose-related clinical signs included pale appearance of skin and mucous membrane (6000 ppm males), alopecia (6000 ppm females) and tissue masses on the skin and in the subcutis (1200 and 6000 ppm males). A reduction in body weight gain was observed at 1200 ppm in males (16%) and females (9%, not statistically significant) and at 6000 ppm in males (27%) and females (12%). A slight reduction in feed consumption was observed in high-dose males and females towards the end of the treatment period. However, on a g feed per kg bw basis, feed consumption was slightly increased.

Treatment-related anaemia (statistically significant decreases in RBC count, haemoglobin, haematocrit, MCV, mean cell haemoglobin and mean cell haemoglobin concentration) was observed in both sexes at 6000 ppm at one or more of the measurements at 3, 6, 12 and 18 months. In the 1200 ppm groups, occasionally statistically significant reductions in haemoglobin in males and MCV and mean cell haemoglobin in females were seen at 3 and/or 12 months. However, as the reductions were seen only occasionally and in one sex only, they were not considered to be toxicologically relevant. Increased platelet counts and white blood cell counts were frequently seen in males of the 6000 ppm group. Differential leukocyte counts remained normal at all test times for all groups.

Blood chemistry revealed statistically significant and dose-related increases in total cholesterol and total protein, and a decrease in albumin to globulin ratio in both sexes of the 1200 and/or 6000 ppm groups at 12 and/or 18 months. Decreased levels of chloride, potassium and decreased activities of lactate dehydrogenase, ALAT, ASAT and creatinine phosphokinase were seen predominantly in females at 1200 and 6000 ppm at 6 and 12 months. In high-dose males, chloride, ALAT and ASAT were decreased after 12 months. Serum cholinesterase activity was slightly increased in high-dose males and slightly decreased in females at 1200 and 6000 ppm. The toxicological relevance of this is unclear. In high-dose males, T_4 and T_3 values were statistically significantly lower than those of the control at 6, 12 and 18 months. TSH values of this group were higher than those of control animals. Similar statistically significant but weaker effects on the thyroid hormones and TSH were also found in females at 6000 ppm and in males at 1200 ppm at 24 months.

The mechanistic study showed that thiophanate-methyl caused decreases in T_4 and T_3 levels and increases in TSH levels and thyroid weights on days 2 and/or 8. The liver weight and amount of total cholesterol in serum were also increased. There was a close correlation between TSH level and thyroid weight. The T_4 supplementation counteracted the hypertrophy of the thyroid and the TSH response, indicating that thiophanate-methyl caused the hypertrophy by negative feedback mechanism. The T_4 supplementation had no influence on the increased liver weight and total cholesterol level. The effect of thiophanate-methyl on the thyroid was reversible after a recovery period without administration. Similar to propylthiouracil, thiophanate-methyl inhibited the thyroid peroxidase, with a median effective dose (ED_{50}) that was 30-fold higher.

Thiophanate-methyl at 6000 ppm caused hypertrophy of the liver, accompanied by a proliferation of liver cells that was observable in rats only after two days but in mice after two and eight days. Thiophanate-methyl induced cytochrome P450 and related drug-metabolizing enzymes, including UDPGT.

This enzyme plays an important role in the clearance of T_4 in the liver. UDPGT activity was increased by 335% in males at 6000 ppm. Phenobarbital, which also induced liver cell proliferation and drug-metabolizing enzymes, induced only a very slight increase of TSH without thyroid hypertrophy. The mechanistic study demonstrates that the thyroid effects resulting from thiophanate-methyl are likely to be the result of inhibition of thyroid hormones.

Urine analysis showed a statistically significant increase of urinary protein in both sexes at 6000 ppm group at various test times. By using a qualitative method (nephelometry), protein was also increased in males of the 1200 ppm dose and the 200 ppm dose (the latter only noted at month 24 and lacking a histological correlate). In high-dose males ketone bodies, urinary volume and water consumption were increased, and pH and specific gravity were decreased significantly at various test times.

Macroscopic examination at the interim kill showed changes in the liver (brownish-black colour) and kidneys (granular surface, brownish-black colour) in both sexes at 6000 ppm. At study termination, males at 1200 ppm showed granular kidneys. In females at 6000 ppm, kidneys were brownish-black and the thyroids were enlarged. In high-dose rats that died during the study, swelling of the thyroid (both sexes) and granular kidney (male) were noted.

Effects of treatment on liver, kidney and thyroid weights are presented in Table 21. Weight changes in other organs were considered to be related to the decrease in body weight.

Table 21. Terminal organ weights in rats in two-year dietary study with thiophanate-methyl [a]

	Organ weights in grams (% change) [b]				
	0 ppm	**75 ppm**	**200 ppm**	**1200 ppm**	**6000 ppm** [c]
Males					
Liver	12.841	13.454	13.811	16.021 (25)***	19.141 (49)
Thyroid	0.033	0.034	0.071 [d] (0.034)	0.041 (24)***	0.320
Kidney (right)	1.623	1.696	1.752	1.943(20)***	1.901
Kidney (left)	1.611	1.655	1.770	1.922 (20)***	1.852
Females					
Liver	7.685	8.038	8.271	9.548 (24)***	10.807 (41)***
Thyroid	0.022	0.027	0.033	0.028 (27)**	0.039 (77)***
Kidney (right)	1.061	1.059	1.093	1.148 (8)*	1.216 (15)***
Kidney (left)	1.062	1.057	1.092	1.128	1.208 (14)***

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; *Source*: Takaori (1993)

[a] Only organs with treatment-related changes that were significant are included in the Table .

[b] Results expressed as weight in g and, in parentheses, the % change in weight.

[c] Statistical analyses were not performed in the high-dose group due to the low number of rats because of mortalities.

[d] High mean value due to a high value (0.994) in one male rat; mean value without this outlier is presented in brackets.

Microscopic examinations showed changes in the thyroid, liver, kidney and adrenals. In the thyroid follicular cell hyperplasia and hypertrophy were noted in both sexes from the 1200 and/or 6000 ppm groups at 12 and 24 months. An increase of focal follicular cell hyperplasia was observed in the 6000 ppm males and females. In males, the incidence of thyroid follicular cell adenoma was increased at 1200 ppm and above, but reached statistical significance only at 6000 ppm (see Table 22). Centrilobular hepatocellular hypertrophy and occurrence of lipofuscin were noted in both sexes of the 1200 and/or 6000 ppm groups at 12 and 24 months and in most of the 6000 ppm males and females that died during the study. Lipidosis of the adrenal cortex was noted in females of the 1200 ppm group and in both sexes at 6000 ppm at 12 months, but not in males and females which died on study or were killed at 24 months. The severity of nephropathy was increased in both sexes of the 6000 ppm group at 12 months and in both sexes of the 1200 and 6000 ppm groups at 24 months. The lesions were more severe in males than in females and grew worse with increasing dose and time. For high-dose males the renal failure was considered to be the main cause of death. Microscopic evidence of renal failure in these rats was accompanied by hyperplasia of the parathyroid, demineralization of the bone and metastatic calcification in various organs.

Table 22. Thyroid changes in rats in two-year dietary study with thiophanate-methyl

	Incidence of thyroid follicular cell changes [a]				
	0 ppm	75 ppm	200 ppm	1200 ppm	6000 ppm
Males					
Diffuse follicular cell hyperplasia/hypertrophy	0/60 (0)	0/58 (0)	0/60 (0)	23/60 (38)**	58/60 (97)***
Focal follicular cell hyperplasia	3/60 (5)	2/58(3)	2/60 (3)	3/60 (5)	15/52 (25)**
Follicular cell adenoma	1/60 (2)	0/58 (0)	0/60 (0)	4/60 (7)	12/52 (20)**
Follicular cell adenocarcinoma	0/60 (0)	0/60 (0)	0/60 (0)	0/60 (0)	3/52 (5)
Females					
Diffuse follicular cell hyperplasia/hypertrophy	1/60 (2)	1/59 (2)	0/60 (0)	28/60 (47)***	59/60 (98)***
Focal follicular cell hyperplasia	0/60 (0)	1/59 (2)	0/60 (0)	4/60 (7)	8/60 (13)*
Follicular cell adenoma	0/60 (0)	0/59 (0)	0/60 (0)	1/60 (2)	2/60 (3)
Follicular cell adenocarcinoma	0/60 (0)	0/59 (0)	0/60 (0)	0/60 (0)	0/60 (0)

ppm: parts per million;*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; *Source*: Takaori (1993)

[a] Results expressed as no. of animals with the finding / no. of animals examined and, in parentheses, as a percentage.

The effects on the thyroid at the two high doses were considered secondary effects resulting from liver enzyme induction which enhances thyroid hormone excretion and leads to perturbations in systemic thyroid hormone levels (i.e. decreased thyroid T_4 and T_3 hormone values and consequently an increase in TSH concentration). The continuous stimulation of the thyroid gland by TSH in rats is known to result in follicular cell hypertrophy or hyperplasia and, depending on dose and time, in follicular cell adenomas or adenocarcinomas.

The NOAEL was 200 ppm (equal to 8.8 mg/kg bw per day) based on tissue masses on the skin and in the subcutis in males; reduced body weight gain in both sexes; increased total cholesterol and total protein and a decreased albumin to globulin ratio in both sexes at 12 and/or 18 months; decreased levels of chloride and potassium and decreased T_4 and T_3 values and increased TSH values in males at 24 months; an increase of urinary protein and granular kidneys in males, follicular cell hyperplasia and hypertrophy in the thyroid in both sexes at 12 and 24 months; a statistically nonsignificant increase in the incidence of thyroid follicular cell adenoma in males, centrilobular hepatocellular hypertrophy and occurrence of lipofuscin in both sexes at 12 and 24 months; lipidosis of the adrenal cortex in females at 12 months; and increased severity of nephropathy in both sexes at 24 months observed at 1200 ppm (equal to 54 mg/kg bw per day) (Takaori, 1993).

In a pre-guideline two-year dietary carcinogenicity study with limited investigations, number of animals per dose and reporting, thiophanate-methyl (purity ~ 94%; batch no. not reported) was administered to Sprague Dawley rats (35/sex per group) at 10, 40, 160 or 640 ppm (equal to 0.5, 2, 7 and 30 mg/kg bw per day for males and 0.5, 2, 8 and 34 mg/kg bw per day for females, respectively). A group of 50/sex served as control. Rats were housed two per cage. The rats were checked daily for mortality and clinical signs. Body weight was recorded weekly. Feed consumption per two rats was recorded every 10 days. Blood and urine of rats (5/sex per group) were sampled at 3, 6, 12 and 18 months, and of all animals at 24 months for determination of a limited number of haematological, clinical chemistry and urine parameters. After 3 and 12 months of administration, five males and five females of each treatment group were killed and the remaining rats were killed at 24 months. All rats were necropsied, weights of brain, heart, lung, liver, spleen, thymus, kidneys, adrenals, thyroid, pituitary and gonads were recorded and a range of organs were histopathologically examined.

There were no effects of treatment on mortality, clinical signs or feed consumption. At 640 ppm, body terminal body weights of male and female rats were 13% and 16% lower than those of controls. No treatment-related effects on haematological, clinical chemistry and urine analysis parameters were

found. Organ weight changes at the high dose were minor and statistically nonsignificant, and were considered secondary to the reduced body weight gains in these rats. In the thyroid of high-dose males hypertrophy of follicular epithelium and a decrease of colloidal substance were observed. In the testes at this dose a slight to moderate reduction in spermatogenesis was observed. There was no increase in the incidence of neoplasia at any dose.

The NOAEL was 160 ppm (equal to 7 mg/kg bw per day) based on reduced body weight gain and histopathological changes in thyroid and testes of males at 640 ppm (equal to 30 mg/kg bw per day) (Hashimoto & Tsubura, 1972).

In view of the limitations in study design and reporting, this study is considered as supporting only in the present risk assessment.

2.4 Genotoxicity

Thiophanate-methyl was tested for genotoxicity in three in vitro assays and one in vivo assay. All tests were negative (Table 23).

Table 23. Overview of genotoxicity tests with thiophanate-methyl

End-point	Test system	Concentrations/ doses tested	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia* coli WP2uvrA	39.1–5000 µg/plate (−S9 *E. coli* WP2uvrA mix, +S9 mix) 0, 16, 50, 158, 500, 1 581, 5000 µg/tube in DMSO (±S9, pre-incubation)	96.6	Negative	Nishibe (1990a)[a]
Mammalian cell gene mutation	V79 Chinese hamster lung cell; HPRT test	6.25–100 µg/mL in DMSO (−S9) (±S9)	96.4	Negative	McSheehy et al. (1984)[b]
Chromosomal aberrations	Chinese hamster ovary cells	100–400 µg/mL in DMSO (−S9, 20 h) 250–1000 µg/mL in DMSO (+S9, 20 h)	95.0	Negative	Murli (1988)[c]
Unscheduled DNA synthesis	Primary rat hepatocytes	5–1000 µg/mL (−S9, 18 h) 250–1000 µg/mL (+S9, 18 h)	NR	Negative	Myhr & Brusick (1981)[d]
Micronucleus	Human peripheral lymphocytes	?–47.5 µg/mL in DMSO (−S9, 18 h) ?–550 µg/mL in DMSO (+S9, 18 h)	98.2	Positive	Marshall (1996a)[e]
Micronucleus (examination of four chromosomes)	Human peripheral lymphocytes	8.5–200 µg/mL in DMSO (range-finding study) 0.05–39.0 µg/mL in DMSO (−S9, 18 h)	96.4	Positive threshold for aneugenicity: 6 µg/mL (−S9 mix)	Marshall (1996b)[f]
In vivo					
Mouse micronucleus	B6D2F1 mouse, bone marrow	500, 1000, 2000 mg/kg bw in 1% aqueous CMC by gavage as single doses	97.3	Weakly positive	Proudlock (1999)[g]
Mouse micronucleus	Swiss albino mouse, bone marrow	1000 mg/kg bw in 70% kaolin by gavage as a single dose	95	Weakly positive	Barale et al. (1993)[h]

End-point	Test system	Concentrations/ doses tested	Purity (%)	Results	Reference
Cytogenetic study	Male Wistar rats	62.5–1000 mg/kg bw in 5% gum arabic vehicle (administered intraperitoneally for 5 days)	94	Negative	Makita et al. (1972)[i]
Dominant lethal test	Male ICR mice	8–500 mg/kg bw in 1% gum arabic vehicle (administered intraperitoneally)	94	Negative	Makita et al. (1972)[j]

bw: body weight; CMC: carboxymethylcelulose; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; HPRT: hypoxanthine-guanine phosphoribosyltransferase; NR: not reported; S9: 9000 *g* supernatant fraction from rat liver homogenate

[a] Batch no. TIF-1016. No increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, neither with nor without metabolic activation.

[b] Batch no. TM-767. In most trials, the lowest percentage of survival was noted at the highest dose with survival rates of 54% and 57% at 100 µg/mL without metabolic activation and of 62% in the second assay at 100 µg/mL in the presence of S9 mix. There were no reproducible increases in mutant numbers or mutation frequency observed at any dose of the test substance, neither in the absence nor in the presence of metabolic activation. Occasional increases were not dose related. The positive control substances gave the expected response.

[c] Batch no. 591. In the absence of S9 mix a precipitate was noted at 400 µg/mL. At >200 µg/mL, cytotoxicity was noted in form of cellular debris, reduction in the number of visible mitotic cells and reductions in the monolayer confluence. In the presence of S9 mix, precipitate was visible at concentrations ≥ 750 µg/mL. Cytotoxicity was noted at the highest dose. No significant increase in chromosomal aberrations was observed in either assay at any dose. The positive control substances induced a marked increase in chromosomal aberration rate.

[d] Batch no. CP 10B, purity not specified. Thiophanate-methyl resulted in a cell survival range of 38% (1000 µg/mL) to 109.6% (25 µg/mL) indicating that sufficient cytotoxicity was achieved in this study. Crystalline test material was observed on the cell monolayer at concentrations ≥ 500 µg/mL. There was no indication that thiophanate-methyl induced increased unscheduled DNA synthesis (UDS) activity. The positive control 2-acetyl aminofluorene demonstrated the sensitivity of the test system by apparent induction of UDS.

[e] Batch no. TEE 2007. Treatment of cultures with the thiophanate-methyl in the absence of metabolic activation resulted in micronucleus frequencies which were statistically significantly greater than those noted in concurrent negative controls at all concentrations and which fell outside the historical negative control range. Although not all cells with micronuclei were evaluated, FISH analysis of the positive samples showed that the majority of the micronuclei induced contained one or more centromeres and were thus formed from whole chromosomes rather than from chromosomal fragments. The increase in the number of cells with centromere-negative micronuclei reaching statistical significance at the lowest concentration level analysed was very small. A clear dose-response was lacking. Thus, this finding is not considered to provide evidence of an additional clastogenic effect. Treatment in the presence of metabolic activation resulted in micronucleus frequencies similar to those seen in concurrent negative controls. A statistically significant increase in cells with micronuclei was apparent at the highest concentration analysed, but the effect was attributable to an increase in a single replicate. FISH analysis of positive samples indicated that the majority of the micronuclei induced contained one or more centromeres, and were thus formed from whole chromosomes rather than from chromosomal fragments. The positive control substances gave the expected increase in the occurrence of micronuclei confirming the sensitivity of the test system. For colchicine (−S9 mix), the increase was only due to the induction of aneuploidy since there were no binucleated cells with micronuclei lacking centromeres. For cyclophosphamide, the respective analysis was not performed. It was concluded that thiophanate-methyl induced a significant increase in micronuclei in cultured human peripheral blood lymphocytes at least without metabolic activation system and that there was evidence for aneuploidy induction (aneugenic potential).

[f] Batch no. TIF-1016. The threshold for the aneugenic activity of thiophanate-methyl was tested in an in vitro assay using human lymphocyte cultures. The distribution of chromosomes between the nuclei of binucleated cells was investigated by FISH technique (simultaneous detection of centromeres). Chromosomes were investigated in the chromosome pairs 1/11 and X/17. Abnormalities were classified under the headings of chromosome loss, chromosome gain, and aneuploidy or polyploidy. A number of closely spaced concentrations were used in order to determine a threshold level and a reliable no-observed-effect concentration (NOEC). For all four chromosomes evaluated, threshold levels and NOECs could be obtained in the first trial. Since there was evidence of an effect (non-disjunction) on chromosomes X and 17 at lower dose levels yet, a second trial was performed which revealed even higher threshold concentrations and NOECs for these chromosomes. Thus, the results of the first trial were considered reliable. The results obtained for the chromosomes 1, 11, 17 and X showed that the lowest threshold for test item–related aneuploidy in cultured human peripheral blood lymphocytes was 6 µg/mL in two independent trials, and the corresponding NOEC was 4 µg/mL. It was concluded that thiophanate-methyl induces aneuploidy in cultured human peripheral blood lymphocytes.

[g] Batch no. TFB-2012. Male and female B6D2F1 mice were treated by single gavage dose of thiophanate-methyl at 500, 1000 or 2000 mg/kg bw. A concurrent negative control group received the vehicle only, while a positive control (PC) group was treated with carbendazim at 1000 mg/kg bw. Bone marrow smears were obtained each from 5 mice/sex per dose group at 24 and at 48 hours after dosing with the exception that mice in the PC group were sampled at the 24-hour time point only. No unscheduled deaths and no clinical signs of toxicity were seen in any of the groups. At all three doses, thiophanate-methyl caused a statistically significant increase in the incidence of micronucleated immature erythrocytes. However, this increase was much smaller than that induced by carbendazim. Carbendazim induced the expected high proportion (68%) of micronuclei containing centromeres, while Mitomycin C caused a low proportion of centromere-positive micronuclei (24%). Thiophanate-methyl appeared to cause an intermediate proportion of centromere-positive micronuclei (34%). It was concluded that thiophanate-methyl has a weak potential to induce the formation of micronuclei in immature mouse erythrocytes. It can be assumed that the mechanism is the same as for carbendazim. The micronucleus incidence was more than six-fold lower in thiophanate-methyl-exposed mice as compared with carbendazim-exposed mice at 1000 mg/kg bw.

[h] The aneugenic potential of thiophanate-methyl (purity: 95%; dispersed in 70% kaolin) was tested in male Swiss albino mice given oral doses of 1000 mg/kg bw. Bone marrow cells were analysed 16, 24, 36, and 48 h after treatment for micronuclei, chromosomal aberrations, hyperdiploidy, and polyploidy. Large micronuclei were significantly induced, but the response was relatively weak, and thiophanate-methyl was less effective than benomyl or carbendazim. There was no increase in the frequency of chromosomal aberrations. At 24 and 36 h, a possibly treatment-related increase in the frequency of polyploid and hyperdiploid cells was observed, which was of borderline significance in view of the very low frequency of changes in ploidy.

i Batch not specified. It is noted that this study has several reporting and study design deficiencies. Thirty male Wistar rats per dose group received intraperitoneal administrations of thiophanate-methyl once daily at doses 0, 62.5, 125, 250, 500 and 1000 mg/kg bw for five consecutive days. Colchicine was administered at 1 mg/kg bw at 4 to 5 hours prior to termination and necropsy. Following necropsy, metaphases of bone marrow cells and of spermatogonial cells were processed and examined microscopically. For each dose, 100 well-spread metaphase chromosomes from the bone marrow and spermatogonial cells were analysed. Signs of toxicity were not reported in this study. According to the study authors, no abnormal chromosome configurations such as breakage, suspected chromatid breakage, chromosome translocation, fragmentation or formation of chromosome bridges were observed in either bone marrow or spermatogonial cells.

j Batch no. not specified. It is noted that this study has several reporting and study design deficiencies. Groups of 10 male ICR mice per dose level were treated with a single intraperitoneal injection of thiophanate-methyl at 0, 8, 40, 200, 400 and 500 mg/kg bw. Following treatment, each male was housed for one week with three primiparous females. The females were replaced after one week with new primiparous ones. This procedure was repeated for a total of eight weeks. Each female was scored for pregnancy, for total number of uterine implants as well as for pre- and postimplantation losses on day 13 after the confirmation of vaginal plugs. Thiophanate-methyl was clearly toxic to the male mice at the higher doses. There were four deaths in the 500 mg/kg bw group and one death in the 400 mg/kg bw group. The incidence of pregnancies in the treated groups did not differ consistently. Total implant number including living fetuses, early fetal deaths and late fetal deaths did not indicate any consistent dose-related differences although a considerable degree of intergroup variation was observed. There was no indication of induction of dominant lethal mutations in male mice by thiophanate-methyl.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation dietary reproductive toxicity study, with a study design resembling OECD TG 416, Sprague Dawley rats (Crl:CD (SD)BR; 25/sex per group) were fed thiophanate-methyl (purity 95.9%; batch no. TIF-01016) at a dietary concentration of 0, 200, 630 or 2000 ppm. The doses were based on a range-finding study. The thiophanate-methyl intake is presented in Table 24.

Table 24. Thiophanate-methyl intake in a two-generation reproductive toxicity study in rats

	Intake per dietary concentration per period (mg/kg bw per day)		
	200 ppm	**630 ppm**	**2000 ppm**
Males			
Premating period	14.6	46.0	147.1
Females			
Premating period	16.8	52.2	164.3
Gestation period	14.3	44.6	138.2
Lactation period	27.2	81.2	247.1
Overall	18.0	55.4	172.9

ppm: parts per million

F_0 adults (P) were treated over a 14-week premating period and throughout the three-week mating period, gestation and 28-day lactation of the F_1 pups. On postnatal day 4, litters were culled to eight pups. After 14 weeks of premating treatment, the F_0 generation was mated for up to 21 days. The F_0 females were allowed to litter and to rear their offspring (F_1 generation) to weaning at postnatal day 21. Following a 14-week maturation period after weaning, the F_1 parental rat (selected from the F_{1a} offspring) were mated for up to 21 days. The F_1 females were allowed to litter and to rear their offspring (F_{2a} generation) to weaning. Because of an unusually high pup loss during the weaning period of the F_{2a} pups, a second mating was performed. The F_1 rats were maintained on thiophanate-methyl for six weeks and then allowed to mate with the same partner as during the first mating to produce the F_{2b} litter. All rats were checked daily for mortality and clinical signs. Body weights of parental rats were recorded weekly. In addition, females were weighed on gestation days 0, 7, 14 and 20 and postnatal days 1, 4, 7, 14 and 21. Individual pup weights were recorded on postnatal days 0 and 4. Parental feed consumption was recorded weekly during the premating period. In addition feed consumption in females was recorded on intervals from gestation day 0–7, 7–14 and 14–20 and postnatal day 0–4, 4–7, 7–14 and 14–21. Blood was sampled for measurement of TSH, T_3 and T_4 in F_0 and F_1 parental rats from 10 males and 10 females per dose during week 1 and 8 of treatment and at necropsy. Fertility parameters and the length of gestation were determined. All litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and gross anomalies. All pups were checked daily for mortality, clinical signs, pinna unfolding, tooth eruption and eye opening. In addition, surface righting reflex (postnatal day 8), grip reflex (postnatal day 17) and pupillary reflex and auditory response (postnatal day 21) were assessed. The culled pups and pups killed at weaning were macroscopically examined for alterations. After weaning of the F_1 and F_{2b} pups, the respective F_0 and F_1 parents were killed and necropsied. In all parental rats (F_0 and F_1) the weights of liver, thyroid epididymides and testes were recorded. In control and high-dose parental rats of the F_0 and F_1 generation, cervix, coagulating gland, epididymides, liver, ovaries, pituitary, prostate, seminal vesicles, testes, thyroids, uterus, vagina and organs which macroscopically showed alterations were histologically examined. A peer review of the histopathological findings was performed by Dr A. Singer in 1995.

No treatment-related mortalities or clinical signs were observed. Terminal body weights in F_0 males at 2000 ppm were slightly lower (6%) than controls. Body weight gain during gestation of the F_{2a} generation was lower in 2000 ppm F_1 females (up to 12%) than in controls. Feed consumption was reduced in high-dose F_1 parental females during the first mating period (9.1%), gestation period (11.3%) and lactation period (18%). The effect of treatment on TSH and thyroid hormone levels are presented in Table 25. In the 2000 ppm group, the males and females of the F_0 and F_1 generations showed elevated TSH levels at the eight-week measuring point. In the males of this dose group from the F_0 generation, lower T_4 values were also considered attributable to treatment. In high-dose F_0 females, reduced T_4 values were noted only in the first week. Effects on T_3 values were marginal or non-existent. The occasional numerical changes in hormone values in the 630 ppm groups were equivocal. No treatment-related macroscopic changes were observed at necropsy in F_0 and F_1 parental rats. The effects on organ weights and histopathological findings are presented in Table 26.

Table 25. Thyroid hormone levels in P and F_1 rats

	Hormone levels per dose and generation							
	0 ppm		200 ppm		630 ppm		2000 ppm	
	P (F_0)	F_1	P (F_0)	F_1	P (F_0)	F_1	P (F_0)	F_1
Males								
T_3								
Necropsy	0.70	0.76	0.79	0.77	0.90*	0.73	0.74	0.70
T_4								
Week 1	4.8	n.m.	4.1*	n.m.	4.3	n.m.	3.5*	n.m.
Week 8	5.2	4.8	5.0	4.9	5.6	4.6	4.5*	4.8
Necropsy	4.2	3.5	3.9	3.5	4.7	3.5	4.3	3.5

(*Continued on next page*)

	Hormone levels per dose and generation							
	0 ppm		200 ppm		630 ppm		2000 ppm	
	P (F_0)	F_1	P (F_0)	F_1	P (F_0)	F_1	P (F_0)	F_1
TSH								
Week 8	4.2	3.4	5.7	4.1	4.6	5.3	7.8*	6.9*
Necropsy	2.8	4.6	3.1	3.6	3.3	3.8	5.0	4.2
Females								
T_3								
Necropsy	0.84	0.79	0.77	0.81	0.76	0.71	0.71*	0.77
T_4								
Week 1	4.5	n.m.	4.2	n.m.	3.7*	n.m.	3.2*	n.m.
Week 8	3.9	3.7	3.4	3.7	3.6	3.5	3.3	3.3
Necropsy	2.7	2.5	2.7	2.7	3.1	2.7	2.8	2.9
TSH								
Week 8	1.4	1.5	1.8	1.8	2.0*	2.2	3.2*	3.6*
Necropsy	3.0	3.4	2.3	3.0	3.0	2.8	3.5	4.3

n.m.: not measured; *: $P < 0.05$; *Source*: Müller (1993)

Table 26. Histopathological findings and organ weights of liver and thyroid of P (F_0) and F_1 rats

	Measure per dose level per generation (ppm)							
	Males				Females			
	0	200	630	2000	0	200	630	2000
P(F_0) generation								
Hepatocyte hypertrophy								
Study pathologist	0/25	9/25	14/25	23/25	0/24	1/23	3/24	20/25
Peer reviewer	0/25	7/25	13/25	24/25	0/25	0/25	2/24	23/25
Liver weight (mg)	24 433	24 993	24 731	28 836*	12 053	12 117	12 142	13 640*
Thyroid hyperplasia								
Study pathologist	0/25	6/25	13/24	17/25	0/24	0/23	2/24	6/25
Peer reviewer	0/25	4/25	11/24	16/25	0/25	0/24	1/25	7/25
Thyroid hypertrophy								
Study pathologist	0/25	9/25	7/24	22/25	0/24	0/23	1/24	4/25
Thyroid weight (mg)	29	31	29	39*	23	22	33	30*
F_1 generation								
Hepatocyte hypertrophy								
Study pathologist	0/25	3/25	6/24	11/24	0/24	1/23	1/24	15/25
Peer reviewer	0/25	1/25	7/25	15/25	0/25	0/24	1/25	18/25
Liver weight (mg)	20 638	25 289*	22 956	26 015*	13 341	13 651	13 445	14 274
Thyroid hyperplasia								
Study pathologist	0/25	4/25	4/24	10/24	0/24	0/23	1/24	2/22
Peer reviewer	0/25	1/25	3/25	9/24	0/24	0/24	1/25	9/22
Thyroid hypertrophy								
Study pathologist	0/25	3/25	2/24	1/24	0/24	0/23	0/24	0/22
Thyroid weight (mg)	28	31	30	36*	23	26*	26*	29*

F_1: first filial generation; ppm: parts per million; *Source*: Müller (1993); Müller and Singer (1995)
*: $P < 0.05$; Statistical significance of histopathological findings not given.

Absolute weights of liver (males 18–26%; females 7–13%) and thyroid (males 29–34%; females 26–30%) were increased in F_0 and F_1 parental rats at 2000 ppm.

The incidences of hyperplasia in the thyroid were markedly increased in F_0 and F_1 males at 630 and 2000 ppm and in F_0 females at 2000 ppm. The hyperplasia was qualified as minimal in most cases and as slight in a few cases. Liver hypertrophy was observed in F_0 males at all doses, in F_1 males at 630 ppm and above and in F_0 and F_1 females at the highest dose.

No effects were observed on mating, fertility, gestation time, live birth index, viability index, weaning index or sex distribution. In the F_1 pups no effects on number of live or dead pups, pup losses viability and weaning indices (number alive at weaning/number alive at day 4 post cull) were found. No effects were observed on physical development and in the grip test, surface righting test, pupillary reflex test and auditory test. Increased pup losses were observed in the F_{2a} generation for all groups after lactation day 4. The weaning indices for the control, low-, intermediate- and high-dose groups were 12%, 24%, 12% and 13%, respectively. No cause for these pup losses could be identified. It was concluded that this was a random finding. The weaning indices for the F_{2b} groups were 94%, 74%, 79% and 87%.

The effects on pup weight are presented in Table 27. No effects were observed from postnatal day 0–7.

Table 27. Pup weights per litter, covariate adjusted means (covariate: number of pups)

	Pup weight per litter per dose level							
	0 ppm		200 ppm		630 ppm		2000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_{1a} generation								
Day 14	28.96	28.46	27.99 (−3%)	27.29 (−4%)	27.03 (−7%)	27.27 (−4%)	26.20 (−10%)	25.29 (−11%)
Day 21	49.26	47.38	48.05 (−2%)	46.63 (−2%)	45.68 (−7%)	46.13 (−3%)	44.62 (−9%)	42.66 (−10%)
F_{2a} generation								
Day 14	29.34	28.74	28.14 (−4%)	23.88 (−17%)	23.23 (−21%)	22.08 (−24%)	25.47 (−13%)	25.02 (−13%)
Day 21	50.12	46.10	44.77 (−11%)	38.24 (−17%)	51.21 (+2%)	43.57 (−6%)	46.76 (−7%)	48.44 (+5%)
F_{2b} generation								
Day 14	33.09	31.38	30.27* (−9%)	30.22 (−4%)	29.79* (−10%)	27.23* (−15%)	28.84** (−13%)	26.46** (−16%)
Day 21	54.73	51.69	52.10 (−5%)	51.23 (−1%)	49.53 (10%)	45.32* (−12%)	48.30* (−12%)	45.30* (−12%)

F: female; M: male; *: $P < 0.05$; **: $P < 0.01$

Source: Müller (1993)

In F_{1a} pups, body weight gain was reduced (up to 11%) in both sexes at the highest dose on days 14 and 21 but not at earlier time points. The reductions did not achieve statistical significance but were considered by the study author to be treatment related. In F_{2a} male and female pups, reductions in body weight gain, without dose dependency, were noted at day 14 in all treatment groups. At day 21 only at the low dose (200 ppm), a marked reduction in body weight gain was noted. None of the changes was statistically significant. Due to the high pup loss in all treatment groups of this generation no conclusions on the toxicological relevance can be drawn. In F_{2b} pups, reduced body weight gain was noted from 630 ppm onwards, with a slight but statistically significant reduction (−9%) also on day 14 at 200 ppm. Necropsy revealed no treatment-related effects.

Mating performance, indicated as duration (in days) of cohabitation until gestation day 0, showed the usual intergroup variations but no treatment-related effect. At 2000 ppm, mating performance was comparable to that of the control group. The slightly higher mean value noted for the high-dose group was due to one mating pair. Exclusion of this value from calculation of mating performance leads to identical results (2.0 days at 2000 ppm and in the control group). Thus, no treatment-related effects were noted on mating performance, insemination, fecundity or fertility index.

The NOAEL for parental toxicity was 200 ppm (equal to 15 mg/kg bw per day) based on thyroid hyperplasia in males and increased TSH levels in females at 630 ppm (equal to 46 mg/kg bw per day).

The NOAEL for offspring toxicity was 200 ppm (equal to 27 mg/kg bw per day) based on maternal intake during lactation and decreased body weights (F_{2b}) during lactation at 630 ppm (equal to 81 mg/kg bw per day).

The NOAEL for reproductive toxicity was 2000 ppm (equal to 147 mg/kg bw per day), the highest dose tested (Müller, 1993; Müller and Singer, 1995).

In a three-generation study conducted prior to OECD guideline implementation and with limitations to study design and reporting, weaning Sprague Dawley rats (10/sex per group) were fed diets containing thiophanate-methyl (batch NF44, purity unspecified) at 0, 40, 160 or 640 ppm (equal to premating doses of 0, 2.7, 10.6 and 43 mg/kg bw per day for males and 0, 3.1, 12.2 and 49 mg/kg bw per day for females, respectively) were mated twice to produce the F_{1a} and F_{1b} litters. Ten male and 20 female offspring were selected from the F_{1b} litters and mated to produce the F_{2a} and F_{2b} litters; 10 male and 20 female F_{2b} offspring were then selected and mated to produce the F_{3a} and F_{3b} litters. The test diets were fed throughout the study – rats in the F_0, F_{1b} and F_{2b} generations being fed for 60 days before mating. In each case, mating lasted for 20 days. The rats were re-mated to produce the second litters about 10 days after weaning of the first litters. The homogeneity, stability and concentrations of thiophanate-methyl in the diets were not reported. The rats were checked regularly for mortality and clinical signs. Body weights and feed consumption of parental rats were measured weekly during the first mating period. Mating performance, pregnancy rate and gestation period were recorded. All litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and gross anomalies. Individual pup weights were recorded on postnatal days 0, 4, 12 and 21. The pups killed at weaning were macroscopically examined for alterations. The pups of the F_{3b} litters were killed at three weeks of age and subjected to a detailed internal and external examination. Skeletal staining and histopathological examination of organs was performed on 10 male and 10 female F_{3b} pups.

In F_0 rats, no treatment-related effects on mortality, feed consumption, or body weight gain or clinical signs of toxicity were seen. No treatment-related effects were observed on mating performance, length of gestation, or pregnancy rate or at terminal autopsy in F_0, F_{1b}, or F_{2b} rats. The total litter sizes and weights at birth were slightly reduced in all rats at 640 ppm except the F_{3a} litter. Viable litter size and weight at weaning were slightly reduced in all generations at 640 ppm. Mean pup weight was not affected. Viable litter size and weight between birth and weaning were slightly reduced in the second F_{2b} litter at all doses, the percentage losses increasing with dose; however, average pup weights were not affected. The observed effects on viable litter size and pup weights did not reach statistical significance. The total litter loss and the incidence of gross malformations of pups were not treatment related. No dose-related effects were seen on macroscopic appearance, organ weight, histopathological parameters, or the skeleton in F_{3b} offspring.

The NOAEL for parental toxicity was 640 ppm (equal to 43 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 640 ppm (equal to 43 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 640 ppm (equal to 43 mg/kg bw per day), the highest dose tested (Palmer et al., 1972).

(b) Developmental toxicity

Mouse

In a limited developmental toxicity study, thiophanate-methyl (batch and purity not indicated), was administrated as a 5% aqueous gum arabic suspension by gavage to 20 pregnant ICR mice from day 1 to day 15 postcoitum at 0, 40, 200, 500 or 1000 mg/kg bw per day. The mice were observed daily for mortality and clinical signs. Body weights were recorded on gestation days 1, 15 and 19. On gestation day 19, all of the dams were autopsied and the implantation sites and number of living, dead and malformed fetuses were counted. Living fetuses were weighed and examined for external gross malformations. Skeletal malformations as well as the state of ossification of the fetuses were examined.

No mortality or treatment-related clinical signs were observed. Body weight of the dams was not affected by treatment. At the highest dose (stated as one third of the oral LD_{50}), the average number of living fetuses (9.7) was slightly but significantly ($P < 0.05$) lower, compared to the control group (10.9). The number of implantation sites, the number of dead fetuses as well as the body weight of living fetuses were comparable to the control values. No treatment-related effect on the incidence of malformations was observed.

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested. In view of the limitations in study design and reporting this study is considered supporting only in the present risk assessment (Noguchi & Hashimoto, 1970d).

Rat

In a developmental toxicity study with a study design resembling OECD TG 414, thiophanate-methyl (batch no. MT-123, purity 97.2%, formulated in 5% aqueous gum arabic), was administrated by gavage to 25 pregnant COBS CD rats from day 6 to day 19 postcoitum at 0, 100, 300 or 1000 mg/kg bw per day. The doses were selected based on the results of a pilot developmental toxicity study in rats (Rodwell, 1981a) in which doses of 0, 250, 500, 1000, 3000 and 5000 mg/kg bw per day were tested in groups of five rats. The results of the pilot study revealed maternal toxicity (decreased body weight gain) at 1000 mg/kg and above. In the main study the animals were observed daily for mortality and clinical signs. Body weights were recorded on gestation days 0, 6, 9, 12, 16 and 20. The fetuses were delivered by caesarean section on gestation day 20 and subjected to gross examination. The uterus was examined and weighed, and the number of live and dead fetuses, corpora lutea, implantations and early and late resorptions were counted. Body weight and sex of the fetuses were recorded. About half of the fetuses from each litter were selected for skeletal examinations, and the other half for visceral examinations.

In the main study, no mortality or treatment-related clinical signs were observed. At the end of treatment, the high-dose rats had an 8% reduction in body weight gain. No effects on number of corpora lutea, number of implantations, post implantation loss and number of viable fetuses was observed. Fetal weights and sex ratio were not affected by treatment. Treatment had no effect on fetal visceral and skeletal development.

The NOAEL for maternal toxicity was 300 mg/kg bw per day, based on a reduced body weight gain during the first days of treatment at 1000 mg/kg bw per day.

The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1981a,b).

Rabbit

In a developmental toxicity study, thiophanate-methyl (batch no. TM-948, purity 96.2% formulated in a suspension of 1% aqueous methylcellulose) was administrated daily by gavage to groups of 15 female New Zealand White rabbits from gestation day 6 to 19 at 0, 2, 6 or 20 mg/kg bw per day. Doses were selected based on the results of a pilot study with four females per dose group in which initially doses of 0, 150, 300 and 600 mg/kg bw per day were used. As severe maternal toxicity was observed at these doses, additional treatment groups using 0, 10, 30 or 100 mg/kg bw/per day thiophanate-methyl were used. In the main study the does were checked daily for mortality and clinical signs. Body weight was

measured daily. Feed and water consumption were recorded over gestation days 0–5, 6–12, 13–19, 20–23 and 24–28. On gestation day 29, the fetuses were delivered by caesarean section. The uterus was examined and weighed, and the number of live and dead fetuses, corpora lutea, implantations, placenta weight and early and late resorptions were counted. Body weight and sex of the fetuses were recorded. The fetuses were macroscopically examined. About half of the fetuses from each litter were selected for skeletal examinations, and the other half for visceral examinations.

In the range-finding study all females at 150, 300 and 600 mg/kg bw per day showed a decline in general health and condition. One female in each of the 150 and 300 mg/kg bw per day groups and two females in the 600 mg/kg bw per day group died or were killed in extremis. The animals showed a pronounced body weight loss and a reduction in feed intake and reduced faeces during the treatment period. Although a marked improvement was recorded in the 150 and 300 mg/kg bw per day groups after the treatment period, the earlier weight loss was not fully recovered. Females of the 30 and 100 mg/kg bw per day groups showed a less severe reduction in feed and water intake and reduced faeces as well as a slight weight loss during the treatment period. Despite some recovery of body weight, the animals failed to attain parity with the control group by gestation day 29. The body weight performance as well as the general health and condition of females receiving 10 mg/kg bw per day corresponded to that of the control group. At 30 mg/kg bw per day, one litter was lost by abortion. This was within the background control range, but involvement with treatment could not be excluded in view of the results obtained at the higher doses. This doe had a body weight on gestation day 20 that was reduced by 21% compared to the control group. At doses of 100 mg/kg bw per day and above, a high level of fetal loss was recorded. Total litter loss was observed in 2/4 does and the remaining two does aborted (gestation day 20 and 21) at 100 mg/kg bw per day. At 150 mg/kg bw per day, total litter loss was seen in 2/4 animals and at 300 mg/kg bw per day three out of four does aborted (gestation days 23, 24 and 25). Pre- and postimplantation loss was recorded for two out of the three pregnant females at 10 mg/kg bw per day, but no such effects were observed at 30 mg/kg bw per day. In the latter group, the fetal weight was marginally reduced. Three grossly abnormal fetuses were observed in one of the three litters at 10 mg/kg bw per day. One fetus with abnormal heart and great vessels was observed at 30 mg/kg bw per day. Such effects were not observed in the main study.

In the main study, five females (two in the control group and one in each of the treated groups) were killed in extremis. Necropsy revealed evidence of respiratory tract and/or gastrointestinal tract disturbance, which was not considered to have been related to treatment. No treatment-related clinical signs were observed. At 20 mg/kg bw per day a statistically significant maternal weight loss on gestation days 6–8 and 8–10 (total loss 180 g) and a significant reduction in maternal weight gain (40 g versus 140 g in controls) for the entire treatment period (gestation days 6–18) was observed. The rabbits at 6 mg/kg bw per day lost 30 g of body weight during the first two days of treatment (not statistically significant). Body weight gain at 2 mg/kg bw per day was unaffected by treatment. Feed consumption at 20 mg/kg bw per day was reduced (115 g/rabbit versus 186 g/rabbit in controls) during gestation days 6–12. No treatment-related effects on water intake were found. At termination no treatment-related macroscopic changes in the does were observed. One rabbit in each of the 2 and 6 mg/kg bw per day groups and two rabbits of the 20 mg/kg bw per day group aborted during the course of study. Total litter resorption was recorded for one control rabbit and one high-dose rabbit. Thus, the total litter loss was slightly higher in the group receiving the highest dose than in the control group.

Necropsy demonstrated a small number of anomalies in all dose groups, the majority of which were of types and incidences previously recorded in rabbits of this strain in these laboratories and showed no relationship to treatment. Skeletal examination revealed a number of skeletal anomalies of which the majority showed no relationship to treatment. However, at 6 and 20 mg/kg bw per day an increased incidence of fetuses with a 13th pair of ribs and 27 presacral vertebrae and/or pelvic asymmetry was recorded (see Table 28). At 20 mg/kg bw per day, a low incidence of fetuses with ribs thickened at the costal cartilages was observed. The most values fell within the historical range.

Table 28. Incidence of skeletal changes in fetuses exposed to thiophanate-methyl

	Observations per dose level [a]				Historical data (8655 fetuses)	
	0 mg/kg bw per day	2 mg/kg bw per day	6 mg/kg bw per day	20 mg/kg bw per day	Mean	Range
13th pair of ribs	40.9 (10)	46.2 (10)	63.2** (11)	60.8* (8)	35.9	11.9–61.0
27 presacral vertebrae	15.9 (7)	17.9 (8)	37.9*** (9)	43.1*** (7)	18.1	7.2–44.3
Ribs thickened at costal cartilage	1.1 (1)	3.8 (3)	2.1 (1)	13.7** (7)	2.4	0.0–12.8
Asymmetric pelvis	3.4 (3)	5.1 (3)	7.4 (5)	9.8 (5)	3.5	0.0–8.9

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Results expressed as % fetuses with the finding and, in parentheses, the number of litters with the finding.

*Sourc*e: Tesh et al. (1986a,b)

The NOAEL for maternal toxicity was 2 mg/kg bw per day, based on the reduction in body weight during the first days of treatment at 6 mg/kg bw per day.

The NOAEL for embryo/fetal development was 2 mg/kg bw per day, based on the increased incidence of skeletal anomalies at 6 mg/kg bw per day. There was no evidence for a teratogenic potential (Tesh et al., 1986a,b).

The data on fetuses from the above study were reanalysed by Christian (1997), who noted a number of deficiencies in the studies of Tesh et al. (1986a,b). For instance, there were too few animals with live litters, a high mortality rate and high abortion/resorption incidences in does associated with intercurrent disease (respiratory and/or gastrointestinal disturbances). Caesarean sectioning at gestation day 29 showed a high incidence of white or milky appearing substances in the amniotic fluid, indicative of intrauterine infections. Furthermore reporting deficiencies were noted. Christian concluded that the slight decrease in viable young and the skeletal abnormalities were probably associated with poor maternal health, technical problems and the relatively few animals available for evaluation. Although the increased incidences of supernumerary ribs were identified by Tesh et al. (1986a,b) as an effect at doses of 6 and 20 mg/bw per day, reanalysis of the data showed that these incidences were within the expected normal range and were not statistically significant. All other variations in skeletal ossification identified in the study also occurred at incidences that were not statistically significant, indicating that they were unrelated to treatment. Based on the re-evaluation the NOAEL for maternal toxicity was 6 mg/kg bw per day, and the NOAEL for developmental toxicity was 20 mg/kg bw per day. Owing to the deficiencies in the Tesh et al. (1986) study, the 1998 JMPR considered that it should be reclassified as a range-finding study. The present Meeting confirmed this view.

In a developmental toxicity study performed according to OECD TG 414, thiophanate-methyl (batch no. TM-948, purity 97.3%, formulated in a suspension of 1% aqueous methyl cellulose), was administrated by gavage to 20 pregnant New Zealand White rabbits from day 6 to day 28 postcoitum at 0, 5, 10, 20 and 40 mg/kg bw per day. The doses were selected based on the results of a pilot developmental toxicity study in rats (York, 1997a) in which doses of 0, 5, 10, 20, 40 and 80 mg/kg bw per day, administered either in the diet or by gavage, were tested in groups of six rabbits. The results of the pilot study revealed maternal toxicity at 40 mg/kg bw per day and above. In the main study the rabbits were observed daily for mortality and clinical signs. Body weights were recorded on gestation days 0 and daily from gestation day 6–29. Feed consumption was measured daily. The fetuses were delivered by caesarean section on gestation day 20 and subjected to gross examination. The uterus was examined and weighed, and the number of live and dead fetuses, corpora lutea, implantations and early and late resorptions were counted. Body weight and sex of the fetuses were recorded. All fetuses were evaluated for skeletal and visceral alterations.

In the range-finding study no or few faeces were observed predominantly at 40 and 80 mg/kg bw per day. In the does exposed to thiophanate through the diet, at doses of 10 mg/kg bw per day or higher, body weight loss was observed from gestation day 6 to 9. In these rabbits a reduced body weight gain was

observed from gestation day 9–12 at doses of 20 mg/kg bw per day or higher. Over the entire treatment period body weight gain was decreased at 80 mg/kg bw per day. In the does exposed to thiophanate by gavage, at doses of 20 mg/kg bw per day or higher body weight loss was observed from gestation day 6 to 9. A reduced body weight gain was observed from gestation day 9 to 12 at doses of 40 mg/kg bw per day or higher. Over the entire treatment period body weight gain was decreased at 40 and 80 mg/kg bw per day. Feed consumption was decreased at 20 mg/kg bw per day and above in the dietary as well as the gavage groups.

In the dietary groups, 1, 3 and 3 does aborted in the 5, 40 and 80 mg/kg bw per day groups, respectively. Body weights, body weight gains and feed consumption values for the does that aborted were comparable to other does in the same dosage groups and there were no clinical observations prior to aborting. The abortion at 5 mg/kg bw per day was considered spontaneous and not treatment related as it was within the historical control range. The abortions in the 40 and 80 mg/kg bw per day groups were considered treatment related as they were not single events and were dose dependent. In the stomach tube dose group, two does aborted at 80 mg/kg bw per day on gestation day 23 and 24, respectively. Clinical findings of no, dry and/or few faeces (gestation days 11–22) and emaciation (gestation days 16–23) were recorded for the first doe and no, dry and/or few faeces (gestation days 12–23) for the second. These does consumed little feed and lost weight throughout most of gestation.

In the 80 mg/kg bw per day dietary group two litters were totally resorbed. This was considered treatment related as the incidence exceeded the historical control range of the testing facility. In the 80 mg/kg bw per day gavage group, one litter was totally resorbed. This group also had an increase in the number of early resorptions and a concomitant decrease in live litter size, relative to control values. These findings were also considered treatment related. Body weights were not affected by treatment. Necropsy revealed no other parameters were affected by treatment in the dietary exposure and gavage groups. Only fetuses from the 0, 5 and 80 mg/kg bw per day stomach tube groups were examined for visceral and skeletal alterations. No fetal malformations attributable to the treatment with the test substance were observed. The number of thoracic vertebrae was increased and lumbar vertebrae were correspondingly decreased in the 80 mg/kg bw per day stomach tube group. Also, the number of rib pairs was increased in this dose group. These increases or decreases in the number of ossification sites were outside the historical control ranges observed at the testing facility and may be attributed to the 80 mg/kg bw per day stomach tube dose.

In the main study, no mortality was observed. At 40 mg/kg bw per day a higher incidence of does with abnormal, scant or no faeces were observed. Maternal body weight gain for the entire dose period was reduced at 20 mg/kg bw per day (12%) and 40 mg/kg bw per day (−76%, $P < 0.01$), as compared with the control group values. Within this period, the 20 mg/kg bw per day dosage group had significantly reduced weight gain on gestation days 12–15, and the 40 mg/kg bw per day dose group had significant weight losses on gestation days 6–12 and significantly reduced weight gain on gestation days 12–15. Reflecting these effects of the test substance, weight gains for the entire gestation period were significantly reduced in the 40 mg/kg bw per day dose group. Maternal body weights were generally significantly reduced in the 40 mg/kg bw per day dose group on gestation days 10–29.

Absolute and relative feed consumption values were significantly reduced at 20 and 40 mg/kg bw per day for the entire treatment period (–12% and –12% at 20 mg/kg bw per day; –44% and –41% at 40 mg/kg bw per day). Within this period, relative feed consumption was significantly reduced at 20 mg/kg bw per day on gestation days 6–18 (from −20% to −16%), and absolute and relative feed consumption was significantly reduced at 40 mg/kg bw per day on gestation days 6–24 (absolute, from –71% to –15%) or gestation day 21 (relative, from –70% to –24%). At necropsy no treatment-related changes were observed.

No abortions occurred during the study. Two premature deliveries occurred at 10 and 20 mg/kg bw per day. These were considered unrelated to treatment. One control group litter consisted of two early resorptions. Because such events may abnormally skew the data distributions, all values for this doe and litter were excluded from statistical analyses. One doe from the 5 mg/kg bw per day dose group had a litter consisting of eight live fetuses, two dead fetuses and one late resorption. No other dead fetuses were observed.

No treatment-related effects on number of corpora lutea and implantations, litter sizes, live and dead fetuses, early and late resorptions, per cent resorbed conceptuses, per cent male fetuses and fetal body weights were found. The number of does with any resorptions or with viable fetuses did not differ significantly in the five dose groups and were within the historical ranges of the testing facility. All placentae appeared normal. No treatment-related fetal malformations were observed. At 40 mg/kg bw per day a significant increase in the average for thoracic ribs (supernumerary ribs), with associated significant increases and reductions in the average for thoracic and lumbar vertebrae, respectively, were found (see Table 29). This variation was considered an effect of treatment because the values were statistically significant and outside the historical ranges of the testing facility. This variation in rib development was considered by the study authors to be a common finding at maternally toxic dosages and to be reversible.

Table 29. Skeletal findings in developmental toxicity study in rabbits

	Ossification sites per fetus per litter (mean ± SD)				
	0 mg/kg bw per day	**5 mg/kg bw per day**	**10 mg/kg bw per day**	**20 mg/kg bw per day**	**40 mg/kg bw per day**
Number of ribs (pairs)	12.45 ± 0.28	12.44 ± 0.25	12.45 ± 0.25	12.58 ± 0.24	12.85 ± 0.15 **
Thoracic vertebrae	12.50 ± 0.28	12.52 ± 0.25	12.53 ± 0.25	12.68 ± 0.22	12.89 ± 0.12 **
Lumbar vertebrae	6.48 ± 0.28	6.47 ± 0.28	6.46 ± 0.24	6.32 ± 0.22	6.09 ± 0.12 **

bw: body weight; SD: standard deviation; **: $P < 0.01$

Source: York (1997a,b)

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on a reduced body weight gain during the first days of treatment at 20 mg/kg bw per day.

The NOAEL for developmental toxicity was 20 mg/kg bw per day based on supernumerary thoracic ribs at 40 mg/kg bw per day (York, 1997a,b).

2.6 Special studies

(a) Liver enzyme induction

Rat

The effects of thiophanate-methyl on the cytochrome P450 enzymes CYP1A1, 1A2, 2B1, 2E1 and 3A (CYP) were investigated in Sprague Dawley rats (6/sex per group) treated intraperitoneally with a single (285 or 570 mg/kg bw) or repeated (daily 285 or 570 mg/kg bw for three consecutive days) of this substance. Several CYP-dependent reactions were monitored in the liver, kidney and lung microsomes of male and female rats following administration of doses of thiophanate-methyl.

No significant changes in absolute or relative liver, kidney and lung weights were observed after thiophanate-methyl injection. In the liver, in males the CYP2B1-dependent pentoxyresorufrin *O*-dealkylase activity was markedly increased following a single (28.0-fold increase, averaged between lower and higher dose) or repeated (27.1-fold increase, averaged between 285 and 570 mg/kg bw) injections. In females receiving three doses of thiophanate-methyl, the CYP2B1-associated activity was increased from 2.6- to 3.9-fold (at lower and higher dose, respectively). An induction of CYP3A-mediated demethylation of aminopyrine (up to 4.3-fold) was observed at the higher dose in males. In the kidney, the *O*-deethylation of ethoxyresorufin (CYP1A1-linked) was increased up to 28.2-fold and the CYP2E1-dependent *p*-nitrophenol hydroxylases were enhanced up to 6.3-fold in females receiving higher repeated thiophanate-methyl administration. In the lung, the CYP3A-associated activity was induced, as demonstrated by the marked increase in the *O*-demethylation of aminopyrine (up to 3.6-fold) in males. A weak, although significant, reduction of CYP2B1-linked oxidases was also observed in repeated treatment in the kidney in males and lung in females (Paolini et al., 1999).

(b) Neurotoxicity

Rat

In an acute neurotoxicity study that complied with United States Environmental Protection Agency (USEPA) and OECD TGs, Crl:CD(SD)IGS BR VAF Plus rats (10/sex per group) were given thiophanate-methyl (purity: 99.7%) at 0, 500, 1000 or 2000 mg/kg bw as a suspension in aqueous 0.5% (w/v) methylcellulose by gavage once on day 1 of the study. In the extension part of the study, conducted to evaluate additional dosages for several parameters affected in the main study, 10/sex per group were given thiophanate-methyl at 0, 50, 125, 500 or 2000 mg/kg bw by gavage on day 1 of the study. The dosage volume was 10 mL/kg in both study phases.

In the main study, viabilities, clinical observations, body weights, feed consumption values, functional observational battery (FOB) evaluations (which included detailed clinical observations) and motor activity evaluations were recorded. Rats were killed on day 15, administered a combination of heparin and an anaesthetic and perfused in situ with neutral buffered 10% formalin. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Brain weights were recorded for all rats after additional fixation of the tissue. Five rats of each sex per group were selected for neurohistological examination; and the tissues from rats selected in the control groups and group at the highest dose were processed for histological examination. In the study extension, viabilities, clinical observations, body weights, feed consumption values, two components of the FOB (behaviour in the home cage and measurement of landing foot splay) and motor activity evaluations (female rats only) were evaluated. These rats were killed on day 3 of the study, and carcasses were discarded without further evaluation.

All rats survived until scheduled necropsy on day 15 (main study) or day 3 (extension study). None of the clinical signs observed in these rats were considered to be related to the test substance. Body weights and feed consumption values were unaffected at 50 or 125 mg/kg bw in the extension study. Transient reductions in body weight gains or body weight losses along with corresponding reductions in absolute and relative feed consumption occurred on the day after dosage in both sexes given thiophanate-methyl at 500 mg/kg bw and higher in the main study, the extension study or both. These changes were followed by increases in body weight gain on the subsequent day.

In the FOB conducted two hours after dosing in the main study, both male and female rats given thiophanate-methyl at 500, 1000 or 2000 mg/kg bw had significantly reduced values for landing foot splay (Table 30). The number of male rats in these groups that appeared to be sleeping when examined in the home cage also was significantly reduced, and the numbers showing other normal patterns (e.g. appeared awake and immobile or showed normal movement) were increased when compared with the control group. Female rats at 500 mg/kg bw and higher also had significantly reduced motor activity during the test that followed the FOB examination on the day of dosing.

There were neither statistically significant nor exceptional differences among the groups in the measures of the FOB or in the motor activity test on the day before dosage and seven days and 14 days after dosing. No gross lesions were observed at necropsy. Brain weights and the ratio of brain weights to terminal body weights were comparable among the groups. No lesions related to the test substance were observed in the microscopic examination of the neural and muscle tissues. The extension study evaluated home cage behaviour and landing foot splay in both sexes and motor activity in females before dosing and two hours after dosing. Landing foot splay values were significantly reduced in both sexes at 50 mg/kg bw and higher (Table 30). The variations in normal home cage behaviour and reduced motor activity observed in the main study were not observed at 50 and 125 mg/kg bw and were not replicated at 500 and 2000 mg/kg bw in the study extension. These changes were considered to be incidental events because they were noted only in one sex in the main study and not reproduced in the extension study. This conclusion is also supported by the evaluations in the short-term study of neurotoxicity in which there were no adverse changes in the FOB, including the home cage observation and motor activity tests at the highest dietary concentration of 2500 ppm at 2, 4, 8 and 13 weeks of exposure.

The toxicological significance of the transient decreases in landing foot splay observed in this study is questionable and considered inappropriate for setting a NOAEL. The biological significance of decreased landing foot splay in the absence of apparent changes in gait, posture or other behavioural responses such as air righting has not been specified in previous research. In addition, the variability observed within the pairs of measurements obtained from individual rats as well as variability among

the averages for rats in the control group in this study and across other studies were comparable to the differences between the control groups and the groups given thiophanate-methyl. This conclusion is also supported by the evaluations in the short-term study of neurotoxicity in which there were no adverse changes in the FOB, including the landing foot splay measurements, at the highest dietary concentration of 2500 ppm (equal to 149.6 mg/kg bw per day for males and 166.3 mg/kg bw per day for females) at 2, 4, 8 and 13 weeks of exposure.

Table 30. Landing foot splay in rats given a single dose of thiophanate-methyl by gavage in a study of acute neurotoxicity

	Landing foot splay in cm					
Study[a] / Time point	**0 mg/kg bw**	**50 mg/kg bw**	**125 mg/kg bw**	**500 mg/kg bw**	**1000 mg/kg bw**	**2000 mg/kg bw**
Males						
Main study						
Before dosing	7.0	NE	NE	7.1	6.6	7.4
2 hours after dosing	6.6	NE	NE	5.2*	4.2**	4.7**
7 days after dosing	6.5	NE	NE	6.9	6.6	7.0
14 days after dosing	6.4	NE	NE	6.8	6.1	6.4
Study extension						
Before dosing	8.4	8.9	8.2	7.6	NE	8.2
2 hours after dosing	8.9	7.0**	6.5**	5.2**	NE	6.1**
Females						
Main study						
Before dosing	6.2	NE	NE	6.5	6.4	7.2
2 hours after dosing	5.6	NE	NE	3.9**	4.2**	4.4**
7 days after dosing	5.5	NE	NE	5.1	5.6	5.8
14 days after dosing	5.3	NE	NE	4.8	5.5	5.9
Study extension						
Before dosing	8.4	8.0	8.3	7.4	NE	7.9
2 hours after dosing	8.4	6.8*	6.0**	6.0**	NE	5.6**

NE: not examined; * P: ≤ 0.05; **: P ≤ 0.01; *Source*: Foss (2005)

[a] Data for historical controls: males 6.5 ± 1.7 cm and 8.7 ± 1.2 cm; females 6.3 ± 1.2 cm and 5.7 ± 1.7 cm.

The NOAEL for general toxicity was 125 mg/kg bw on the basis of transient reductions in body weight gains (including body weight losses) and feed consumption at 500 mg/kg bw and above.

The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested. The toxicological significance of the transient decreases in landing foot splay observed at all doses was questionable and was considered to be inappropriate for identifying a NOAEL for neurotoxicity (Foss, 2005).

In a 13-week USEPA and OECD TG-compliant neurotoxicity study, Crl:CD(SD)IGS BR VAF Plus rats (10/sex per group) were fed diets containing thiophanate-methyl (purity: 99.7%) at a concentration of 0, 100, 500 or 2500 ppm (equal to 0, 6.2, 30 and 150 mg/kg bw per day for males and 0, 6.8, 35 and 166 mg/kg bw per day for females, respectively). Viability, clinical observations, body weights, feed consumption, FOB evaluations (which included detailed clinical observations) and motor activity evaluations were recorded. At termination rats were administered a combination of heparin and an anaesthetic and perfused in situ with neutral buffered 10% formalin. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Liver and individual kidney weights were recorded at necropsy, and brain weights were recorded after additional fixation of the tissue. Five rats of each sex per group were selected for neurohistological examination; and the tissues from rats selected in the control group and the group receiving the highest dietary concentration were evaluated.

None of the clinical signs observed in the daily examinations were considered to be related to the test substance. Body weights, body weight changes and absolute and relative feed consumption values were significantly decreased at 2500 ppm in females, while these values were unaffected in males (Table 31). Other than the body weights in the female rats, none of the parameters evaluated in the FOB and motor activity test sessions were affected by the dietary concentrations of thiophanate-methyl administered in this study. The decreases in the forelimb and hind limb grip in females at 100 ppm and higher during week 2 of exposure were considered to be incidental events unrelated to the test substance, because the differences were not dose dependent and there were no effects on other FOB parameters that might be affected by muscle weakness. There were no gross lesions in either sex, but the absolute weights of the liver and thyroid as well as ratios of these weights to terminal body weight and to brain weight were significantly increased at 2500 ppm in male rats; the ratios of these weights to terminal body weight were also significantly increased in female rats at this concentration. No microscopic lesions were observed in the neural or muscle tissues of the rats in the control group or at 2500 ppm.

Table 31. Selected findings from a 13-week dietary study of neurotoxicity in rats with thiophanate-methyl

	Findings per dose level							
	Males				Females			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**2500 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**2500 ppm**
Body weight (g), day 91	540.6	532.8	544.5	519.9	309.9	290.1	296.3	280.2
Body weight gain (g), days 1–91	329.1	323.6	335.0	309.7	140.0	121.7*	127.3	110.6**
Feed consumption (g/day)	24.7	25.1	24.9	23.8	18.7	16.8**	17.6	15.9**
Limb grip								
Fore limb, maximum (g), week 2	284.5	335.5	335.5	340.0	291.5	201.0**	264.0	222.5*
Fore limb, average (g), week 2	257.2	295.8	303.2	289.2	259.8	177.8**	235.0	204.5*
Hind limb, maximum (g), week 2	296.5	338.5	366.5	291.5	279.5	202.0**	206.5*	213.0*
Hind limb, average (g), week 2	259.2	315.5	325.2	262.0	236.8	184.5*	187.8*	187.2*
Liver								
Absolute weight (g)	22.95	20.75	23.91	27.66	12.95	11.80	12.88	13.92
Relative weight (g/100 g bw)	4.24	3.89	4.37	5.32**	4.19	4.06	4.34	4.97**
Thyroid								
Absolute weight (g)	29	30	34	57**	28	21	26	35
Relative weight (mg/100 g bw)	5.45	5.72	6.25	10.97**	8.87	7.03	8.84	12.45**

bw: body weight; ppm: parts per million; * $P \leq 0.05$; ** $P \leq 0.01$

Source: Foss (2005b)

The NOAEL for general toxicity was 500 ppm (equal to 30 mg/kg bw per day) on the basis of decreased body weights and feed consumption in females and increased liver and thyroid weights in both sexes at 2500 ppm.

The NOAEL for neurotoxicity was 2500 ppm (equal to 150 mg/kg bw per day), the highest dose tested (Foss, 2007).

(c) Studies on metabolites and impurities

Carbendazim is the major toxicologically relevant metabolite of thiophanate-methyl. No studies on carbendazim were provided by the sponsor. The sponsor did provide studies on acute toxicity and genotoxicity for some other metabolites of thiophanate-methyl.

Acute toxicity

Acute toxicity studies with metabolites or impurities of thiophanate-methyl were available. Studies with well-described study designs and reporting are presented in Table 32.

Methyl *N*-[2-(*N*′-methoxycarbonylthioureido)phenylaminocarbonyl]carbamate (DX-105) is a metabolite found in mice and rats. DX-189 and FH-613 are described as impurities of technical thiophanate-methyl. CM-0237 is a major plant metabolite. In addition, a very concise report was available that indicated that the animal metabolites FH-624, CF-44 and FH-622, the plant and animal metabolites dimethyl [(1,2-phenylene)bis(iminocarbonyl)]bis(carbamate) (FH-432) and FH-278 and the impurities TFY-61, FH-46, AV-1951 and FH-73 have low acute toxicity in mouse and/or rat (Anonymous, 1988).

Table 32. Results of studies of acute toxicity with metabolites of thiophanate-methyl

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference [a]
Rat	CD(SD)	M/F	Oral	DX-105 [b]	99.4	> 5000 (M/F)	Nishibe (1988)
Rat	CD(SD)	M/F	Oral	DX-189 [c]	98.0	> 5000 (M/F)	Nishibe (1988)
Rat	CD(SD)	M/F	Oral	FH-613 [d]	99.6	1776 (M/F) 2007 (F)	Nishibe (1988)
Rat	Crj:CD(SD)IGS D-1	M/F	Oral	CM-0237 [e]	98.3	> 2000 (M/F)	Sasaki (2002)

bw: body weight; F: female; M: male;
OECD TG: Organisation for Economic Co-operation and Development Test Guideline

[a] Statements of adherence to quality assurance and good laboratory practice were included in all studies.

[b] Batch no. MO-8904. DX-105 = methyl *N*-[2-(*N*′-methoxycarbonylthioureido)phenylaminocarbonyl]-carbamate. Study design resembles OECD TG 401. A dose of 5000 mg/kg bw was administered by gavage. Vehicle was distilled water containing a small volume of Tween 80. No mortalities, clinical signs or abnormalities at necropsy were observed.

[c] Batch no. MO-8902. DX-189 = 1,4-*bis*(3-methoxy carbonyl-2thioureido)benzene. Study design resembles OECD TG 401. A dose of 5000 mg/kg bw was administered by gavage. Vehicle was distilled water containing a small volume of Tween 80. No mortalities, clinical signs or abnormalities at necropsy were observed.

[d] Batch no. MO-8901. FH-613 = 2-methoxycarbonylamino-1-(3-methoxy carbonyl-2-thioureido)-benzene. Study design resembles OECD TG 401. Doses of 0, 500, 1000, 2000, 4000 and 5000 mg/kg bw were administered by gavage. Vehicle was distilled water containing a small volume of Tween 80. Mortalities numbered 0, 2, 3, 3 and 5 in males and 0, 2, 2, 3 and 5 in females at 500, 1000, 2000, 4000 and 5000 mg/kg bw, respectively. Decreased motor activity, hypotonia, ataxia and low sensitivity appeared in many rats after 20–30 minutes. In addition, convulsion, Straub tail and paralysis of anterior legs and posterior legs were also observed after three hours. Lateral position, bradypnoea and urine incontinence and salivation appeared in a few rats. These signs disappeared in surviving rats after 1–4 days. Decrease of body weight was observed at 1000, 4000 mg/kg in males, and at 2000, 4000 mg/kg in females after 1–2 days. Dark reddish lung, dark reddish stomach and intestine, and hydrothorax were found in dead rats. Hypertrophy of spleen and atrophy of thymus were also found in one rat at termination.

[e] Batch no. 31-02122-I. TAKEUCHI. CM-0237 = methoxy-*N*-{4-[(methoxycarbonylamino)carbonylamino]-benzothiazol-2-yl}carboxamide. Study performed according to OECD TG 401. A dose of 2000 mg/kg bw was administered by gavage. Vehicle was ion-exchanged water. No mortalities, clinical signs or abnormalities at necropsy were observed.

Genotoxicity

The results of genotoxicity studies with metabolites of thiophanate-methyl are summarized in Table 33. CM-0237 is a major plant metabolite. FH-73 is a plant metabolite.

Table 33. Results of studies on the genotoxicity of metabolites of thiophanate-methyl

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference [a]
In vitro						
CM-0237	Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2uvrA	12.5–400 µg/plate (± S9)	98.3	Negative	Fujii (2002) [b]
FH-73	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2uvrA	62–5000 µg/plate (± S9)	99.9	Negative	Kuboki (2014) [c]

DMSO: dimethyl sulfoxide; OECD TG; Organisation for Economic Co-operation and Development Test Guideline; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Positive and negative (solvent) controls were included in all studies. In all studies, statements of adherence to GLP and quality assurance were included.

[b] Batch no. 31-02122-I.TAKEUCHI. Study was performed according to OECD TG 471. Test compound was dissolved in DMSO.

[c] Batch no. YS-9128. Performed in accordance with OECD TG 471. Test compound was dissolved in DMSO.

Observations in humans

Thiophanate-methyl has been commercially produced since 1969. Once yearly a health examination on all employees in the manufacturing plant was performed and results were reported on a regular basis. No health effects related to thiophanate-methyl have been reported in plant personnel or from agricultural use (Mori, 1972; Ikeda, 1978; Aizawa, 1991; Ota, 2014).

Comments

Biochemical aspects

Thiophanate-methyl is rapidly and almost completely absorbed (88–89%) after oral administration of a dose of 14 mg/kg bw (Noguchi, 1970; Noguchi & Kosaka, 1971). Thiophanate-methyl is rapidly excreted (approximately 47% in urine and approximately 40% in bile) within 48 hours of administration (Bernard, 2011b). Plasma half-lives were 1.6–2.8 hours after a dose of 13 mg/kg bw and 2.4–7.8 hours after a dose of 140–170 mg/kg bw (Tanoue et al, 1992a,b). Absorption and excretion patterns were similar in male and female rats (Bernard, 2011b). There is no potential for accumulation (Kosaka et al., 1975). Thiophanate-methyl is widely distributed, with highest levels in liver and thyroid (Tanoue et al., 1992a,b). The major urinary metabolite was 5-hydroxycarbendazim sulfate (5-OH-MBC-S). Minor metabolites were 5- and 4-hydroxythiophanate-methyl, each representing approximately 2% of the administered radiolabel. The major faecal metabolites were 4-hydroxythiophanate-methyl (6–10%), 5-OH-MBC-S (2–5%) and carbendazim (2–3%). Unchanged thiophanate-methyl accounted for approximately 20–24% and 50% of the administered radiolabel after repeated low and high doses, respectively (Nabetani & Mori, 1993).

Toxicological data

In rats, the oral LD_{50} was greater than 5000 mg/kg bw (Nishibe, 1990b); in rabbits the dermal LD_{50} was greater than 2000 mg/kg bw (Nishibe, 1990c); and in rats, the inhalation LC_{50} was 1.7–1.98 mg/L (Nishibe, 1987). Thiophanate-methyl was not irritating to the skin or eyes of rabbits (Nishibe, 1987, 1986).

Thiophanate-methyl was a skin sensitizer in a Magnusson and Kligman test in guinea pigs (Nishibe, 1989), but not in a Buehler test (Nishibe & Mochizuiki, 1993).

In repeated-dose oral toxicity studies with thiophanate-methyl in mice, rats and dogs, the most sensitive organs were the liver and thyroid.

In a pre-guideline six-month dietary toxicity study (with limited investigations) in mice administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 2, 10, 50, 250 and 1240 mg/kg bw per day for males and 0, 2, 11, 52, 231 and 1630 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 231 mg/kg bw per day) based on decreased body weight gain and haematological changes indicative of slight anaemia in males and females at 8000 ppm (equal to 1240 mg/kg bw per day) (Noguchi & Hashimoto, 1970b).

In a 13-week dietary toxicity study in rats administered dietary thiophanate-methyl concentrations of 0, 200, 2200, 4200, 6200 or 8200 ppm (equal to 0, 13.9, 155, 293, 427 and 565 mg/kg bw per day for males and 0, 15.7, 173, 323, 479 and 647 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 13.9 mg/kg bw per day) based on haematological changes indicative of slight anaemia, increased thyroid and liver weights, follicular hyperplasia and hypertrophy of the thyroid and hepatocellular hypertrophy and increased lipofuscin pigment in both sexes and increased severity of glomerulonephrosis in males observed at 2200 ppm (equal to 155 mg/kg bw per day) (Nishibe & Takaori, 1990).

In a pre-guideline six-month dietary toxicity study (with limited investigations) in rats administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 1, 4, 20, 95 and 500 mg/kg bw per day for males and 0, 1, 5, 22, 110 and 660 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 95 mg/kg bw per day) based on decreased body weight gain, haematological changes indicative of slight anaemia, decreased glucose levels and increased cholesterol levels, increased thyroid weights and histological changes in the thyroid in males and females at 8000 ppm (equal to 500 mg/kg bw per day) (Noguchi & Hashimoto, 1970c).

In a three-month toxicity study in dogs, administering thiophanate-methyl by gelatine capsule at doses of 0, 50, 200 and 800/400 mg/kg bw per day, the LOAEL was 50 mg/kg bw per day based on the hypertrophy of the follicular epithelial cells of the thyroid at this dose level (Auletta, 1991).

In a pre-guideline two-year toxicity study (with limited investigations) in dogs administered thiophanate-methyl by gelatine capsule at doses of 0, 2, 10, 50 or 250 mg/kg bw per day, the NOAEL was 10 mg/kg bw per day based on effects on thyroid weight and histopathology of the thyroid in both sexes at 50 mg/kg bw per day (Hashimoto et al., 1972).

The overall NOAEL for the studies in dogs was 10 mg/kg bw per day, and the overall LOAEL was 40 mg/kg bw per day.

In a pre-guideline two-year dietary carcinogenicity study (with limited investigations) in mice administered dietary thiophanate-methyl concentrations of 0, 10, 40, 160 and 640 ppm (equal to 0, 1.2, 4.4, 20 and 82 mg/kg bw per day for males and 0, 1.3, 5.0, 19 and 82 mg/kg bw per day for females), the NOAEL was 160 ppm (equal to 20 mg/kg bw per day) based on reduced body weight gain and histopathological changes in testes of males at 640 ppm (equal to 82 mg/kg bw per day) (Nishibe et al., 1973).

In an 18-month dietary carcinogenicity study in mice administered dietary thiophanate-methyl concentrations of 0, 150, 640, 3000 or 7000 ppm (equal to 0, 24, 99, 468 and 1079 mg/kg bw per day for males and 0, 29, 123, 558 and 1329 mg/kg bw per day for females, respectively), the NOAEL was 150 ppm (equal to 29 mg/kg bw per day) based on the hepatocellular centrilobular hypertrophy at nine months and hepatocellular adenomas in females observed at 640 ppm (equal to 123 mg/kg bw per day) (Tompkins, 1992).

In a two-year combined chronic toxicity/carcinogenicity study in rats administered dietary thiophanate-methyl concentrations of 0, 75, 200, 1200 or 6000 ppm (equal to 0, 3.3, 8.8, 54 and 281 mg/kg bw per day for males and 0, 3.8, 10.2, 64 and 335 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 8.8 mg/kg bw per day) based on reduced body weight gain in both sexes; increased total cholesterol and total protein in both sexes; decreased albumin to globulin ratio in both sexes at 12 and/or 18 months; decreased levels of chloride and potassium; decreased T_4 and T_3 and

increased TSH in males at 24 months; increased urinary protein and granular kidneys in males; follicular cell hyperplasia and hypertrophy in the thyroid in both sexes at 12 and 24 months; a possible increase in the incidence of thyroid follicular cell adenoma in males; centrilobular hepatocellular hypertrophy and occurrence of lipofuscin pigment in both sexes at 12 and 24 months; lipidosis of the adrenal cortex in females at 12 months; and increased severity of nephropathy in both sexes at 24 months observed at 1200 ppm (equal to 54 mg/kg bw per day). In males, the incidence of thyroid follicular cell adenoma was increased at 1200 ppm and above, but reached statistical significance only at 6000 ppm (Takaori, 1993).

A mechanistic study showed that thiophanate-methyl induced cytochromes CYP450 (not further specified) and CYPB5 as well as uridine glucuronosyltransferase (UDPGT), an enzyme that plays an important role in the clearance of T_4 in the liver. Thiophanate-methyl also inhibited porcine thyroid microsomal peroxidase, an enzyme involved in thyroid hormone synthesis. T_4 supplementation counteracted the hypertrophy of the thyroid and the TSH response, indicating that thiophanate-methyl caused the hypertrophy by negative feedback mechanism. The mechanistic study demonstrates that the thyroid effects resulting from thiophanate-methyl are likely to be the result of a reduction in thyroid hormones. In another study, thiophanate-methyl induced increases in CYP3A and CYP2B1 in the liver of rats (Takaori, 1993; Paolini et al., 1999).

The Meeting concluded that thiophanate-methyl is carcinogenic in mice and rats. The Meeting considered the different modes of action that might underlie the observed tumour induction. The effects on the thyroid, including the induction of thyroid follicular cell adenoma may be secondary effects resulting from liver enzyme induction that enhances thyroid hormone excretion and leads to perturbations in systemic thyroid hormone levels (i.e. decreased thyroid T_4 and T_3 hormone values and, as a consequence, an increase in TSH concentration). The continuous stimulation of the thyroid gland by TSH is known to result in follicular cell hypertrophy/hyperplasia and, depending on dose and time, in follicular cell adenomas/adenocarcinomas. Rats are particularly sensitive to decreases in T_4 and T_3 levels resulting from liver enzyme induction; these reductions eventually lead to thyroid tumour formation. This is a well-established adverse outcome pathway without relevance for humans. The thiophanate-methyl–induced increase in hepatocellular adenomas may be a consequence of activation of nuclear receptors involved in the induction of the cytochrome P450 drug metabolizing system. Another possible mode of action for the carcinogenic effect may be the interference of the thiophanate-methyl metabolite carbendazim with mitotic spindle proteins leading to aneuploidy (see below).

Thiophanate-methyl was tested in an adequate range of in vitro and in vivo assays for genotoxicity. Thiophanate-methyl does not cause gene mutations or structural chromosomal aberrations; however, it causes changes in chromosome number (aneuploidy) both in vitro and in vivo. Induction of micronucleus formation in mice was seen after single doses (500 mg/kg bw and above), but the response was weak (about six times lower) when compared with that for the metabolite of thiophanate-methyl, carbendazim (Myhr & Brusick, 1981; McSheehy et al., 1984; Murli, 1988; Nishibe, 1990; Barale et al, 1993; Marshall, 1996a,b; Proudlock, 1999).

Carbendazim causes changes in chromosome number (aneuploidy) both in vitro and in vivo (in somatic cells and germ cells) as a result of its interference with mitotic spindle proteins. The mechanism by which aneuploidy is induced by carbendazim is well understood and consists of inhibition of the polymerization of tubulin, the protein that is essential for the segregation of the chromosomes during cell division. The nature of the mechanism is thus consistent with the identification of a threshold dose below which no toxicological effect would occur. Like thiophanate-methyl, carbendazim does not cause gene mutations or structural chromosomal aberrations.

The Meeting concluded that the genotoxic effect of thiophanate-methyl is a threshold phenomenon and is likely related to the production of carbendazim.

The Meeting concluded that thiophanate-methyl is unlikely to pose a carcinogenic risk to humans at dietary doses.

In a two-generation dietary reproductive toxicity study in rats administered thiophanate-methyl at dietary doses of 0, 200, 630 or 2000 ppm (equal to premating doses of 0, 14.6, 46.0 and 147.1 mg/kg bw per day for males and 16.8, 52.2 and 164.3 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 200 ppm (equal to 14.6 mg/kg bw per day) based on thyroid hyperplasia in males and increased TSH levels in females at 630 ppm (equal to 46 mg/kg bw per day). The NOAEL

for offspring toxicity was 200 ppm (equal to 16.8 mg/kg bw per day) based on decreased body weights (in F_{2b}) during lactation at 630 ppm (equal to 52.2 mg/kg bw per day). The NOAEL for reproductive toxicity was 2000 ppm (equal to 147 mg/kg bw per day), the highest dose tested (Müller, 1993; Müller & Singer, 1995).

In a developmental toxicity study in rats administered gavage doses of thiophanate-methyl of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 300 mg/kg bw per day based on a reduced body weight gain at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1981a,b).

In a developmental toxicity study in rabbits administered gavage doses of thiophanate-methyl of 0, 5, 10, 20 and 40 mg/kg bw per day, the NOAEL for maternal toxicity was 10 mg/kg bw per day based on a reduced body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day based on supernumerary thoracic ribs at 40 mg/kg bw per day. This effect was considered unlikely to be an effect of a single dose (York, 1997a,b).

The Meeting concluded that thiophanate-methyl is not teratogenic.

In a study of acute neurotoxicity in rats administered gavage doses of thiophanate-methyl of 0, 50, 125, 500, 1000 or 2000 mg/kg bw, the NOAEL for general toxicity was 125 mg/kg bw based on transient reductions in body weight gains (including body weight losses) and feed consumption at 500 mg/kg bw. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Foss, 2005).

In a 13-week study of neurotoxicity in rats administered thiophanate-methyl at dietary doses of 0, 100, 500 or 2500 ppm (equal to 0, 6.2, 30 and 150 mg/kg bw per day for males and 0, 6.8, 35 and 166 mg/kg bw per day for females, respectively), the NOAEL for general toxicity was 500 ppm (equal to 30 mg/kg bw per day) based on decreased body weights and feed consumption in females and increased liver and thyroid weights in both sexes at 2500 ppm (equal to 150 mg/kg bw per day). The NOAEL for neurotoxicity was 2500 ppm (equal to 150 mg/kg bw per day), the highest dose tested (Foss, 2007).

The Meeting concluded that thiophanate-methyl is not neurotoxic.

No immunotoxicity tests with thiophanate-methyl were available. However, the data from the available toxicity studies did not indicate an immunotoxic potential of thiophanate-methyl.

The Meeting concluded that thiophanate-methyl is unlikely to be immunotoxic.

Toxicological data on metabolites and/or degradates

Studies of acute oral toxicity and genotoxicity were performed with metabolites and impurities of thiophanate-methyl. For DX-105, a photodegradation product and a metabolite also found in mice and rats, the acute oral toxicity LD_{50} was greater than 5000 mg/kg bw; for the impurities of technical thiophanate-methyl DX-189 and FH-613, the acute oral toxicity LD_{50} was greater than 5000 and 1776 mg/kg bw, respectively; and for CM-0237, a major plant metabolite, the acute oral toxicity LD_{50} was greater than 2000 mg/kg bw. In addition, a very concise report was available that indicated that the animal metabolites FH-624, CF-44 and FH-622, the plant and animal metabolites FH-432 and FH-278, and the impurities TFY-61, FH-46, AV-1951 and FH-73 have low acute toxicity in mouse and/or rat. Negative results in an in vitro bacterial gene mutation test were obtained for CM-0237 and for FH-37, a plant metabolite of thiophanate-methyl (Anonymous, 1988).

The major residues in crops and livestock were thiophanate-methyl, carbendazim, 5-OH-MBC and 5-OH-MBC-S.

Carbendazim itself is used as a pesticide, and JMPR established an ADI in 1995 and an ARfD in 2005. No specific toxicity studies on the metabolites 5-OH-MBC and 5-OH-MBC-S of thiophanate-methyl, which are also metabolites of carbendazim, were available. However, 5-OH-MBC-S was found in rats at more than 40% of the absorbed dose in a toxicokinetic study with thiophanate-methyl and at 21–43% of the absorbed dose in a toxicokinetic study with carbendazim. 5-OH-MBC is an intermediate in the metabolic pathway leading to the formation of 5-OH-MBC-S. The toxicities of the rat metabolites 5-OH-MBC and 5-OH-MBC-S are therefore considered to be covered by that of thiophanate-methyl and carbendazim.

Human data

Thiophanate-methyl has been commercially produced since 1969. No health effects related to thiophanate-methyl have been reported in manufacturing plant personnel or from agricultural use.

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

Toxicological evaluation

Thiophanate-methyl

The Meeting established an ADI of 0–0.09 mg/kg bw for thiophanate-methyl on the basis of a NOAEL of 8.8 mg/kg bw per day based on reduction in body weight gain and clinical chemistry, urine analysis and histopathological changes in the kidney, thyroid, liver and adrenals in a two-year study in rats. This ADI is supported by the overall NOAEL of 10 mg/kg bw per day based on increased thyroid weight and histopathological changes in the thyroid observed in three-month, one-year and two-year toxicity studies in dogs . A safety factor of 100 was applied.

The upper bound of the ADI provides a margin of exposure of 600 relative to the LOAEL for thyroid follicular cell adenoma in male rats (54 mg/kg bw per day) and about 3100 relative to the LOAEL for hepatocellular adenomas in female mice (280 mg/kg bw per day).

The Meeting established an ARfD of 1 mg/kg bw for thiophanate-methyl on the basis of a NOAEL of 125 mg/kg bw for transient reductions in body weight gains (including body weight losses) and feed consumption in an acute neurotoxicity study in rats, using a safety factor of 100.

Carbendazim

Since plant and food residues are expressed as carbendazim, the ADI and ARfD established for that compound must be taken into consideration.

No toxicological studies on carbendazim were available for the present evaluation.

JMPR last evaluated carbendazim to establish an ADI in 1995. The Meeting established an ADI of 0–0.03 mg/kg bw based on a NOAEL of 2.5 mg/kg bw per day, on the basis of hepatotoxicity observed at 12.5 mg/kg bw per day in a two-year study in dogs. A safety factor of 100 was applied.

The need for an ARfD for carbendazim was considered in 2005. At that time the Meeting established an ARfD of 0.1 mg/kg bw based on an overall NOAEL of 10 mg/kg bw per day for developmental toxicity from three studies in rats and one study in rabbits, and a safety factor of 100. The 2005 Meeting concluded that this ARfD applies only to women of childbearing age.

For the general population, including children, the Meeting established an ARfD of 0.5 mg/kg bw based on the NOAEL of 50 mg/kg bw in the study of toxicity to the male reproductive system in rats and supported by the studies on micronucleus or aneuploidy induction in vivo, using a safety factor of 100.

An additional safety factor for the severity of the effects was considered to be unnecessary, since the underlying mechanism is clearly understood and there is a clear threshold for these effects.

Levels relevant to risk assessment of thiophanate-methyl

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity [a]	Toxicity	150 ppm, equal to 29 mg/kg bw per day	640 ppm, equal to 123 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 29 mg/kg bw per day	640 ppm, equal to 123 mg/kg bw per day
Rat	Acute neurotoxicity study [b]	Toxicity Neurotoxicity	125 mg/kg bw 2 000 mg/kg bw [b,c]	500 mg/kg bw –
	Thirteen-week neurotoxicity study [a]	Neurotoxicity	2500 ppm, equal to 150 mg/kg bw per day [b,c]	–
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	200 ppm, equal to 8.8 mg/kg bw per day	1200 ppm, equal to 54 mg/kg bw per day
		Carcinogenicity	200 ppm, equal to 8.8 mg/kg bw per day	1200 ppm, equal to 54 mg/kg bw per day
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	2000 ppm, equal to 147 mg/kg bw per day [c]	–
		Parental toxicity	200 ppm, equal to 14.6 mg/kg bw per day	630 ppm, equal to 46 mg/kg bw per day
		Offspring toxicity	200 ppm, equal to 16.8 mg/kg bw per day	630 ppm, equal to 52.2 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	300 mg/kg bw per day	1000 mg/kg bw per day
		Embryo/fetal toxicity	1000 mg/kg bw per day [c]	–
Rabbit	Developmental toxicity study [b]	Maternal toxicity	10 mg/kg bw per day	20 mg/kg bw per day
		Embryo/fetal toxicity	20 mg/kg bw per day	40 mg/kg bw per day
Dog	Thirteen-week, one-year and two-year studies of toxicity [d,e]	Toxicity	10 mg/kg bw per day	40 mg/kg bw per day

[a] Dietary administration.

[b] Gavage administration.

[c] Highest dose tested.

[d] Two or more studies combined.

[e] Capsule administration.

Estimate of acceptable daily intake (ADI)

0–0.09 mg/kg bw

Estimate of acute reference dose (ARfD)

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 thiophanate-methyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and almost complete (rats)
Dermal absorption	≥ 53% at 0.3 mg/rat; ≥ 23% at 32 mg/rat
Distribution	Widespread distribution, highest concentrations found in liver and thyroid
Potential for accumulation	Low potential for accumulation
Rate and extent of excretion	Rapid; 87% in 48 hours
Metabolism in animals	Extensively metabolized, major metabolite is 5-OH-MBC-S
Toxicologically significant compounds in animals and plants	Thiophanate-methyl, carbendazim
Acute toxicity	
Rat, LD_{50}, oral	> 5000 mg/kg bw
Rat, LD_{50}, dermal	> 2000 mg/kg bw
Rat, LC_{50}, inhalation	> 1.7 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Guinea-pig, dermal sensitization	Sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Liver, thyroid, haematological effects
Lowest relevant oral NOAEL	10 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	Body weight, clinical chemistry, urine analysis, histopathology of liver, thyroid, kidney, adrenal
Lowest relevant NOAEL	8.8 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice and rats [a]
Genotoxicity	Genotoxic, threshold phenomenon (aneuploidy) [a]
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	14.6 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	16.8 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	147 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Supernumerary ribs
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	20 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	166 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data

Studies on toxicologically relevant metabolites	
Carbendazim	Major toxicologically relevant metabolite is carbendazim. Last evaluated by JMPR in 1995 (ADI: 0.03 mg/kg bw) with an addendum in 2005 (ARfD: 0.1 mg/kg bw)
Other metabolites	Low acute toxicity for DX-105, DX-189, FH-613 and CM-0237. Negative results in an in vitro gene mutation test for CM-0237 and FH-37
Human data	No adverse effects reported

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI	0–0.09 mg/kg bw	Two-year study (rats)	100
ARfD	1 mg/kg bw	Acute neurotoxicity study (rats)	100

Residue and analytical aspects

The Meeting did not receive any information on the toxicology of carbendazim. The Meeting was unable to complete its evaluation for residues.

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TRIFLUMEZOPYRIM

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Explanation

Triflumezopyrim is the ISO-approved common name for 2,4dioxo-1-(pyrimidin-5-ylmethyl)-3-[3-(trifluoromethyl)phenyl]-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-1-ium-3-ide (International Union of Pure and Applied Chemistry [IUPAC] name), with the Chemical Abstracts Service (CAS) number 1263133-33-0.

Triflumezopyrim is a new insecticide belonging to the novel class of mesoionics that is intended to control a number of insect species in rice, including white-backed planthoppers, small brown planthoppers, green leafhoppers and, in particular, the brown planthopper *Nilaparvata lugens*, which has developed strong resistance to neonicotinoids such as imidacloprid. Triflumezopyrim acts by binding to and inhibiting the orthosteric site of the nicotinic acetylcholine receptor, deviating from action of neonicotinoids and other receptor agonists which, in contrast, stimulate the receptor, leading to over-excitation of the insect nervous system (Cordova et al., 2016; Holyoke et al., 2016). Application timing is early in the growth of the rice, as soon as the population of hoppers reaches the economic threshold level.

Triflumezopyrim has not been evaluated previously 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 current test guidelines.

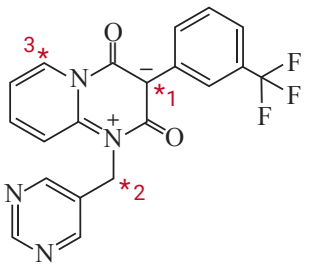
Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution, metabolism and excretion (ADME) as well as toxicokinetics of triflumezopyrim have been investigated in Sprague Dawley [Crl:CD®(SD)IGS BR] rats following single oral administration of 10 or 200 mg/kg body weight (bw). For these experiments, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was radiolabelled in one of three positions (see Fig. 1). A separate repeated-dose study with triflumezopyrim at the low dose level of 10 mg/kg bw per day was performed with only one (i.e. the pyridine-2,6-^{14}C) radiolabelled form and only in female rats since no major differences in the overall disposition of triflumezopyrim had been observed between the three radiolabelled forms and between sexes. Results of this second study are reported below. Metabolism of triflumezopyrim in the rat was studied in samples obtained in both studies and is described in the next subsection.

Figure 1. Structure of triflumezopyrim and position of radiolabels



* Denotes position of the radiolabel at:

1 [fused pyrimidine-3-^{14}C]triflumezopyrim

2 [methylene-^{14}C]triflumezopyrim

3 [pyridine-2,6-^{14}C]triflumezopyrim

(a) Oral route

Single-dose experiments

For all experiments, the doses were prepared with 0.1% Tween 80 in 0.5% methylcellulose and administered by gavage at an approximate volume of 4 mL/kg bw. The radioactive doses ranged from 26.7 to 58.1 µCi/rat (0.99–2.15 MBq/rat) for male rats and 20.2 to 38.1 µCi/rat (0.75–1.41 MBq/rat) for female rats.

In a pilot study, one male and one female per radiolabel received a low dose of the test material. Besides recovery of triflumezopyrim in urine and faeces and its analysis in whole blood, radiolabelled CO_2 and other exhaled volatiles were measured for up to 168 hours. All three radiolabels showed similar results. Total recovery for both sexes ranged from 84.7% to 114%. Similar portions of the administered dose were recovered in the urine (38.1–49.4%) and faeces (40.4–57.4%) with no apparent difference between sexes. Radioactivity recovered from exhalation breath was extremely low, ranging from 0.000 13% to 0.07% of the dose for all three radiolabels. Thus, the exhaled portion was considered negligible and was not measured in the subsequent experiments. The residual ^{14}C in the carcass ranged from 0.375% to 1.22% in male rats and 0.234% to 0.568% in female rats, suggesting efficient elimination.

The complex design of the main study is summarized in Table 1 with regard to the experiments using one of the three radiolabels only. Nearly the whole test programme was repeated with fused [pyrimidine-3-^{14}C]- and [methylene-^{14}C]-labelled triflumezopyrim. In addition, a vehicle control group of one male and one female was included in all experiments except for the tissue distribution experiments after 0.5, 2 or 24 hours. A large number of groups, comprising a variable number of animals of both sexes, was employed. The time intervals after which the animals were killed ranged from 0.5 hours to seven days post dosing. From many groups, samples for subsequent analysis for metabolites (see below) were taken.

Table 1. Summary of the different groups and treatment regimens in the single dose ADME study[a] with [pyridine-2,6-^{14}C]-labelled triflumeropyrim

End-point under investigation	Single dose (mg/kg bw)	No. and sex of animals	Termination post dosing (h)	Samples
Material balance and tissue distribution	10	4 M, 4 F	168	U, F, T, C, CW, FR
	200	4 M, 4 F	168	U, F, T, C, CW, FR
Biliary excretion	10	4 M, 4 F	48	B, U, F, C, CW, GIT
	200	4 M, 4 F	48	B, U, F, C, CW, GIT
Pharmacokinetics	10	1 M, 1 F	2	Plasma, RBC
	200	1 M, 1 F	2	Plasma, RBC
	10	4 M, 4 F	48	Plasma, RBC
	200	4 M, 4 F	48	Plasma, RBC
Tissue distribution	10	4 M, 4 F	0.5	T
	200	4 M, 4 F	2	T
	10	4 M, 4 F	24	T
	200	4 M, 4 F	24	T

B: bile; C: carcass; bw: body weight; CW: cage wash; F: faeces; *Source*: Himmelstein (2015)
FR: residues in feed; GIT: gastrointestinal tract (including contents); RBC: erythrocytes; T: tissues; U: urine

[a] Pilot study data not included.

Absorption

Absorption of radiolabelled material was rapid and quantitative. Mean absorption half-lives of radiolabel in plasma ranged from three to eight minutes and from 17 to 66 minutes after low and high dose administration, respectively. Mean time to reach maximum concentration (T_{max}) values at the low dose for all three labels ranged from 0.3 to 0.8 hours. T_{max} at the high dose ranged from 1.8 to 2.5 hours for all three radiolabels. The extent of oral absorption was calculated from data obtained in the experiments in bile-cannulated rats (n = 4/sex per dose, termination 48 hours post dosing). Based on urinary and biliary excretion, and including also the radioactivity in cage wash, carcass and whole blood, an overall mean per cent absorption of 84 ± 5% was calculated for the low dose, across both sexes and the three radiolabels. Absorption of the high dose was lower, with an overall mean of 63 ± 12%. Table 2 depicts the mass balance data for the pyridine-2,6-^{14}C label.

Table 2. Elimination and recovery of [pyridine-2,6-^{14}C]triflumezopyrim in male and female rats over 48 hours

Matrix	Measure (mean ± SD)			
	Low dose (10 mg/kg bw)		High dose (200 mg/kg bw)	
	Males	Females	Males	Females
Urine	54.0 ± 4.0	68.0 ± 9.1	33.8 ± 7.54	39.7 ± 6.59
Bile	31.3 ± 4.37	16.1 ± 5.83	23.1 ± 17.6	25.5 ± 7.76
Faeces	10.3 ± 3.52	12.4 ± 3.97	34.6 ± 15.8	22.9 ± 8.24
Cage wash	1.5 ± 1.17	1.02 ± 0.425	3.82 ± 2.28	2.38 ± 1.62
Carcass	0.405 ± 0.0473	0.542 ± 0.106	0.588 ± 0.482	0.932 ± 0.86
Whole blood	0.004 57 ± 0.000 55	0.002 28 ± 0.000 495	0.006 86 ± 0.003 41	0.008 34 ± 0.009 22
GI tract	0.001 96 ± 0.000 198	0.002 33 ± 0.000 817	0.029 1 ± 0.033 3	0.191 ± 0.371
GI contents	0.011 4 ± 0.001 7	0.019 6 ± 0.015	0.958 ± 1.41	7.05 ± 13.9
Total (48 h)	97.8 ± 0.914	98.9 ± 0.768	97.5 ± 5.43	98.6 ± 2.67
Absorbed[a]	87.2 ± 3.9	85.7 ± 3.8	61.3 ± 14.4	68.6 ± 8.4

bw: body weight; GI: gastrointestinal; SD: standard deviation

[a] Sum of elimination/recovery in urine, bile, cage wash and residues in carcass, gastrointestinal tract and its contents.

Elimination via urine was slightly higher in females than in males and was lower in bile and/or faeces. However, these findings were partly specific to the radiolabel.

Distribution

A wide and rapid distribution of ^{14}C-triflumezopyrim throughout the rat body was observed at T_{max} (around 30 minutes after administration of the low dose), but absolute organ and tissue concentrations showed a marked decline over time and were generally low when ^{14}C equivalents per gram of tissue were measured (at 24 hours post dosing),,at scheduled kill (168 hours post dosing), or when expressed as the percentages of the administered dose or the tissue to plasma concentration ratios. For example, the time course of tissue distribution is depicted in Table 3 for the low dose administration of the pyridine-2,6-^{14}C label since no major radiolabel differences were observed in the overall distribution.

At study termination, highest concentrations were found in liver and kidneys in both sexes, in skin and bone marrow in males, as well as in gastrointestinal (GI) tract and its contents in females. Apart from that, there were no notable sex differences. A similar distribution pattern was observed in tissues of male and female rats administered the high dose of 200 mg/kg bw of each radiolabel. The total tissue burden, including the carcass, ranged from 0.116% to 0.586% of the administered dose at termination, that is, it was rather low. Based on distribution, in particular when the tissue to plasma concentration ratios were taken into account, there was no evidence of a potential for accumulation. The data obtained using bile-cannulated rats, as well as metabolite profiles (section 1.2), suggest significant enterohepatic circulation. Radioactive residues in plasma and red blood cells (RBCs) were similar throughout all sampling times, indicating the absence of any affinity to the erythrocytes.

Table 3. Mean tissue concentration and tissue to plasma ratio in male and female rats following single low dose administration of [pyridine-2,6-^{14}C]triflumezopyrim [a, b]

	Measures per time post dosing and sex [c]											
	0.5 hours [c]				24 hours				168 hours			
	Males		Females		Males		Females		Males		Females	
	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P
Plasma	3.93	1.0	3.75	1.0	0.081 5	1.0	0.022 1	1.0	0.005 79	1.0	0.002 36	1.0
RBC	2.84	0.71	2.09	0.59	0.022 4	0.28	0.012 8	0.51	0.007 15	1.2	0.003 30	1.4
Whole blood	3.89	0.98	3.62	0.97	0.059 3	0.73	0.017 6	0.80	0.006 86	1.2	0.003 44	1.5
Skin	3.18	0.81	3.47	0.91	0.124	1.6	0.026 1	1.2	0.034 1	5.7	0.003 33	1.4
Bone marrow	4.64	1.2	4.44	1.2	0.030 5	0.38	0.019 1	0.71	0.018 8	2.9	0	NA
Brain	0.264	0.068	0.290	0.076	0.003 84	0.046	0.001 35	0.058	0.001 51	0.31	0	NA
Fat	2.02	0.51	2.09	0.56	0.012 0	0.15	0	NA	0	NA	0	NA
Heart	5.99	1.5	6.05	1.6	0.024 7	0.30	0.013 7	0.54	0.003 99	0.70	0.002 12	0.90
Lungs	7.84	2.0	9.00	2.5	0.044 8	0.55	0.023 4	1.1	0.004 85	0.87	0.004 55	1.9
Spleen	4.96	1.3	4.82	1.3	0.026 5	0.33	0.013 3	0.54	0.004 08	0.72	0.004 07	1.8
Liver	18.9	4.8	17.1	4.8	0.252	3.1	0.100	4.5	0.035 2	6.1	0.015 2	6.5
Kidney	15.1	3.8	14.6	4.0	0.144	1.8	0.068 3	3.0	0.013 9	2.4	0.008 66	3.7
GI tract	42.8	11	47.0	14	0.316	3.9	0.240	9.6	0.005 54	0.90	0.008 23	3.2
GI contents	134	36	104	31	4.85	60	3.00	120	0.006 43	1.1	0.019 6	7.5
Pituitary	9.46	2.5	7.86	2.1	0.175	2.4	0	NA	0	NA	0	NA
Thyroid	8.62	2.2	8.71	2.2	0	NA	0	NA	0	NA	0	NA
Thymus	3.70	0.95	3.78	1.0	0.020 1	0.25	0.012 3	0.50	0.003 85	0.68	0.003 00	1.2
Testes	1.13	0.29	–	–	0.016 4	0.21	–	–	0.001 87	0.33	–	–

(*Continued on next page*)

	Measures per time post dosing and sex [c]											
	0.5 hours [c]				24 hours				168 hours			
	Males		Females		Males		Females		Males		Females	
	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P	Conc. (µg eq/g)	T:P
Ovaries	–	–	4.02	1.1	–	–	0.022 5	0.83	–	–	0	NA
Pancreas	7.74	2.0	7.88	2.1	0.031 8	0.39	0.019 8	0.82	0.004 63	0.80	0.002 92	1.2
Adrenals	12.9	3.3	11.1	3.0	0.059 1	0.71	0.030 2	1.2	0	NA	0	NA
Uterus	–	–	3.81	1.0	–	–	0.018 2	0.73	–	–	0.004 33	2.0
Muscle	3.82	0.96	3.79	1.0	0.014 6	0.18	0.008 51	0.34	0.002 69	0.46	0.002 02	0.86
Bladder	20.9	5.5	21.8	5.0	0.403	5.0	0.055 8	2.0	0	NA	0	NA
Bone	1.65	0.42	1.61	0.44	0.015 9	0.20	0.010 7	0.44	0.003 34	0.58	0.002 84	1.2

conc: concentration; eq: equivalents; GI: gastrointestinal; *Source*: Himmelstein (2015)
NA: not applicable; RBC: red blood cells; T:P: tissue to plasma ratio

[a] Mean of four rats/sex per dose.

[b] Single low dose of 10 mg/kg bw.

[c] Time to reach maximum concentration (T_{max}).

Excretion

The major part of excretion occurred within the first 12 or at least 24 hours and was substantially complete by 48 hours post dose administration as demonstrated by the mass balance experiments (see Table 2); by the short tissue distribution half-lives of 1.4–5.4 hours; and by the high area under the concentration–time curve (AUC) partial area percentages (82–96%) over this interval of radiolabelled material. The remaining residues (4–18%) were eliminated with an average elimination half-life of 46 ± 7 hours. A similar biphasic elimination was observed for all three radiolabels without significant sex differences. The overall mass balance was also similar across sexes and radiolabels. The mean percentage of administered dose recovered from all groups by 168 hours post dose administration ranged from 89.0 ± 5.1% to 102 ± 3%. Recovery ranged from 43.3% to 52.6% in faeces and 39.9% to 47.8% in urine across all low-dose treatment groups. The recovery in faeces increased slightly after administration of high dose, which was in line with the lower absorption. The mean percentage detected in faeces ranged from 56.4% to 68.8%, representing a 1.1- to 1.4-fold increase compared to the low dose. Concomitantly, the ^{14}C recovery in urine declined slightly. The mean values ranged from 25.0% to 35.3% (i.e. a 1.3- to 1.7-fold decrease relative to the respective low dose groups for each radiolabel). Biliary elimination was also significant (see the "Absorption" section above) (Himmelstein, 2015).

Repeated-dose study

Kinetics of pyridine-2,6-^{14}C-triflumezopyrim were studied in female Sprague Dawley rats during and following multiple oral administration. The dose rate was 10 mg/kg bw per day for 14 subsequent days by gavage (days 0–13) followed by a 5-day depuration phase. The radiolabelled test substance was adjusted to a measured specific activity of 4.67 µCi/mg (0.17 MBq/mg) with non-radiolabelled triflumezopyrim and suspended in 0.1% Tween 80 in 0.5% methylcellulose as the dose vehicle. The approximate dose volume was 2 mL/kg bw. The mean daily radioactive dose was 10.2 µCi/animal, corresponding to 0.3774 MBq/animal (n = 16). Thus, in contrast to many older studies of this type, the animals received a radiolabelled dose on all days of treatment and not only once on the final day.

Absorption

Plasma and tissue ^{14}C concentrations were measured to assess the extent of absorption at 30 minutes after the last (14th) dose administration. The maximum plasma concentration (C_{max}) (µg equiv./g) at that time point ranked as follows: liver (11.1) > kidney (9.91) > plasma (3.20) ~ uterus (3.17) > whole blood (2.94) ~ RBCs (2.69) > muscle (2.40) > fat (1.20). Tissue concentrations were similar to C_{max} values (0.5 hours) after a single dose. For example, plasma concentrations were 3.75 ± 1.10 at 0.5 hours following the single dose and 3.20 ± 1.30 µg eq./g in the repeated-dose study.

Distribution

Radioactivity (^{14}C) was determined in selected organs/tissues at 0.5, 8, 24 and 120 hours after the last (14th) dose administration to determine if any difference occurred relative to single gavage administration. A comprehensive comparison of ^{14}C residues in 21 different tissues was made at 120 hours after the final dose. The profile of distribution observed for tissue concentrations, tissue to plasma concentration ratios, and per cent of dose were consistent with those observed in the single-dose study even though there were some notable differences. For instance, mean plasma concentration had increased 6-fold (0.133 µg equiv./g) compared to the mean concentration (0.0221 µg equiv./g) at 24 hours after single-dose administration, suggesting a greater retention of ^{14}C residues after multiple dosing. This could be due to loading of peripheral tissues; there might also be differences in absorption and intestinal transit times in non-fasted animals in the multiple-dose study. Similar increases in tissue concentrations were apparent for other selected tissues at 24 hours after the final dose relative to the single dose experiment. These increases ranged from 3.6-fold for kidneys to 8.7-fold for RBCs.

As in the single-dose experiments, the concentration in plasma and the selected tissues showed a rapid rate of decline between 0 and 24 hours after the last dose, followed by transition to a slower terminal elimination phase between 24 and 120 hours. Again, the liver and kidney were the tissues with the highest measured concentrations. In line with that, tissue to plasma ratios were highest in those organs that had values ranging from 2:1 to 5:1. There was no evidence of accumulation in fat. At termination, overall tissue burden was very low in this study as well. The organ concentrations ranged from 0.005 48% (uterus) to 0.733% (muscle) of the administered dose 0.5 hours after the last multiple-dose administration and subsequently declined to less than the limit of quantification (LOQ) (fat), 0.000 06% (uterus) and 0.008 5% (muscle) of the total administered dose by 120 hours after the last dose.

Excretion

The cumulative excretion of ^{14}C residues in faeces and urine was determined from the first dose until five days after the final dose. The overall mean material balance was 90.7% of the cumulative dose administered (Table 4). The dose was excreted in the faeces (46.4%) and urine (38.7%) in similar proportions. Total excretion in faeces and urine combined accounted for 85.1%. Retention of ^{14}C residues in tissues was low (0.47%). On balance, the pattern of recovery was very similar to that in the single-dose study.

Table 4. Mass balance in repeated-dose experiment with [pyridine-2,6-^{14}C]triflumezopyrim in female rats

Matrix	Mean % of dose[a]	SD
Urine	38.7	3.02
Faeces	46.4	5.59
Cage wash	4.97	0.507
Carcass	0.470	0.137
Total	90.7	4.38

bw: body weight; SD: standard deviation

[a] Low dose of 10 mg/kg bw per day.

In plasma, the observed terminal elimination half-life (48 h) after multiple-dose administration was comparable to the mean plasma elimination halflives (35–54 h) in the single-dose study measured for all three radiolabels ([pyridine-2,6-^{14}C], [fused pyrimidine-3-^{14}C] or [methylene-^{14}C]).The exception was the high-dose [methylene-^{14}C] label, which showed a shorter terminal elimination half-life of between 12 and 14 hours. Again, elimination was biphasic showing a rapid decline between 0.5 and 24 hours after the last dose administration which transitioned to a slightly slower rate of elimination between 24 and 120 hours (Himmelstein, 2016).

1.2 Biotransformation

The proposed metabolic pathway for triflumezopyrim is based on metabolites identified in faeces, urine, bile and plasma obtained in the single-dose study (Himmelstein, 2015) and in faeces, urine and plasma from the repeated-dose study (Himmelstein, 2016).

A significant portion of the low and high dose was excreted in faeces and urine metabolically unchanged as intact triflumezopyrim. Following administration of the low dose, the unchanged parent accounted for 31–41% of the amount excreted in urine and for 12–18% in faeces. Following administration of the high dose, 20–26% of urinary excretion was in the form of triflumezopyrim, as was 25–37% of the faecal excretion. Parent triflumezopyrim was also the major component in plasma.

The metabolism of triflumezopyrim was similar across dose, sex and the three radiolabels. The proposed metabolic pathway included a series of hydroxylation, oxidation, hydrolysis, decarboxylation and rearrangement reactions at various locations throughout the molecule. The most significant reaction was hydroxylation of the triflumezopyrim trifluoromethyl phenyl ring to form IN-R6U70, which was found in faeces (20–27% of the administered low dose and 14–26% of the administered high dose) and to a lesser extent in urine (0.7–2.2% of the administered dose). Sulfation and glucuronidation of IN-R6U70 were also observed. In bile, the sulfate conjugate of IN-R6U70 appeared to be the most dominant component.

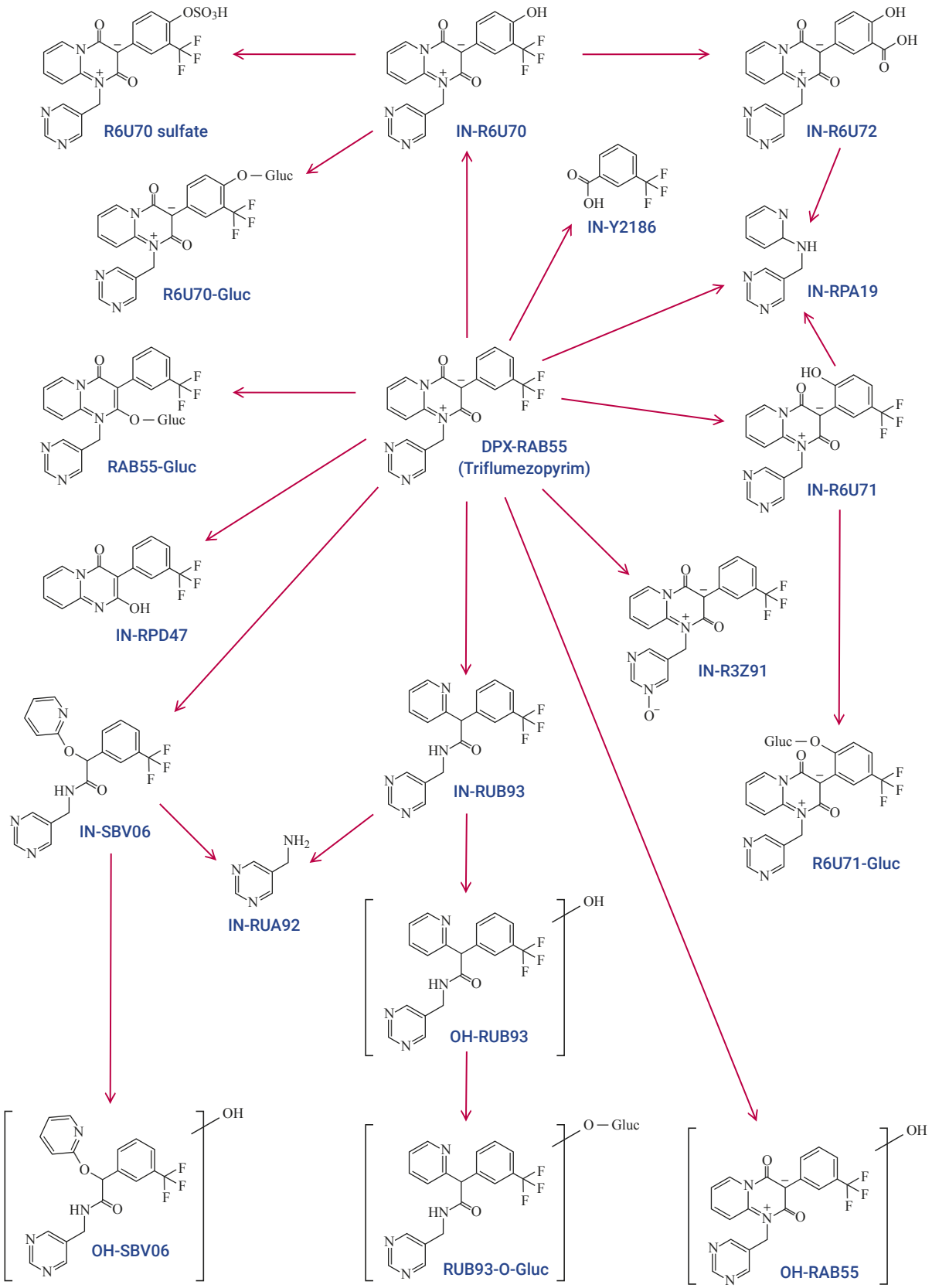
Further metabolic reactions on triflumezopyrim were proposed but are expected to be of minor importance. They include hydroxylation (IN-R6U71 and four isomers of OH-RAB55, hydroxylated parent), *N*-oxidation (IN-R3Z91), oxidative hydrolysis (IN-RPD47 and IN-Y2186), sequential oxidative hydrolysis, decarboxylation and rearrangement (IN-SBV06 and IN-RUB93) and hydrolytic cleavage (IN-RPA19). IN-RPA19 may also be formed by hydrolytic cleavage of IN-R6U70, IN-R6U71 or IN-R6U72. In addition, hydrolytic defluorination of IN-R6U70 (IN-R6U72), amide hydrolysis of IN-SBV06 or IN-RUB93 (IN-RUA92), hydroxylation of IN-SBV06 and IN-RUB93, and glucuronidation of OH-RAB55, INR6U71 and OH-RUB93 were proposed to occur.

With very few exceptions, these additional metabolites were present at percentages of less than 1% in the different matrices and never exceeded 4%.

The only six metabolites of [pyridine-2,6-^{14}C]triflumezopyrim observed in the repeated-dose study (IN-R6U70, IN-R6U72, IN-R3Z91, IN-RPA19, IN-RPD47 and one OH-RAB55 isomer) had all been previously identified in the single-dose radioactive study. The material balance and profile of metabolites were consistent with what was observed in the single-dose study. However, not all metabolites in the metabolism pathway were detected after repeated-dose administration. This was attributed to the lower specific activity of the dose (4.67 µCi/mg; 0.17 MBq/mg) administered in the multiple-dose study, approximately 3.7-fold lower than the adjusted specific activity (17.2 µCi/mg; 0.64 MBq/mg; [pyridine-2,6-^{14}C] label) used in the single-dose study, thus reducing the overall sensitivity for identification and quantitation of metabolites accounting for approximately <1% of the dose.

The proposed metabolic pathway for triflumezopyrim in the rat is depicted on the following page in Fig. 2.

Figure 2. Proposed metabolic pathway of triflumezopyrim (DPX-RAB55) in the rat



2. Toxicological studies

The toxicological studies were performed with different (in total seven) batches of triflumezopyrim. These batches and their purities are reported for the individual studies. For some end-points, studies with different batches are available and, sometimes, partly contradictory results were obtained. An overview of the batches used is given in Appendix 2, as well as examples of deviations in the results when another batch was applied but the same end-point was investigated.

2.1 Acute toxicity

Summaries of acute toxicity, irritation and skin sensitization studies are presented in Table 5. Two different batches were used.

Table 5. Summary of acute toxicity, skin and eye irritation and skin sensitization studies with triflumezopyrim

Study type and route	Species	Batch no. (purity)	Result	Reference
Acute oral	Rat	RAB55-037 (99.4%)	LD_{50} > 5000 mg/kg bw in males; 4930 mg/kg bw in females	Carpenter (2013a)
Acute oral	Rat (females only)	RAB55-037 (99.4%)	LD_{50} > 5000 mg/kg bw	Merrill (2014a)
Acute oral	Rat (females only)	RAB55-104 (97.04%)	LD_{50} > 5000 mg/kg bw	Fallers (2014)
Acute dermal	Rat	RAB55-037 (99.4%)	LD_{50} > 5000 mg/kg bw	Lowe (2012a)
Acute inhalation	Rat	RAB55-037 (99.4%)	LC_{50} > 5 mg/L air (four-hour nose-only exposure)	Lowe (2012b)
Skin irritation	Rabbit	RAB55-037 (99.4%)	Non-irritant	Lowe (2012c)
Skin irritation	Rabbit	RAB55-104 (97.04%)	Non-irritant	Merrill (2014b)
Eye irritation	Rabbit	RAB55-037 (99.4%)	Very mild irritation	Lowe (2012d)
Eye irritation	Rabbit	RAB55-104 (97.04%)	Non-irritant	Merrill (2014c)
Skin sensitization (Magnusson–Kligman test)	Guinea pig	RAB55-037 (99.4%)	Negative	Lowe (2012e)
Skin sensitization (Magnusson–Kligman test)	Guinea pig	RAB55-104 (97.04%)	Negative	Merrill (2014d)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

(a) Lethal doses

Three oral studies, a dermal and an inhalation study were available. They show that triflumezopyrim is of low acute toxicity via all three routes.

In a study performed according to a Chinese guideline, a single oral dose of triflumezopyrim (batch no. RAB55-037, purity 99.4%) at 5000 mg/kg bw was administered by gavage to fasted Crl:CD(SD) rats (5/sex). The test item was suspended in a 0.1% solution of Tween 80 in 0.5% aqueous methylcellulose. Since deaths occurred in female but not male rats, three additional groups (5 fasted female rats/group) were dosed at 2500, 3750 or 4375 mg/kg bw. (Further testing of male rats was considered not necessary.) Because of the volume administered (20 mL/kg bw), the rats receiving 5000 or 3750 mg/kg bw were dosed twice, at least 15 minutes apart. All animals on study were observed for

clinical signs of toxicity, body weight effects and mortality for 14 or 15 days post dosing. Following unscheduled or scheduled deaths, the rats were necropsied and examined for gross pathological findings.

Four out of the five female rats receiving 5000 mg/kg bw were found dead or were euthanized for humane reasons at latest on day 2 post dosing. No other mortalities were observed. In males, only minor signs of toxicity, apart from yellow staining of the bedding, were observed. In contrast, high-dose females exhibited spasms, ataxia, decreased muscle tone, eyelid ptosis, high or low posture and slower breathing and were cold to the touch. In 3/5 males and the surviving high-dose female, body weight loss was noted. In the additional groups of female rats receiving lower doses of triflumezopyrim, clinical signs were confined to high posture on the day of dosing, occasional and transient body weight losses and yellow staining of bedding and faeces. Gross findings that could be attributed to test substance administration were confined to the females that died or were prematurely killed following exposure to 5000 mg/kg bw and included stomach ulcer or erosion and staining about the face and eyes.

The oral LD_{50} in rats was greater than 5000 mg/kg bw in male rats and was calculated to be 4930 mg/kg bw in female rats (Carpenter, 2013a).

Taking into account the outcome of the Carpenter (2013a) study, a single oral dose of the same batch of triflumezopyrim (batch no. RAB55-037, purity 99.4%), suspended in a 0.1% solution of Tween 80 in 0.5% aqueous methylcellulose, was administered by gavage to a fasted female rat at a dose of 4390 mg/kg bw. Since this animal survived and did not exhibit either clinical signs or body weight loss over the 14-day post-dosing observation period, three additional female Sprague Dawley rats were administered a single dose of 5000 mg/kg bw, following an up-and-down procedure. These animals were observed for clinical signs of toxicity, body weight effects and mortality for 14 days after dosing and were examined grossly at necropsy for evidence of organ or tissue damage.

No mortalities were observed. Reduced faecal volume was the only finding in all animals receiving 5000 mg/kg bw but disappeared by day 3. No treatment-related effects on body weight were noted and no gross lesions were observed at necropsy.

The oral LD_{50} in female rats was greater than 5000 mg/kg bw (Merrill, 2014a).

A single oral dose of triflumezopyrim, suspended in a 0.1% solution of Tween 80 in 0.5% aqueous methylcellulose, was administered by gavage to three fasted female Crl:CD(SD) rats at a dose of 5000 mg/kg bw. A different batch of triflumezopyrim, RAB55-104 (lot no. SG0314011, purity 97.04%) was used. The animals were observed for clinical signs of toxicity, body weight effects and mortality for 14 days after dosing. All animals were necropsied and gross examination made for evidence of organ or tissue damage.

No mortalities were observed. The only clinical sign of toxicity observed was staining of the bedding with the yellow colour of the test substance for all animals between days 2 and 4. No test substance-related body weight effects were noted. No test substance-related gross lesions were observed at necropsy.

The oral LD_{50} in rats was greater than 5000 mg/kg bw for female rats (Fallers, 2014).

In a limit test via the dermal route, a single dose of triflumezopyrim (batch no. RAB55-037, purity 99.4%) was applied to the shaved, intact skin of Sprague Dawley rats (5/sex) at a dose of 5000 mg/kg bw. The test item had been moistened with deionized water to form a dry paste before administration. The application site covered approximately 10% of each animal's body surface area and was semi-occluded for 24 hours, after which the test substance was removed. The rats were observed for clinical signs, body weight effects, dermal effects and mortality for 14 days post application and, following scheduled kill, were examined for gross pathological changes.

No mortalities were observed. All animals survived, appeared active and healthy, and gained body weight during the study. Apart from mechanical damage due to unwrapping around the dose site of all rats between days 1 and 3, there was no dermal irritation recorded for any animal over the 14-day observation period. No gross abnormalities were noted in any of the animals at necropsy.

The dermal LD_{50} in rats was > 5000 mg/kg bw (Lowe, 2012a).

In an acute inhalation study, Sprague Dawley rats (5/sex) were exposed, nose-only, for a four-hour period to triflumezopyrim (batch no. RAB55-037, purity 99.4%) suspended as an aerosol in air. The actual (gravimetric) concentration was 5.04 ± 0.16 mg/L (mass median aerodynamic diameter [MMAD] 2.8 µm, geometric standard deviation [GSD] 2.17). Animals were observed for clinical signs of toxicity, body weight effects and mortality for up to 14 days after treatment and were examined for gross pathological changes.

No mortalities were observed. The clinical sign of toxicity most often observed in male and female rats was irregular respiration, but all animals appeared normal by day 3. No test substance-related gross lesions were observed at necropsy.

The inhalation median lethal concentration (LC_{50}) in rats was > 5 mg/L (Lowe, 2012b).

(b) Dermal irritation

Triflumezopyrim (batch no. RAB55-037, purity 99.4%) was applied as 75% dry paste to give a single dermal dose of 0.5 g to the shaved intact skin of three young adult New Zealand White rabbits (two males and one female). The rabbits were exposed to the test substance for four hours, after which it was removed. The exposure was commenced in the female rabbit and, after no signs of irritation or corrosion had been observed, continued in the two males. Test sites were evaluated using Draize criteria for signs of dermal irritation immediately after test substance removal (only in the female animal) and after between 30 and 60 minutes and 24, 48 and 72 hours after the triflumezopyrim was removed.

No signs of dermal irritation were noted in any animal at any time point of reading during the study (Lowe, 2012c).

Under conditions similar to those in the Lowe (2012c) study, another batch of triflumezopyrim, RAB55-104 (purity 97.04%) was applied as a dry paste (70% weight per weight [w/w] mixture in water) to the shaved intact skin of three young adult New Zealand White rabbits. The total dose was also 0.5 g. In this case, one male and two females were employed and the male rabbit was exposed first. The rabbits were exposed to the test substance for four hours, after which it was removed. Test sites were evaluated using Draize criteria for signs of dermal irritation immediately after test substance removal (male animal only) and after between 30 and 60 minutes and 24, 48 and 72 hours after the test substance was removed in all three animals.

There was no dermal irritation noted for any treated site in any animal during the study (Merrill, 2014b).

(c) Ocular irritation

A single dose of 0.07 g (equivalent to 0.1 mL) of triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered, under ocular and systemic analgesia, into the conjunctival sac of the right eye of one female young adult New Zealand White rabbit. No significant irritation was seen. Two additional female rabbits were tested in the same way to confirm the result. The eyes of all test animals were not rinsed after treatment. The conjunctiva, iris and cornea of each treated eye were evaluated for evidence of irritation approximately 1, 24, 48 and 72 hours after administration of the test substance.

The test substance produced corneal opacity (score of 1) in one rabbit; conjunctival redness (score of 1) and discharge (scores of 1 and/or 2) in all animals; and chemosis (scores of 1 and/or 2) in two rabbits. Signs of irritation completely resolved in one rabbit by 24 hours and by 48 hours in the remaining two rabbits.

As a result of the weak and transient eye irritation, triflumezopyrim was characterized as a very mild eye irritant (Lowe, 2012d).

A single dose of 0.08 g (equivalent to 0.1 mL) of triflumezopyrim was administered, under ocular and systemic analgesia, into the conjunctival sac of the right eye of one female young adult New Zealand White rabbit. A different batch of triflumezopyrim, RAB55-104 (purity 97.04%) was used. No significant irritation was seen. Two additional female rabbits were tested in the same way to

confirm the result. The eyes of all test animals remained unwashed after treatment. The conjunctiva, iris and cornea of each treated eye were evaluated for evidence of irritation approximately 1, 24, 48 and 72 hours after administration of the test substance.

Conjunctival redness (score of 1) was observed in all rabbits and slight discharge (score of 1) in one rabbit. These minor signs of irritation were noted only one hour after treatment and had resolved completely in all rabbits by 24 hours. In contrast to the Lowe (2012d) study, neither corneal opacity nor chemosis were observed, that is, triflumezopyrim proved almost not irritating to the eyes (Merrill, 2014c).

(d) Dermal sensitization

The dermal sensitization potential of triflumezopyrim (batch no. RAB55-037, purity 99.4%) was evaluated using a Magnusson–Kligman maximization test in male Hartley albino guinea pigs. Preliminary irritation testing was performed on 12 animals to determine appropriate concentrations of test substance to be used for both the intradermal and topical induction and the topical challenge. Based on these results, 20 animals were intradermally induced on day 1 with pairs of injections (six in total) of the test substance in a 2% weight per volume (w/v) solution of carboxymethylcellulose (CMC) in distilled water (1% w/w), the test substance (1% w/w) combined with an emulsion of Freund's Complete Adjuvant, as well as an emulsion of Freund's Complete Adjuvant alone. Approximately one week later, animals were topically induced with a 65% w/w mixture of the test substance in a 2% w/v solution of CMC in distilled water. Due to a lack of irritation produced during preliminary testing, all animals received a pretreatment of sodium lauryl sulfate prior to test substance application. Approximately two weeks later, animals were challenged with 0.5 mL of a 65% w/w mixture in a 2% w/v solution of CMC in distilled water and 0.5 mL of a 22% w/w mixture of triflumezopyrim in a 2% w/v solution of CMC in distilled water at two separate test sites (corresponding to the highest non-irritating concentration and 33% of this highest non-irritating concentration). At a third site, 0.5 mL of a 2% w/v solution of CMC in distilled water was applied. All challenge treatments were made under occlusion. Approximately 24 and 48 hours after the challenge, the test sites were evaluated for signs of a dermal reaction suggestive of sensitization. Throughout the study, animals were also evaluated for body weight development and clinical signs.

No test substance-related dermal irritation scores greater than 2 (moderate erythema) were observed during the induction phase. No test substance-related dermal irritation scores greater than 0.5 (very faint erythema) were observed at 24 hours for the animals challenged with the 65% or 22% w/w mixtures. Nno signs of erythema were visible after 48 hours in all six animals. Likewise, no test substance-related dermal irritation scores greater than 0.5 (very faint erythema) were noted in the negative control animals. Only two animals were affected, and no signs were visible after 48 hours. Appropriate historical control data from studies in the laboratory using alpha-hexylcinnamaldehyde as positive control demonstrated a positive response.

Triflumezopyrim did not produce a skin sensitization response in guinea pigs (Lowe, 2012e).

The dermal sensitization potential of another batch of triflumezopyrim, RAB55-104 (purity 97.04%), was also evaluated using a Magnusson–Kligman maximization test in male Hartley albino guinea pigs. The study was similar to Lowe (2012e), but used another vehicle and slightly different applied concentrations of the test substance. Again, preliminary irritation testing was performed on 12 animals to determine appropriate concentrations of test substance to be used for both the intradermal and topical induction and the topical challenge. Based on these results, 20 animals were intradermally induced on day 1 with pairs of injections (six in total) of a 1:1 emulsion of water and Freund's Complete Adjuvant, a 1% (w/w) test article suspension in polyethylene glycol (PEG, MW 400) and 1% (w/w) test article suspension in a 1:1 emulsion of Freund's Complete Adjuvant and water. Approximately one week later, the animals were topically induced with a 53% (w/w) suspension of test substance in polyethylene glycol. Two weeks later, animals were challenged with 0.5 mL of a 70% (w/w) suspension, which had proved to be the highest non-irritating concentration, and a 23% (w/w) suspension of test substance in polyethylene glycol, under occlusion. Approximately 24 and 48 hours after the challenge, the test sites were evaluated for signs of a dermal response suggesting sensitization. Animals were also evaluated for body weight and clinical signs.

No test substance-related dermal irritation scores greater than 2 (mild) were observed during the induction phase. No test substance-related dermal irritation scores greater than 0.5 (very faint erythema) were observed at 24 and/or 48 hours for the animals challenged with the 70% or 23% w/w mixture. In total, not more than four animals were affected. No responses were noted in the negative control (vehicle) animals. Appropriate data from a validation study in the laboratory using alpha-hexylcinnamaldehyde demonstrated a clear positive response.

Triflumezopyrim was not sensitizing to the skin in guinea pigs under the conditions of the study (Merrill, 2014d).

2.2 Short-term studies of toxicity

(a) Oral administration

Mouse

In a non-GLP-compliant subacute feeding study, triflumezopyrim (batch no. RAB55-028, purity: 99%) was administered to Crl:CD1(ICR) mice (10/sex per dose level) at dietary concentrations of 0, 200, 800, 2500 or 7000 ppm. According to the study report, the actual duration of treatment was 33 and 34 days in males and females, respectively. The calculated mean daily intakes were 0, 34, 129, 416 and 1104 mg/kg bw per day for males and 0, 41, 161, 504 and 1343 mg/kg bw per day for females. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology including coagulation, clinical chemistry, urine analysis, selected the following biochemical parameters in liver: cytochrome P450 (CYP), uridine diphosphoglucuronosyltransferase (UDPGT, formerly known as UDP), and beta-oxidation. Also evaluated were gross pathology, organ weights and histopathology.

There were no test substance-related deaths or clinical signs of toxicity at any dietary concentration either in males or in females. Furthermore, there were no test substance-related effects on body weights, body weight gain, feed consumption or feed efficiency.

Haematological examination revealed an impact on RBC parameters in males and females fed 7000 ppm. Mean RBC count, haemoglobin and haematocrit were lower in these groups, reduced to 88–91% of control values (with variable statistical significance) in males and to 91–94% of control values in females (not statistically significant). In females, however, these changes were associated with a slight but significant increase in absolute reticulocyte count by 39% as compared to the control group, suggesting a regenerative response. In line with these haematological findings, a small increase in the incidence of minimal to mild splenic extramedullary haematopoiesis was observed in the top-dose males: extramedullary haematopoiesis was noted in 1/10, 2/10, 1/10, 3/10 and 4/10 male mice at 0, 200, 800, 2500 and 7000 ppm, respectively. This effect correlated with increased absolute and relative splenic weights. However, higher mean spleen weights were also observed in males at 2500 ppm and females at 7000 ppm, even though extramedullary haematopoiesis was not reported in these groups. On balance, a treatment-related and potentially adverse impact on RBCs was confined to the top dose level in both sexes.

The liver was found to be another target organ of triflumezopyrim in this study. At scheduled kill, serum cholesterol concentrations were significantly elevated in male and female mice fed 7000 ppm, reaching 161% and 155% of the respective control means. At necropsy, a significant and dose-related increase in mean absolute and relative liver weights was observed in both males and females at 2500 and 7000 ppm. Gross liver changes were confined to dark discoloration of this organ in four males at 7000 ppm. An increased incidence of hepatocellular hypertrophy was observed in males and females at 7000 ppm and in males at 2500 ppm, corroborating the higher organ weight in these groups. In males at 2500 ppm, the hypertrophy was graded minimal (grade 1 of 4), whereas in males and females at 7000 ppm it was graded moderate (grade 3 of 4).

In addition, triflumezopyrim caused statistically significant, though in a number of cases relatively modest, increases in the following hepatic biochemical parameters: peroxisomal β-oxidation activity (7000 ppm in females), total microsomal CYP content (2500 and 7000 ppm, males and females), immunoreactive levels of CYP1A2 (2500 and 7000 ppm, males and females), CYP2B1/2 (7000 ppm, males), CYP2E1 (2500 and 7000 ppm, males; all dose levels in females), and CYP4A1/2/3 (2500 and 7000 ppm, males and females). These alterations in the levels

of microsomal liver enzymes are not necessarily adverse but rather suggest a physiological response of the organism to xenobiotics. In contrast, there were no effects on UDPGT activity (*p*-nitrophenol as substrate) attributable to test substance exposure in either sex.

A decrease in accessory sex organ weights was observed in males at 2500 and 7000 ppm, but there were no correlative microscopic changes, and there was no confirmation of this effect in other longer studies in mice (although not the same batches of test substance were used).

In summary, the liver effects in the study were most likely treatment related, but in the absence of more severe histological lesions, were considered adaptive rather than adverse. Potential effects of concern were haematological changes and increased cholesterol levels, even though the latter was not associated with a change in triglyceride levels.

The sponsor considered the no-observed-adverse-effect level (NOAEL) to be the highest dose level of 7000 ppm. However, the Meeting considered the haematological effects and the increase in cholesterol as potentially adverse, and determined the NOAEL to be 2500 ppm (equal to 416 and 504 mg/kg bw per day in males and females, respectively) (Anand, 2015).

In a similar but longer feeding study, triflumezopyrim (batch no. RAB55-031, purity 98.8%) was administered to male and female Crl:CD1®(ICR) mice (10/sex per dose) for 13 weeks at the same concentrations of 0, 200, 800, 2500 or 7000 ppm. The calculated mean daily intakes were 0, 31.4, 125.4, 416.7 and 1127.8 mg/kg bw per day for males and 0, 44.1, 177.3, 476.2 and 1526 mg/kg bw per day for females, respectively. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology, clinical chemistry, ophthalmology, organ weights and gross and microscopic pathology. A full range of tissues was histologically examined in the control and top dose groups, whereas examinations in the low, intermediate low and intermediate high dose groups were confined to liver, kidneys and all gross lesions.

Four additional male and female mice per dose level were employed for sampling blood for toxicokinetic investigations. At scheduled kill, 0.5 mL blood was taken by cardiac puncture under isoflurane anaesthesia and shipped to the sponsor. No information is available on what use was made of these samples.

No test substance-related clinical signs of toxicity were observed and there were no test substance-related unscheduled deaths at any concentration. One male mouse in the control group was euthanized in extremis on day 24 and one female at 200 ppm was found dead on day 76. The deaths were considered unrelated to test substance administration. No cause of death could be determined microscopically for either animal.

There were no test substance-related effects on body weights, body weight gains, feed consumption or feed efficiency. No test substance-related ophthalmological changes were observed for any dietary concentration in either males or females.

There were no test substance-related differences in haematological parameters in male or female mice, that is, the effects on RBC parameters in the subacute mouse study (Anand, 2015) were not reproduced. Mice at 7000 ppm had a significant decrease in leukocytes, but this finding was not considered toxicologically relevant, as it was of small magnitude and values remained within expected and historical ranges.

There were no adverse, test substance-related differences in clinical chemistry parameters in male or female mice. Male mice at 7000 ppm had a decrease in total plasma bilirubin, which, in contrast to an increase, is generally not considered adverse.

No test substance-related gross lesions were observed at necropsy.

Absolute and relative liver weights were significantly increased in males at 2500 ppm and males and females at 7000 ppm, and were most probably linked to the minimal hepatocellular hypertrophy at the same dose levels. Hypertrophy was observed in 6/10 males at 2500 ppm and 7/10 males at 7000 ppm, but not in the control or lower dose levels. In females, hypertrophy was detected in two mice at 7000 ppm only. When stimulation of microsomal liver enzymes, as demonstrated by Anand (2015), is taken into consideration, the liver findings were considered adaptive rather than adverse.

Absolute and relative adrenal gland weights were higher in males and females at 7000 ppm, but the difference from the control groups was not statistically significant. It is also questionable whether there was a histological correlate. The only finding in adrenal glands was minimal subcapsular hyperplasia in 3/10 males at 7000 ppm.

Other microscopic findings comprised an increased incidence or more pronounced severity of cysts in the kidneys of males and females at 7000 ppm and more frequent lymphoid depletion in the thymus of males at 7000 ppm. However, statistical significance was not achieved and such lesions are common in mice of this strain and age. Unusually for a study of this type, a malignant histiocytic sarcoma that had affected the liver, bone marrow and mandibular lymph node was detected in a female in the control group.

The study author proposed the highest tested dose of 7000 ppm (1127.8 and 1526 mg/kg bw per day for males and females, respectively) as the NOAEL because all the possibly treatment-related effects on liver and adrenals were not adverse. The Meeting shared this view with regard to the liver; however, uncertainty remained with regard to the adrenals. As such, the NOAEL was identified as 2500 ppm (416.7 and 476.2 mg/kg bw per day in males and females, respectively), the effects on the adrenals at the lowest-observed-adverse-effect level (LOAEL) being of equivocal toxicological significance (Papagiannis, 2015a).

Rat

In a non-GLP-compliant 14-day study, triflumezopyrim (batch no. RAB55-014, purity > 99%) was dissolved in 0.5% methylcellulose and 0.1% Tween 80 and administered to male and female Crl:CD(SD) rats by gavage at doses of 0, 25, 300 or 1000/600 mg/kg bw per day. For the main segment of the study, 5 rats/sex per dose were employed. An additional 3 rats/sex per dose were included in each treatment group to provide samples for toxicokinetic evaluations (blood and fat tissue); these rats were also administered triflumezopyrim for 14 days. Due to excessive weight loss during the first four days of dosing, the high dose was decreased from 1000 to 600 mg/kg bw per day on day 5. An additional group of 5 rats/sex was designated for a genetic toxicity component (i.e. micronucleus assay in blood; see also section 2.4b) and were dosed only once, on day 12, with triflumezopyrim at 2000 mg/kg bw.

The rats were evaluated for mortality, clinical signs, body weight and body weight gain, haematological (including coagulation) parameters, blood and urine clinical chemistry parameters and organ weights. In addition, thyroid hormones triiodothyronine (T_3) and tetraiodothyronine (T_4; thyroxine) in blood were measured. All rats underwent gross and histopathology. Blood and fat tissue samples from the rats in the toxicokinetic group were used for analysis of routine pharmacokinetic parameters such as T_{max} and AUC. Genotoxicity was investigated by means of flow cytometrical evaluation of reticulocytes in peripheral blood for micronuclei. This analysis was performed in all control and high-dose (1000/600 mg/kg bw per day) groups and in the group that had received 2000 mg/kg bw as a single dose two days prior to scheduled kill.

Repeated administration of 1000 mg/kg bw clearly exceeded the maximum tolerated dose (MTD). Two females from this group were euthanized on day 8 due to excessive weight loss (> 20%) compared to their weight on day 0, despite lowering the dose on day 5. All other high-dose rats survived to scheduled kill, but there were clear test substance-related effects on body weights, body weight gains and clinical signs in male and female rats at this dose level. Both males and females at 1000 mg/kg bw per day lost weight; after the dose was reduced to 600 mg/kg bw per day, the animals in this group started to gain weight, and body weight gain was comparable to the control group over most of the rest of the study. However, due to the early body weight loss, final body weights (on day 15) and overall body weight gains (on days 1–15) in the high-dose group were less than controls (variable statistical significance). Adverse clinical observations in the animals at 1000/600 mg/kg bw per day occurred between days 2 and 9 and included abnormal gait, dark eye, decreased muscle tone, high posture, stained fur/skin (females only) and yellow-stained bedding/cage.

Haematological, clinical chemistry and urine analysis parameters were not affected, but there was a statistically significant reduction in T_4 in high-dose males. Although not statistically significant, T_4 concentrations were also lower in both sexes at the mid dose as well as in females at the high dose. T_3 levels were also statistically nonsignificantly lower in females at 300 mg/kg bw and in both sexes

at 1000/600 mg/kg bw per day compared to control and the low-dose group values (Tables 6 and 7). Histopathological examination did not reveal an increase in thyroid lesions. With regard to the decline in thyroid hormones, in the absence of microscopic changes, the excessive toxicity of the high dose should be taken into account; in addition, the observed reduction in thyroid hormones would be in line with an induction of hepatic microsomal enzymes. These were not measured in this study, but higher liver weights and hypertrophy, along with experimental data obtained in the 28-day feeding study in rats (Anand, 2013) as well as in mice (Anand, 2015), suggest that this hypothesis is not implausible.

Absolute and relative liver weights were significantly increased in both males and females at 300 and 1000/600 mg/kg bw per day (Table 6). These increases were accompanied by centrilobular liver cell hypertrophy. Although hypertrophy is often considered not adverse but rather an adaptive response, it should be noted that the increase in the high-dose group was appreciable. In contrast to the study author, these findings are regarded as potentially adverse. In addition, minimal to mild periportal vacuolation of the liver was observed only in the treated groups and the frequency was highest at 300 and 1000/600 mg/kg bw per day. On its own, this finding would not be considered adverse; along with the strong liver weight increase, it might point to a potentially adverse impact on the liver. No pathological findings in other organs could be attributed to triflumezopyrim administration.

The half-life of triflumezopyrim was three and four hours in the low-dose males and females, respectively. AUC and terminal half-life could not be determined for the 300 and 1000/600 mg/kg bw per day dose levels because triflumezopyrim was not at the terminal elimination phase by 24 hours.

There did not appear to be a difference in toxicokinetics between males and females at the 25 mg/kg bw per day dose level based on similar C_{max} and AUC values. Values for T_{max} were 1, 3 and 9 hours in male rats and 1, 2 and 7 hours in female rats at 25, 300 and 1000/600 mg/kg bw per day, respectively. Steady state was achieved by day 7. Triflumezopyrim did not appear to preferentially partition into the fat; fat to plasma ratios were generally below 1.0 at all dose levels in both male and female rats. These findings are in line with other ADME studies (Himmelstein, 2015, 2016).

Table 6. Body weight, thyroid hormone, liver weight and liver histopathology in a 14-day study with triflumezopyrim in male and female rats [a]

	Measure per dose level			
Parameter	**0 mg/kg bw per day**	**25 mg/kg bw per day**	**300 mg/kg bw per day**	**1000/600 mg/kg bw per day**
Males				
Mean body weight, day 15 (g)	357.7	368.6	358.5	324.5
Mean body weight gain, days 1–15 (g)	27.4	41.6	37.8	4.5
T_3 (ng/dL)	49.000	50.540	47.422	37.454
T_4 (µg/dL)	4.495	4.494	4.229	3.009
Absolute liver weight (g) [b]	10.345	10.723 (3.7%)	12.710* (22.9%)	13.899* (34.4%)
Relative liver weight [c]	2.893	2.946 (1.8%)	3.654* (26.3%)	4.614* (59.5%)
Liver histopathology [d]				
Hypertrophy, centrilobular	0	1	5	5
Minimal	0	1	5	5
Vacuolation, periportal	0	1	3	3
Minimal	0	1	3	2
Mild	0	0	0	1

Parameter	Measure per dose level			
	0 mg/kg bw per day	25 mg/kg bw per day	300 mg/kg bw per day	1000/600 mg/kg bw per day
Females				
Mean body weight, day 15 (g)	231.9	230.3	225.3	210.5
Mean body weight gain, days 1–15 (g)	11.4	9.4	8.3	−5.2
T_3 (ng/dL)	53.475	55.748	46.895	35.841
T_4 (µg/dL)	2.691	2.911	2.188	2.058
Absolute liver weight (g)[b]	6.570	6.746 (2.6%)	7.795* (18.6%)	9.842* (49.8%)
Relative liver weight[c]	2.825	2.947 (4.3%)	3.614* (27.9%)	4.824* (70.8%)
Liver histopathology[d]				
Hypertrophy, centrilobular	0	0	5	5
Minimal	0	0	5	2
Mild	0	0	0	3
Vacuolation, periportal	0	2	4	5
Minimal	0	2	4	3
Mild	0	0	0	2

bw: body weight; T3: triiodothyronine; T4: tetraiodothyronine (thyroxine); *: $P < 0.05$ (Dunnett test, two-sided)

[a] Mean of 5 rats/sex per dose.

[b] Results expressed as weight in g and, in parentheses, % change from the control value.

[c] Results expressed as %, and in parentheses, % change from the control value.

[d] Results expressed as number of animals with the finding.

Source: Nabb (2015a)

The micronucleus assay showed no evidence of genotoxicity in both male and female rats.

The NOAEL for males and females was 25 mg/kg bw per day, based on liver toxicity at 300 mg/kg bw per day. The study author had proposed to set the NOAEL at 300 mg/kg bw per day, based on reduced body weight and body weight gain and clinical signs of toxicity in males and females at the highest dose of 1000/600 mg/kg bw per day, and, apparently, regarding the liver effects as not adverse (Nabb, 2015a).

In a non-GLP-compliant 28-day feeding study, triflumezopyrim (batch no. RAB55-028, purity 99%) was administered to male and female Crl:CD(SD) rats (5/sex per dose) at concentrations of 0, 200, 800, 4000 or 20 000 ppm. Due to body weight loss, the highest concentration was reduced to 10 000 ppm from day 3 onwards. The overall mean daily intakes were 0, 17, 65, 309 and 653 mg/kg bw per day for males and 0, 16, 64, 317 and 627 mg/kg bw per day for females, respectively. The end-points evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology, coagulation, clinical chemistry, urine analysis, gross pathology, organ weights and histopathology. Furthermore, enzyme induction in liver tissue was investigated and thyroid hormones in blood were measured.

No test substance-related deaths or clinical signs were noted. Test substance-related, adverse effects on body weight, body weight gain, feed consumption and efficiency were noted in females at 20 000/10 000 ppm. In males, significant and dose-related reductions in body weight gain and feed consumption were observed at 800 ppm and higher concentrations. Lower mean body weight was apparent at 4000 and 20 000/10 000 ppm. The sponsor considered that the findings at 200 and 800 ppm were not adverse because of the inconsistent dose response, but this argument is unconvincing when the figures in Table 7 are taken into account.

Decreases in erythrocyte parameters (red blood cell count, haemoglobin, and haematocrit) were noted in male and female rats at 20 000/10 000 ppm and in females at 4000 ppm (Table 8). Reticulocyte counts did not significantly change. No adverse, test substance-related effects were observed on coagulation, clinical chemistry or urine analysis parameters.

Absolute and relative liver weights were increased in males and females at the two highest dose levels. Pathological examination found pale discoloration of the liver and hepatocellular hypertrophy in both sexes at 4000 ppm and higher. The latter finding, as well as the higher organ weight, are well in line with the results of special biochemical investigations, that is, an increase in total CYP content and, in particular, in the immunoreactive content of CYP1A2 and CYP2B1/2, in UDPGT activity (towards *p*-nitrophenol), and increased beta-oxidation rate. These changes were statistically significant at the highest dose level in males and at the two highest dose levels in females.

The only other possible test substance-related effects were noted in males at 4000 ppm and higher, and affected the accessory sex organs. The findings included small seminal vesicles and decreased fluid in the seminal vesicles and coagulating glands in 4/5 males at 20 000/10 000 ppm and a reduction in accessory sex organ weights also at 4000 ppm. The meaning and relevance of these findings is not clear. The sponsor considered that these findings were not adverse because there was no other microscopic effect except for the lower fluid content and the findings were not reproduced in the subsequent 90-day feeding study in rats at concentrations up to 6000 ppm.

In contrast to the 14-day gavage study in rats (Nabb, 2015a), there were no test substance–induced alterations in thyroid hormone (T_3, T_4 or thyroid-stimulating hormone [TSH]) levels in blood.

According to the study author, the NOAEL for males was 4000 ppm (309 mg/kg bw per day) based on the effects on body weight and nutritional and red cell mass parameters at 20 000/10 000 ppm and the NOAEL for females was 800 ppm (64 mg/kg bw per day) based on the effects on red cell mass parameters at 4000 ppm. However, based on the decreases in body weight gain and feed consumption in male rats, the overall NOAEL was identified at 200 ppm (17 mg/kg bw per day) (Anand, 2013).

Table 7. Body weights, body weight gain, feed consumption and feed efficiency in a 28-day feeding study with triflumezopyrim in rats

	Measure per dose level of triflumezopyrim				
Parameter	**0 ppm**	**200 ppm**	**800 ppm**	**4000 ppm**	**20 000/10 000 ppm**
Males					
Body weight, day 28 (g)	416.2	406.8	383.1	370.6*	303.4*
Body weight gain, days 0–28 (g)	201.9	193.5	172.6*	152.8*	87.8*
Feed consumption (g/animal per day), days 0–28	24.9	25.0	23.1** [b]	22.1** [b]	16.0** [b]
Feed efficiency, days 0–28 (g/day) [a]	0.289	0.277	0.267	0.246	0.195*
Females					
Body weight, day 28 (g)	243.7	238.8	238.1	230.5	201.7*
Body weight gain, days 0–28 (g)	69.5	67.9	63.3	55.7	29.1*
Feed consumption, days 0–28 (g)	17.0	16.5	16.0	15.7	10.8**
Feed efficiency, days 0–28 (g/day) [a]	0.146	0.147	0.141	0.128	0.096*

ppm: parts per million;

*: $P < 0.05$ (Dunnett two-sided test); **: $P < 0.05$ (Dunnett non-parametric two-sided test).

[a] Feed efficiency calculated as average weight gain / average amount feed consumed.

Source: Anand (2013)

Table 8. Haematological findings in a 28-day feeding study in rats with triflumezopyrim in rats

	Measure per dose level of triflumezopyrim				
Parameter	**0 ppm**	**200 ppm**	**800 ppm**	**4000 ppm**	**20 000/10 000 ppm**
Males					
RBC (× 10^6/µL)	7.80	7.65	7.59	7.74	7.16*
Haemoglobin (g/dL)	14.7	14.4	14.5	14.7	13.4*
Haematocrit (%)	45.6	44.1	44.1	43.8	40.1*
RBC distribution width (%)	12.0	11.8	12.0	11.9	13.1*
Reticulocytes (× 10^3/µL)	274.8	256.8	271.2	254.9	298.4
Females					
RBC (× 10^6/µL)	7.88	7.47	7.51	7.25*	7.24*
Haemoglobin (g/dL)	14.9	14.1	14.4	13.7*	13.5*
Haematocrit (%)	44.3	42.6	42.8	40.1*	39.5*
RBC distribution width (%)	10.9	11.3	11.5	11.7*	12.0*
Reticulocytes (× 10^3/µL)	196.4	256.7	250.5	232.8	203.9

ppm: parts per million; RBC: red blood cell; *: $P < 0.05$ (Dunnett test, two-sided)

Source: Anand (2013)

In the first 90-day feeding study, triflumezopyrim (batch no. RAB55-031, purity 98.8%) was administered to male and female CD®[Crl:CD(SD)] rats (16 /sex per group) at concentrations of 0, 100, 400, 1500 or 6000 ppm (corresponding to 0, 4.5, 18, 70 and 274 mg/kg bw per day for males and 0, 6.0, 23, 83 and 316 mg/kg bw per day for females, respectively). End-points under investigation included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology, clinical chemistry, urine analysis, ophthalmology, organ weights and gross and microscopic pathology. Designated animals in each group were evaluated for neurobehavioural functions (functional observational battery, locomotor activity) and neuropathology.

There were no effects of treatment on survival, clinical signs including ophthalmoscopic findings, neurological functions (in FOB and measurements of locomotor activity), haematology including coagulation or clinical chemistry. Urine analysis as well as gross pathological examination did not reveal any remarkable treatment-related findings. One female at 100 ppm died on day 88; the cause of death was undetermined but not attributable to triflumezopyrim when dose selection is considered.

Effects on body weight and feed consumption parameters were observed in male and female rats but were confined to the highest dose level of 6000 ppm. Body weight at the end of the study males and females at 6000 ppm were 11% and 6% below corresponding control group values, respectively, although achieving statistical significance only in males. Overall (weeks 1–13) mean body weight gain of male rats at 6000 ppm was 21% lower than in the control group. In females, the weight gain at this highest dose was only 6% lower than in the controls, and this difference was not statistically significant. Mean feed consumption of males and females at 6000 ppm was statistically significantly lower (by 11% and 16%, respectively) compared to control. Weekly feed efficiency values were generally similar to control, except for statistically significantly lower feed efficiency over week 1 in males and females at 6000 ppm. These findings suggest a lower weight gain due to reduced feed consumption. The effects in males at lower dose levels (800/4000 ppm) in a previous 28-day study (Anand, 2013) with another batch of triflumezopyrim were not reproducible when other animals were treated for a longer time. What was confirmed, in contrast, was the higher susceptibility of male rats to alterations of nutritional parameters.

Absolute and relative liver weights were increased in males and females at 6000 ppm but not at lower dose levels. The increased liver weights correlated with minimal microscopic centrilobular hepatocellular hypertrophy in 4/10 males at 6000 ppm and were considered non-adverse and consistent with an adaptive increase in liver enzyme activity (which was not measured). Further treatment-related pathological changes were not observed in any other organ, and neuropathological examinations did not reveal any remarkable findings.

The NOAEL for subchronic toxicity was 1500 ppm (corresponding to 70 and 83 mg/kg bw per day in males and females, respectively) based on adverse effects on body weight and nutritional parameters at 6000 ppm. It should be emphasized that no evidence of neurotoxicity was obtained in this study by means of appropriate tests (Papagiannis, 2013a).

Another 90 day feeding study was performed in CD®[Crl:CD®(SD)] rats using another batch of triflumezopyrim, RAB55-104 (purity 97.04%). The test substance was administered to male and female rats (10/sex per dose) at concentrations of 0, 100, 400, 1500 and 6000 ppm (corresponding to 0, 4.2, 17, 63.9 and 257.1 mg/kg bw per day for males and 0, 5.1, 20.4, 74.23 and 278.1 mg/kg bw per day for females, respectively). End-points under evaluation included body weight, body weight gain, feed consumption, feed efficiency, clinical (including ophthalmoscopic) signs, haematology, clinical chemistry, urine analysis, organ weights and gross and microscopic pathology.

There was no impact on survival, clinical and ophthalmoscopic signs, blood coagulation, clinical chemistry, urine parameters or on gross pathological findings.

Body weight and body weight gain were significantly reduced in both sexes at 6000 ppm, confirming previous results. In females, feed consumption was also lower at the mid dose, but this finding did not result in a body weight effect and was not accompanied by a lower efficiency. Therefore, it is not considered adverse.

In contrast to the Papagiannis (2013a) 90-day study, but in line with the Anand (2013) 28-day study, decreases in erythrocyte parameters (RBC count, haematocrit, haemoglobin) were observed in both sexes at 6000 ppm (Table 9). In females, RBC count was also significantly reduced at 1500 and 400 ppm but with no dose response. In addition, the eosinophils were reduced at 6000 ppm in both sexes; however, the pathological meaning of this effect is not clear. In contrast to the more clear-cut high-dose effects on the RBCs, this effect on eosinophils is not considered adverse in isolation. The same holds true for the lower but not statistically significantly decreased haemoglobin and haematocrit values.

Table 9. Haematological findings in the second 90-day feeding study in rats

	Measure per dose level of triflumezopyrim				
Parameter	**0 ppm**	**100 ppm**	**400 ppm**	**1500 ppm**	**6000 ppm**
Males (day 92)					
RBC (10^6/µL)	8.718	8.940	8.588	8.487	8.038**
Hb (g/dL)	15.36	15.76	15.44	15.08	14.57**
Haematocrit (%)	45.89	47.41	46.12	44.96	43.50*
Eosinophils (10^3/µL)	0.132	0.127	0.131	0.094	0.052**
Females (day 92)					
RBC (10^6/µL)	7.934	7.862	7.502*	7.513*	7.267**
Hb (g/dL)	14.42	14.69	14.46	14.08	13.70*
Haematocrit (%)	43.03	43.09	42.38	41.47	40.20**
Eosinophils (10^3/µL)	0.083	0.095	0.077	0.076	0.032**

Hb: haemoglobin; ppm: parts per million; RBC: red blood cells; *: $P < 0.05$ (Dunnett test); **: $P < 0.01$ (Dunnett test)
Source: Ford (2015)

As in previous short-term studies with triflumezopyrim, liver weight was increased at the high-dose level, although the effect was less pronounced in this study and was statistically significant only for relative organ weights in both sexes. In addition, uterus with cervix weights were increased at least in high-dose females (Table 10). Neither the liver nor the uterus weight increases were accompanied by histological findings.

The NOAEL was 1500 ppm (63.9 and 74.23 mg/kg bw per day in males and females, respectively) based on reductions in body weight and nutritional parameters and haematological effects in both sexes at 6000 ppm. The uterus was identified as potentially another target organ of triflumezopyrim, along with blood and liver, given that absolute and relative organ weights were increased even though statistical significance was only achieved for relative organ weights (Ford, 2015).

Table 10. Organ weight changes in the second 90-day feeding study in rats

Parameter	Absolute and relative weights per dose level of triflumezopyrim [a]				
	0 ppm	100 ppm	400 ppm	1500 ppm	6000 ppm
Males					
Absolute liver weight (g)	14.610	16.056 (+9.90%)	15.085 (+3.25%)	15.256 (+4.42%)	16.178 (+10.73%)
Relative liver weight (%)	2.894 4	2.904 2 (+0.34%)	2.849 1 (−1.57%)	3.005 2 (+3.83%)	3.510 6* (+21.29%)
Females					
Absolute liver weight (g)	8.567	8.248 (−3.72%)	9.337 (+8.99)	8.863 (+3.46)	9.352 (+9.16)
Relative liver weight (%)	2.926 7	2.853 6 (−2.50%)	3.177 4 (+8.57%)	3.164 9 (+8.14)	3.682 1* (+25.81)
Absolute uterus with cervix weight (g)	0.611	0.653 (+6.87%)	0.695 (+13.75%)	0.708 (+15.88%)	0.793 (+29.79%)
Relative uterus with cervix weight (%)	0.208 1	0.226 2 (+8.70%)	0.236 5 (+13.65%)	0.253 4 (+21.77%)	0.312 1* (+49.98%)

ppm: parts per million; *: $P < 0.01$ (Dunnett test)

[a] Expressed as absolute weight (g) or relative weight (%) and, in parentheses, the % increase (+) or decrease (−) relative to the controls.

Source: Ford (2015)

Dog

In a 28-day feeding study that was intended to identify potential toxicity but also palatability problems and to choose appropriate doses for the subsequent 90-day study, triflumezopyrim (batch no. RAB55-031, purity 98.8%) was administered to male and female beagle dogs (2/sex per dose) at concentrations of 0, 300, 3000 or 30 000 ppm. After one week, the high-dose group dietary concentration was reduced due to very low feed consumption and severe body weight loss, first to 15 000 ppm (week 2), and then (because the adverse effects continued) to 10 000 ppm (weeks 3 and 4). Taking this reduction and the lower intake into account, the mean daily compound intakes for male dogs were calculated to be 0, 6.1, 49.4 and 55.1 mg/kg bw per day for males and 0, 8.2, 67.1 and 145 mg/kg bw per day for females. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs with special attention paid to neurobehavioural findings, haematology, blood and urine clinical chemistry, organ weights and gross and microscopic pathology.

No test substance-related deaths occurred. One high-dose male was found moribund and euthanized on day 24 for humane reasons; the dog's condition was attributed to bacterial pneumonia and not to test substance exposure. Test substance-related clinical and neurobehavioural observations noted in the male and female dogs receiving the highest dose were generally considered secondary to the reduced feed consumption and body weight loss. Adverse, test substance-related reductions in body weight and nutritional parameters were noted in both sexes at 10 000 ppm and, to a lesser extent, at 3000 ppm. These body weight effects correlated with thin appearance noted in most dogs, including controls. The reduction in the highdose group dietary concentration was generally associated with less severe effects on body weight and nutritional parameters but, nonetheless, these parameters remained below those of control, especially in males. Based on these findings, it was concluded that even the reduced high-dose concentration of 10 000 ppm exceeded the MTD and should not be included in longer studies in dogs.

No test substance-related effects were detected upon ophthalmoscopic examination in any dose group.

There were no test substance-related effects on haematology, coagulation or urine analysis parameters or adverse effects on clinical chemistry parameters. Males and females at 10 000 ppm

showed mild increases in serum alanine aminotransferase activity and concentration of bile acids as well as decreases in total protein and albumin. These changes were considered indicative of possible hepatic dysfunction but might be secondary responses to the severe body weight and nutritional effects.

At necropsy, there were no test substance-related macroscopic observations or organ weight changes that could be attributed to treatment. Histopathological examination found a number of lesions, in particular in individual high-dose animals, that were considered secondary to the severe body weight loss and/or stress rather than to specific target organ toxicity. These findings comprised, for example, fatty changes in liver and kidney tubules of high-dose females.

The NOAEL was 300 ppm (calculated to be 6.1 and 8.2 mg/kg bw per day for males and females, respectively) based on effects on body weight and nutritional parameters at the LOAEL of 3000 ppm (calculated to be 49.4 and, 67.1 mg/kg bw per day for males and females, respectively (Papagiannis, 2013b).

In a 90-day feeding study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to male and female beagle dogs (4 dogs/sex per concentration) at concentrations of 0, 100, 400, 1000 or 4000 ppm (equal to 0, 3.05, 12.20, 26.60 and 114.94 mg/kg bw per day for males and 0, 2.69, 12.15, 26.87 and 131.13 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology, clinical chemistry, ophthalmology, organ weights, urine analysis and gross and microscopic pathology.

A single female at 4000 ppm was euthanized in extremis on day 77. The assumed cause of death was haemorrhage of undetermined origin. The relationship between the test substance administration and the haemorrhage/moribundity was uncertain, but cannot be discounted in terms of identification of the NOAEL.

Test substance-related and sometimes statistically significant reductions in body weight parameters were noted in males and females at 1000 ppm and higher concentrations. The reductions in body weight were associated with reduced feed consumption and/or feed efficiency at these concentrations (Table 11), and some dogs receiving the highest dose looked thin.

At 4000 ppm, there were mild, adverse decreases in red cell mass, alterations to erythrocyte morphology and associated microscopic findings in the bone marrow, spleen and liver at study termination that provide some evidence of a haemolytic process. There was also evidence of a mild inflammatory pattern among serum proteins (mild decreases in albumin and calcium and increases in globulin) and an increase in alkaline phosphatase activity at this concentration, with no definitive histologic correlates. No effects were observed on coagulation or urine analysis parameters.

Gross changes observed at the two highest dose levels were considered secondary to the body weight and nutritional effects. These secondary effects included macroscopic findings of small thymus in one male per group with correlative increased severity of microscopic generalized lymphoid depletion in both sexes at 1000 ppm and higher concentrations. Moderate body fat depletion was observed in a few dogs at 4000 ppm, which correlated with the body weight reductions and microscopic findings.

A number of organ weight differences (with a variable statistical significance) noted at the highest dose level were considered secondary to the marked body weight decrements. These differences included increased adrenal gland weights (males) and reduced thymus, epididymides, testes, prostate, ovary and uterus with cervix weights. Increased absolute and relative spleen weights in females at 4000 ppm were considered secondary to haematological effects observed in this group. Thymus weights were also reduced in males and females at 1000 ppm.

Table 11. Body weights, body weight gain, feed consumption and feed efficiency in a 90-day feeding study in dogs

	Measure per dose level [a]				
	0 ppm	**100 ppm**	**400 ppm**	**1000 ppm**	**4000 ppm**
Males					
Body weight, day 1 (kg)	10.163	9.875 (↓2.8)	10.213 (↑0.5)	9.800 (↓3.6)	9.475 (↓6.8)
Body weight, day 91 (kg)	10.688	9.625 (↓10.0)	9.913 (↓7.3)	9.575 (↓10.4)	8.225 (↓23.0)
Body weight gain, weeks 1–13 (kg)	0.525	−0.250	−0.300	-0.225	−1.250*
Feed consumption, weeks 1–13 (g/animal per day)	304.19	314.79	318.27	270.88	234.85
Feed efficiency, weeks 1–13 [b]	1.85	−0.79	−1.19	−1.11	−5.84#
Females					
Body weight, day 1 (kg)	8.100	8.100 (NA)	8.375 (↑3.4)	7.713 (↓4.8)	8.238 (↑1.7)
Body weight, day 91 (kg)	8.575	8.313 (↓3.1)	8.613 (↑0.4)	7.200 (↓16.0)	6.950 (↓19.0)
Body weight gain, weeks 1–13 (kg)	0.475	0.213	0.238	−0.513	−1.000**
Feed consumption, weeks 1–13 (g/animal per day)	247.21	238.82	271.88	203.47	229.52
Feed efficiency, weeks 1–13	1.91	0.85	0.77	−2.55	−5.28##

NA: not applicable; ppm: parts per million; ↓: decreased; ↑: increased;

*: $P < 0.05$ (Levene test); **$P < 0.01$ (Levene test); #: $P < 0.05$ (Dunnett test); ##: $P < 0.01$ (Dunnett test)

[a] Expressed as weight (g) and, in parentheses, the per cent decrease (↓) or increase (↑)..

[b] Feed efficiency calculated as average weight gain /average amount feed consumed.

Source: Papagiannis (2013c)

Microscopic lesions were confined to the dose levels causing systemic toxicity and comprised the following findings: diffuse cortical hypertrophy of the adrenals in both sexes at 4000 ppm; generalized lymphoid depletion of the spleen, thymus, mandibular and/or mesenteric lymph nodes of both sexes at 4000 ppm; and increased severity of generalized lymphoid depletion of the thymus of males and females at 1000 ppm and higher concentrations; immaturity of the reproductive tract of males and females at 4000 ppm (testes, epididymides, prostate, ovary and uterus with cervix). Other microscopic findings reflect the haemolytic process: erythrocytic hyperplasia of the bone marrow (femur, rib and/or sternum) in males at 1000 ppm and greater concentrations and in females at 4000 ppm; increased Kupffer cell pigmentation and/or extramedullary haematopoiesis in the livers of males and/or females at 4000 ppm; and increased incidence and/or severity of pigmented macrophages and/or extramedullary haematopoiesis in the spleen of males and females at 4000 ppm.

The NOAEL was 400 ppm (equal to 12.2 mg/kg bw per day for both males and females), based on effects on body weight, lower thymus weight and lymphoid depletion in thymus at 1000 ppm (equal to 26.6 and 26.9 mg/kg bw per day in males and females, respectively). Based on the severity of effects observed at 4000 ppm, including haemolysis, this exposure level was considered to have exceeded the MTD (Papagiannis, 2013c).

In a one-year feeding study, triflumezopyrim (batch no. RAB55-064, purity 98.5%) was administered to male and female beagle dogs (4 dogs/sex per dose) at concentrations of 0, 40, 100, 400 and 2000 ppm (equal to 1.5, 3.3, 11.1 and 53.2 mg/kg bw per day for males and 1.2, 3.4, 10.8 and 55.9 mg/kg bw per day for females). High-dose female dogs received the test substance at a concentration of 1000 ppm for 13 weeks, and at 2000 ppm for the remainder of the study. Parameters evaluated included body weight,

body weight gain, feed consumption, feed efficiency, clinical signs, haematology, coagulation, clinical chemistry, urine analysis, ophthalmology, organ weights and gross and microscopic pathology.

There were no test substance-related effects on survival, clinical findings, body weight or body weight change or nutritional parameters (feed consumption and feed efficiency). One female dog at 400 ppm was euthanized on day 310 because of polyarteritis, which is common in beagle dogs and was not related to treatment. Ophthalmoscopic and physical examinations, clinical pathology evaluations (haematology, coagulation, clinical chemistry or urine analysis), organ weight determinations, gross or histopathological evaluations did not find evidence of any treatment-related effects.

The NOAEL was 2000 ppm (equal to 53.2 and 55.9 mg/kg bw per day for males and females, respectively), the highest concentration tested (Papagiannis, 2015b). The effects on body weight, thymus and blood as observed in the 90-day dog study (Papagiannis, 2013c) even at lower dose levels were not confirmed. However, different batches of triflumezopyrim were used in the two studies.

(b) Dermal administration

In a 28-day dermal study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was applied to the shaved, intact dorsal skin of male and female Crl:CD(SD) rats (10/sex per dose). The test substance was applied on 29 consecutive days for six hours per day at dose levels of 0, 100, 350 or 1000 mg/kg bw per day. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, as well as the occurrence of clinical signs. Clinical pathology (haematology, clinical chemistry, urine analysis) and ophthalmological examinations were also performed. At termination, the rats were necropsied, organ weights were determined and selected organs examined histopathologically.

No deaths occurred, and no clinical or ophthalmological observations were attributable to exposure to the test substance. There was no evidence of irritation or other local skin effects. No adverse or test substance-related effects on body weight, organ weights or nutritional parameters were observed. At the top dose level, differences in body weight and body weight gain were noted but these were not statistically significant. Moreover, both were lower in male rats compared to the control group but higher, in contrast, in females. There were no alterations in clinical pathology parameters. Neither gross nor histopathology revealed any remarkable findings that could be attributed to test substance administration.

The NOAEL for systemic effects as well as for local effects in both sexes was 1000 mg/kg bw per day since no adverse effects were observed at any dose level (Carpenter, 2013b).

2.3 Long-term studies of toxicity and carcinogenicity

The chronic toxicity and carcinogenicity of triflumezopyrim were investigated in long-term feeding studies in mice and rats.

Mouse

In a carcinogenicity study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to male and female Crl:CD1®(ICR) mice (60/sex per dose level) for 18 months at nominal dietary concentrations of 0, 200, 800, 2500 and 7000 ppm. These dose levels were based on results from a 90-day feeding study in the same mouse strain (Papagiannis, 2015a; section 2.2a). The overall (week 1–77) mean daily intakes in male mice were 0, 20, 84, 248 and 727 mg/kg bw per day. The mean daily intakes in female mice were 0, 22, 88, 283 and 810 mg/kg bw per day.

An additional group (5 animals/sex and dose level) received the test substance for only one year and were used for toxicokinetic investigations (results not been submitted). Twenty mice of each sex were co-housed as sentinel animals on which health screens were performed pretest and at 6, 12 and 18 months.

The study was run in compliance with OECD TG 451 in its 2009 version. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, ophthalmology, differential blood counts (confined to all animals euthanized in extremis and all control and high-dose mice at terminal kill), organ weights, and gross and microscopic pathology.

There were no clinical signs and no premature deaths that could be attributed to treatment. Survival in males was lowest in the high-dose group (44/60 at 7000 ppm vs 52/60 in the control, 53/60 at 200 ppm and 50/60 at 800 and 2500 ppm). There was no impact on body weight, body weight gain, feed consumption or feed efficiency or on differential blood counts. At necropsy, an increase in the number of animals with masses or nodules in the liver was grossly noted in males at 7000 ppm. Liver weights were statistically significantly higher in both sexes at 2500 ppm and higher concentrations. Enlarged spleens noted in females at 7000 ppm correlated with increased spleen weights at the same dose level (Table 12).

Table 12. Significant organ weight changes in the 18-month feeding study in mice

	Mean relative or absolute weight (% change from control) per dose level[a]				
Weight	**0 ppm**	**200 ppm**	**800 ppm**	**2500 ppm**	**7000 ppm**
Males					
Absolute liver with gall bladder weight (g)	2.161	2.220 (+2.73%)	2.247 (+3.98%)	2.500 (+15.69%)*	3.185 (+47.39%)*
Relative liver with gall bladder weight (%)	5.040 7	4.888 8 (−3.01%)	5.079 4 (+0.77%)	5.636 3 (+11.82%)	7.468 9 (+48.17%)*
Females					
Absolute liver with gall bladder weight (g)	1.880	1.872 (−0.43%)	1.938 (+3.09%)	2.173 (+15.59%)*	2.604 (+38.51%)*
Relative liver with gall bladder weight (%)	4.865 2	4.902 9 (+0.77%)	4.769 1 (−1.98)	5.592 8 (+14.96%)*	6.982 9 (+43.53%)*
Absolute spleen weight (g)	0.203	0.279 (+37.43%)	0.195 (−3.94%)	0.245 (+20.69%)	0.397 (+95.57%)*
Relative spleen weight (%)	0.534 0	0.717 4 (+34.34%)	0.491 2 (−8.01%)	0.630 0 (+17.98%)	1.058 5 (+98.22%)*

ppm: parts per million; *: $P < 0.01$ (Levine's test)

[a] Results expressed as mean absolute weight (g) or weight relative to body weight (%) and, in parentheses, % change from the respective control value.

Source: Papagiannis (2015d)

Histopathological examination revealed an increase in centrilobular hepatocyte hypertrophy in male mice at the two highest dose levels. The occurrence of centrilobular hepatocyte hypertrophy in females was also confined to these doses, but was much more rare (Table 13). In contrast, the increase in incidence of extramedullary haematopoiesis in the liver was clearly seen in females at 7000 ppm.

In both sexes, the number of animals with extramedullary haematopoiesis in the spleen was high in all groups but there was a trend towards greater severity. There was an increase in the number of male and female mice with the diagnosis of "mild" or "moderate" extramedullary haematopoiesis and a concomitant decrease in the grading as "minimal" at the two highest dose levels in males even though the differences were not statistically significant. In females, the affected number was higher at least at the top dose level. In fact, the number was also higher as compared to the control group from 200 ppm onwards but with no dose response. Even though haematological parameters that could be indicative of possible RBC effects were not measured in this study, and although there were no correlated histopathological changes in the bone marrow, the increase in extramedullary haematopoiesis suggests a possible adverse effect of the test substance. This would be in line with similar observations in short-term feeding studies in the mouse as well as in rats or dogs (for example, Anand, 2015; Ford, 2015; Papagiannis, 2013c).

Table 13. Incidences of selected non-neoplastic microscopic findings in the 18-month feeding study in mice with triflumezopyrim

Organ / finding	Number of animals with the finding per dose level				
	0 ppm	200 ppm	800 ppm	2500 ppm	7000 ppm
Males					
Number of males evaluated	60	60	60	60	60
Liver					
Hypertrophy, hepatocyte, centrilobular	2	3	3	7	10
Haematopoiesis, extramedullary	2	2	1	2	2
Spleen					
Haematopoiesis, extramedullary	46	48	46	52	53
Haematopoiesis, extramedullary, mild or moderate degree	24	27	27	35	38
Duodenum					
Mucosal hyperplasia, total	10	10	7	6	13
Mucosal hyperplasia, moderate degree	0	0	0	0	3
Females					
Number of females evaluated	60	60	60	60	60
Liver					
Hypertrophy, hepatocyte, centrilobular	0	0	0	1	1
Haematopoiesis, extramedullary	0	3	0	1	11*
Spleen					
Haematopoiesis, extramedullary	44	49	50	46	53
Haematopoiesis, extramedullary, mild or moderate degree	22	33	32	35	44
Mammary gland					
Dilatation, duct	7	11	10	14	23*

ppm: parts per million; *: $P < 0.01$ (Fisher exact test)

Source: Papagiannis (2015d)

Increased occurrence of ductal dilation of the mammary gland in females was observed at the highest dose level but, because of minimal severity, this was not considered adverse. The same, along with low incidence of the finding, holds true for an increase in severity of mucosal hyperplasia of the duodenum in male mice.

In males, there was a positive trend for hepatocellular adenoma with a clear increase over the control value at 7000 ppm (see Table 14). A numerically higher incidence was also noted at 2500 ppm but the difference from the control group was small. A similar difference, but in the opposite direction, was seen in the low- and mid-dose groups. The increase in centrilobular liver cell hypertrophy noted in male mice at the two highest dose levels (Table 13) suggests that both findings were part of a pattern that might be associated with induction of hepatic microsomal enzymes as observed in short-term feeding studies. It is notable that neither hypertrophy nor adenoma were increased in females. In males, which were clearly more markedly affected by the hepatic effects, there was no progression to carcinoma.

Table 14. Tumour incidences of concern and related pre-neoplastic lesions in the long-term feeding study in mice

Parameter	Number of animals with the finding per dose level				
	0 ppm	200 ppm	800 ppm	2500 ppm	7000 ppm
Males (*n* = 60/group)					
Hepatocellular adenoma	9	6	4	12	17**
Hepatocellular carcinoma	1	0	0	0	1
Females (based on PWG re-evaluation, *n* = 60 per group)					
Bronchoalveolar adenoma	6	8	9	9	10
Bronchoalveolar carcinoma	2	0	1	4	6*
Bronchoalveolar adenoma and carcinoma, combined	8	8	7	11	15***
Bronchoalveolar hyperplasia	2	10	8	5	3
Sum of lung tumours and hyperplasia	10	17	15	16	18

ppm: parts per million; PWG: pathology working group;
*: $P < 0.05$, Cochran–Armitage trend test and Peto test; **: $P < 0.05$ (Cochran–Armitage trend test), and $P < 0.01$ (Peto test);
***: $P < 0.05$ (Cochran–Armitage trend test)
Source: Papagiannis (2015d)

An increase in the incidence of bronchoalveolar carcinoma and in the combined incidence of adenoma and carcinoma in the lungs was observed in females mice at 7000 ppm. A positive trend has been demonstrated even though a pairwise comparison did not reveal a statistically significant difference from the control group.

Historical control data in ICR mice from the performing laboratory were not available because 18-month carcinogenicity studies were not routinely conducted there. It is known that lung tumours are a common neoplasm in CD-1 mice and occur with variable incidence. The sponsor provided a summary Table on 10 studies in CD-1 mice with a duration of 78 weeks. As these data appear to be from different laboratories, it is not clear if they apply to the ICR mice employed in this study; and as more detailed information on the 10 included studies is lacking, these historical data cannot be used for the assessment of lung tumours in this study. What was obvious, however, was a frequent occurrence of lung carcinoma in female CD-1 mice since, with such tumours being observed in 6/10 studies. On the other hand, their incidences are usually low since in five of these studies, only one female was affected (1/50–1/60), and in four other studies the control incidence was 0. The only exception was one study with a higher frequency of 6/50 in the control group. The overall mean was about 1.9% (11/575). The combined incidence of adenoma and carcinoma ranged from 1/60 up to 9/50 with a total mean of 6.3% (36/575).

A working group of six pathologists performed a re-review of the slides (reflected in Table 15) and weighed the evidence for and against an association of lung tumours with the administration of the test substance. This group included the pathologists who had performed the first histopathological evaluation or had first peer-reviewed the slides.

A strong argument against the assumption that the lung tumours were treatment-related is the absence of a dose response with regard to non-neoplastic but tumour-precedent lesions such as hyperplasia. Even the numeric increase in adenoma is not convincing when the dose spacing is taken into account. For a tumour caused by a chemical agent, one would expect a much stronger increase in pre-neoplastic changes or benign tumours at dose levels as high as 7000 ppm.

No increase in bronchoalveolar adenoma was observed in male mice in the same study and the difference in the number of males with carcinoma (8/60 at the highest dose level as compared to 5/60 in the control group) was small and did not attain statistical significance when compared with the controls. In contrast, the absence of a higher frequency in lung tumours in rats is not considered a valid argument since certain types of neoplasia are often unique to one of the rodent species (and may be still relevant to humans).

Mechanistic considerations are valuable in deciding on potential carcinogenicity. A genotoxic mode of action may be excluded since triflumezopyrim is devoid of such a potential (see section 2.4). Additional mechanistic studies (see section 2.6c) indicated that there was no metabolism of triflumezopyrim in mouse (and human) lung microsomes and no activation by Cyp2f2, a prominent CYP in mouse lungs that metabolizes a number of primary lung toxicants (Nabb, 2015c). In addition, there was no evidence of increased proliferation of the bronchoalveolar epithelium in female mouse lung following three or seven days of dietary administration (Mukerji, 2015b). These results do not provide a possible mode of action to explain the higher tumour incidence in female mice. On the other hand, they are not suitable for rejecting the hypothesis of a long-term carcinogenic effect. It is worth noting that minor lung effects (but no tumours) were also seen in the long-term study in rats (Papagiannis, 2015c) suggesting that the lung might be an additional target organ.

The NOAEL in this study was 800 ppm (equal to 84 and 88 mg/kg bw per day in males and females, respectively) based mainly on liver effects (increase in liver weight of up to 16% in both sexes and increased occurrence of centrilobular hypertrophy of hepatocytes in males) at 2500 ppm (equal to 248 mg/kg bw per day). In determining this NOAEL, the Meeting considered the liver weight increase and hepatocellular hypertrophy as adverse rather than adaptive, taking into account the extent of organ weight increase and also the progression of the lesion to (benign) liver tumours. This NOAEL is also supported by an increase in extramedullary haematopoiesis suggestive of weak haematological effects.

Triflumezopyrim was not carcinogenic at dietary concentrations up to 2500 ppm in males and females. There was a clear carcinogenic effect of triflumezopyrim on the liver in high-dose male mice even though these tumours were benign. An additional carcinogenic effect on the lungs in female mice upon long-term exposure to high doses was only equivocally related to treatment but cannot be completely discounted (Papagiannis, 2015d).

Rat

In a two-year chronic toxicity and carcinogenicity feeding study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to CD®[Crl:CD(SD)] rats (70/sex per dose) at dietary concentrations of 0, 100, 500, 2000 or 8000 ppm (corresponding to 0, 3, 15.9, 70.6 and 283.8 mg/kg bw per day for males and 0, 3.2, 17.3, 73.8 and 395.9 mg/kg bw per day for females, respectively). Additional satellite animals (10/sex per dose) were designated for interim kill and received the control or test diet for approximately 52 weeks. End-points investigated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, ophthalmology, organ weights, gross and microscopic pathology. Clinical pathology was performed on satellite animals at 3, 6 and 12 months and comprised haematology including coagulation parameters and clinical chemistry. Urine analysis was performed in 10 animals/sex per dose at 3, 6 and 12 months. In addition, blood smear differentials for white blood cell counts were collected from all animals at 12, 18 and 24 months and from all those euthanized in extremis. However, evaluation of this end-point was confined to the control and high-dose groups at termination and the premature deaths in order to identify a possible cause of sickness and death. In addition, 25 animals/sex were employed as health sentinels and co-housed with study rats for the whole study period. Serological health screens conducted on these animals pretest and at 6, 12, 18 and 24 hours showed no evidence of infections.

In general, survival was low, but there was no significant difference between the control and treated groups and no clear dose response. In males, only 23 out of 70 control rats were still alive at scheduled kill. In the treated groups, the numbers of surviving males were as follows: 17 (100 ppm), 16 (500 ppm), 18 (2000 ppm) and 28 (8000 ppm). In females, 18 out of 70 control rats survived until scheduled kill. The numbers of surviving treated females were as follows: 15 (100 ppm), 24 (500 ppm), 20 (2000 ppm) and 19 (8000 ppm). There were no deaths that could be attributed to treatment and no impact of substance administration on mortality/survival was evident. In all male groups, survival fell below 50% after week 80 and, in some groups, approached 25% around week 100. In females, more than 50% of animals were still alive around week 90, followed by a sharp decline in all groups. The poor survival resulted in a low number of animals for organ weight analysis. For histopathological evaluation including tumour analysis, all 70 animals from the main study groups were taken into consideration, independent of their individual time points and causes of death.

Adverse effects on body weight, body weight gain, feed consumption and feed efficiency were noted during the study in males and females at 2000 ppm and higher concentrations, with females more severely affected than males. A greater number of top-dose females appeared thin. This was the only treatment-attributable clinical sign of note.

Respective mean body weights for males at 2000 and 8000 ppm at week 51 were 6.8% and 19.9% lower than that of control males. At week 104, the respective differences were 8% and 19.2% lower than that of controls, that is, the difference remained nearly the same over the second year. No impact on body weight was seen at the two lower doses. Mean overall body weight gain at 2000 ppm was 11.4% below control and at 8000 ppm, 26.9% below control. Even though only the effects at 8000 ppm were statistically significant, the body weight findings in males are considered test substance related and indicative of an adverse effect at the two highest dose levels, 2000 and 8000 ppm. Feed consumption in males was reduced only at the top dose, as was feed efficiency, even though this was not always statistically significant. Also, at 2000 ppm, feed efficiency tended to be lower and might have caused the lower body weight (gain), which was apparently not solely related to the reduced feed intake.

In females, mean body weights at 2000 and 8000 ppm at week 51 were 17.9% and 32.7% below control. At week 103, the respective differences were even more pronounced, approximately 23% and more than 45%, respectively, below control. The large differences are obvious in Table 16, in the weights at necropsy. These decreases were due to a strongly reduced mean overall body weight gain at 2000 ppm of 31.9% below control and of 61.5% below control at 8000 ppm. All of these differences were statistically significant. Mean feed consumption at 8000 ppm was 12% and 11% below control over 1 and 2 years (both statistically significant). A significantly lower feed consumption was also observed at 2000 ppm. In addition to the reduced feed intake, the feed efficiency was clearly compromised in female rats; at 8000 ppm, mean feed efficiency over the entire study period was 52.7% below control and at 2000 ppm, it was 21.2% below control.

Based on the magnitude of effects on nutritional parameters, it can be concluded that the MTD for systemic toxicity was greatly exceeded in females at 2000 and 8000 ppm and in males at 8000 ppm.

No adverse test article-related effects on body weight or body weight gain were observed in rats at 500 ppm and below. In females at 500 ppm, however, final (week 103) mean body weight and overall (weeks 1–103) body weight gain were still statistically significantly reduced, at 11.6% and 15.9% below control, respectively. This reduction was attributed to a transient reduction in body weight gain over the ~12- to 18-month interval. During this interval (days 357–539), mean body weight was 22.7% below control but returned to the control level afterwards. The reason for this transient lower body weight gain is not known, but it was also seen in other male and female groups during this same general time interval. As this period of lower body weight gain was transient, differences during most intervals were not statistically significant and no other evidence of toxicity was observed in this group, these differences in females at 500 ppm were considered non-adverse.

No adverse effects were observed on clinical pathology parameters (haematology, clinical chemistry, urine analysis, coagulation, differential white blood cell count).

At interim necropsy after one year, no gross lesions were seen. Mean relative liver weights were significantly increased in both sexes at 2000 and 8000 ppm. This increment reflects the lower body weight in these groups but, on the other hand, it could be due to centrilobular hypertrophy of hepatocytes that was observed microscopically in all top-dose males and females but in none at lower doses or in the controls. In addition, interstitial cell hyperplasia of the testes was noted in 6/10 males at 8000 ppm. This finding was not seen in any other group. In females, there was also a (nonsignificant) increase in alveolar histiocytosis of the lungs at 8000 ppm that is discussed below in greater detail.

Due to the severely compromised body weights at 2000 and 8000 ppm, the changes in relative organ weights noted at scheduled kill after two years were not considered to be a direct consequence of treatment or an adverse response. The increase in relative liver weight in females at 2000 and 8000 ppm, however, might also be related to histological liver findings (see Table 15). In addition, there was a marked and statistically significant increase in absolute and relative uterus weight at the maximum dose (Table 15), reflecting the enlarged appearance of this organ in the two highest dose groups at gross examination. A higher uterus weight was also noted at 2000 ppm, but this was not statistically significant. The low number of animals surviving until scheduled kill must be taken into account when assessing statistical comparisons in this study.

Macroscopic examination found an increased incidence of tan or white foci in the lungs of males and females at 8000 ppm and females at 2000 ppm, which correlated with microscopic findings of alveolar histiocytosis, which is common in ageing rats. This finding was characterized by the presence of grey foamy macrophages within alveolar spaces. There was no significant increase in the incidence of this finding, but a progression towards greater severity was obvious (see Table 16). Normally, this finding is indicative of an increased phagocytic activity of lung macrophages, the pathological relevance of which is not clear. On the one hand, it could suggest uptake of, for example, xenobiotic particles, which would be part of an adaptive immunological response; on the other hand, the foamy appearance suggests damage to the macrophages with the possibility that, for example, lysosomes were released. This could mean that the lung macrophages themselves were an additional target of triflumezopyrim. However, when the incidences were taken into account, the treatment-related effect was confined to doses that were unequivocally toxic to the animals and at which other findings were of greater importance.

Table 15. Selected[a] body weight and organ weight changes in female rats in the two-year feeding study with triflumezopyrim

	Weight (% control) per dose level[b]				
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**2000 ppm**	**8000 ppm**
Number of female rats examined	18	15	24	20	19
Mean body weight (g) at necropsy (day 723 or 725)	537	544	491	425**	299**
Absolute liver weight (g)	14.279	14.028 (98.2%)	13.163 (92.2%)	13.019 (91.2%)	13.100 (91.7%)
Relative liver weight (%)	2.649 6	2.577 6 (97.3%)	2.706 3 (102.1%)	3.118 9* (117.7%)	4.407 4** (166.3%)
Absolute uterus weight (g)	1.129	1.210 (107.2%)	1.100 (97.4%)	1.513 (134.0%)	4.568* (404.6%)
Relative uterus weight (%)	0.222 1	0.229 6 (103.4%)	0.232 4 (104.6%)	0.363 0 (163.4%)	1.557 1** (701.1%)

ppm: parts per million; *: $P < 0.05$ (Welch's t-test); **: $P < 0.01$ (Welch's t-test)

[a] Changes could be indicative of an adverse effect of triflumezopyrim.

[b] Expressed as mean absolute weight (g) or mean relative to body weight (%) and, in parentheses, as a % of the respective control value.

Source: Papagiannis (2015c)

Non-neoplastic findings are summarized in Table 16. Liver, lungs, testes and uterus were likely affected by treatment; however, the increases in incidence and/or severity were confined to the maximum dose in males and to the two highest dose levels in females which, as indicated above, exceeded the MTD.

Table 16. Incidence of non-neoplastic findings in rats in the two-year feeding study with triflumezopyrim

	Number of animals with the finding per dose level				
Organ/Finding	**0 ppm**	**100 ppm**	**500 ppm**	**2000 ppm**	**8000 ppm**
Males					
Number of animals evaluated	70	70	70	70	70
Liver					
Degeneration, cystic, focal	17	15	17	19	29**
Hyperplasia bile duct	33	31	33	44	54*
Hypertrophy, centrilobular	0	0	0	0	7**
Testes					
Hyperplasia, interstitial cell	3	7	8	8	16*

Organ/Finding	Number of animals with the finding per dose level				
	0 ppm	100 ppm	500 ppm	2000 ppm	8000 ppm
Lung					
Histiocytosis, alveolar	30	21	14	22	37
Minimal	28	21	14	19	26
Mild	2	0	0	3	11
Females					
Number of animals evaluated	70	70	70	70	70
Liver					
Necrosis, single cell	0	0	0	0	3#
Hypertrophy, centrilobular	0	0	0	5#	47**
Lung					
Histiocytosis, alveolar	25	16	17	37	52#
Minimal	24	13	14	22	17
Mild	1	3	3	15	32
Moderate	0	0	0	0	3
Uterus					
Dilation/inflammation/hyperplasia	0	1	0	0	6*
Hyperplasia, cystic, endometrial	1	0	1	2	8*
Hyperplasia, squamous cell	2	0	3	1	6
Polyp, stromal (benign)	3	3	2	2	6

ppm: parts per million; *Source*: Papagiannis (2015c)

*: $P < 0.05$ (Fisher exact test); **: $P < 0.01$ (Fisher exact test); #: $P < 0.05$ (Cochran–Armitage trend test)

No statistically significant increase in any tumour type was found in male rats. In particular, it was worth noting that the testicular hyperplasia did not progress to tumours.

In contrast, there were carcinogenic findings in females that could be attributed to substance administration. Significant increases in uterine and liver tumours were observed at 8000 ppm even though total incidences were low (Table 17).

Table 17. Uterine and liver tumours in female rats in the two-year feeding study with triflumezopyrim

Tumour type	Number of animals with the finding per dose level				
	0 ppm	100 ppm	500 ppm	2000 ppm	8000 ppm
Number of females evaluated	70	70	70	70	70
Uterus					
Carcinoma, squamous cell	0	0	0	1	5*,#
Adenocarcinoma	0	0	0	0	2*
Granular cell tumour (benign)	0	1	1	0	4*,#
Sarcoma, endometrial stromal	0	2	1	2	1
Haemangiosarcoma	0	0	0	1	0
Schwannoma (malignant)	2	0	0	0	1
Liver					
Adenoma	0	0	0	0	2*

ppm: parts per million; *: $P < 0.05$ (Cochran–Armitage trend test); #: $P < 0.05$ (Peto trend test)

Source: Papagiannis (2015c)

The higher incidence of uterine squamous cell carcinoma (affecting mainly the cervix region and, in two animals, also the vagina) exceeded the historical control range of up to 1.5% as given by the sponsor. Accordingly, the single carcinoma at 2000 ppm was still within the historical control range. However, two other types of tumours in the same organ were also significantly increased. The incidence of adenocarcinoma (2/70) at the highest dose level was close to the upper limit of the historical range (3.3%). The (benign) granular cell tumours at this dose, in contrast, were well within the historical control range (0–15%). In addition, very few tumours of other types were observed in the uterus and, due to the absence of a dose response, were not considered treatment related.

All the uterine tumours were observed in different animals. Thus, 11 rats were affected by a uterine tumour of concern as compared to none in the control group and one each in the groups receiving the lower doses. All of the rats bearing malignant tumours were euthanized for humane reasons or died spontaneously prior to scheduled kill. In all cases, their malignant uterine tumours were identified as the cause of death. Day 589 was the earliest time point of death. No uterine tumours were observed at interim necropsy with subsequent histopathological examination. Thus, there is no evidence of an early onset of carcinogenic effects.

Of the rats with a benign granular cell tumour, only one survived to scheduled kill; the other three were euthanized in extremis on days 449, 596 or 707, but for other (mammary or pituitary tumours) or for unknown reasons. In two of these females, the tumour site also comprised the cervix; in the others, localization was not clearly reported.

Mechanistic studies suggest that uterine tumours were related to endocrine activity of triflumezopyrim, basically to a reduction in prolactin levels and a skewed estrogen to progesterone ratio (see section 2.6c), but the information was far from being entirely convincing.

The incidence of liver cell adenoma (see Table 17) was slightly above the upper limit of historical controls, that is, 1.7%. No hepatocellular carcinoma was observed in any group. The adenoma is considered part of a pattern that is characterized by induction of microsomal liver enzymes, centrilobular hypertrophy and higher organ weight, which has been observed in shorter studies.

The malignant and benign uterine tumours and liver cell adenoma were considered treatment-related as they occurred in two organs that had been identified as target organs of triflumezopyrim in rats; females were more severely affected. There were concomitant or preceding non-neoplastic findings in both organs (see Table 16). However, significant increases were confined to 8000 ppm, which clearly exceeding the MTD in this strain and sex.

The NOAEL was 500 ppm (corresponding to 15.9 and 17.3 mg/kg bw per day in males and females, respectively) based on reductions in body weight, feed consumption and feed efficiency, increased organ weights, gross pathological and histopathological findings at 2000 ppm (corresponding to 70.6 and 73.8 mg/kg bw per day in males and females, respectively).

Under the conditions of this study, triflumezopyrim was not carcinogenic at dietary concentrations up to and including 8000 ppm for male rats and up to 2000 ppm for female rats. Test substance-related neoplastic findings were observed in females at 8000 ppm (equal to a mean daily intake of approximately 396 mg/kg bw) (Papagiannis, 2015c).

2.4 Genotoxicity

Triflumezopyrim was evaluated in a range of in vitro and in vivo genotoxicity studies using different batches of the compound.

(a) In vitro studies

In vitro genotoxicity studies are summarized in Table 18, with more details given below.

Table 18. Summary of in vitro genotoxicity studies with triflumezopyrim

Type of study	Target	Concentration range tested	MA (±S9)	Batch no./ purity (%)	Result	Reference
Reverse gene mutation test in bacteria (Ames test)	*Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and *Escherichia coli* (WP2*uvrA*)	1.5–5000 µg/plate	+/−	RAB55-037 99.4%	Negative	Wagner & VanDyke (2012)
Reverse gene mutation test in bacteria (Ames test)	*S. typhimurium* (TA98, TA100, TA1535, TA1537) and *E. coli* (WP2*uvrA*)	1.5–5000 µg/plate	+/−	RAB55-064 98.5%	Negative	Wagner & VanDyke (2013)
Reverse gene mutation test in bacteria (Ames test)	*S. typhimurium* (TA98, TA100, TA1535, TA1537) and *E. coli* (WP2*uvrA*)	66.7–5000 µg/plate	+/−	RAB55-104 97.04%	Negative, precipitation observed at 4000 µg/plate and above	Myhre (2015a)
Reverse gene mutation test in bacteria (Ames test)	*S. typhimurium* (TA98, TA100, TA1535, TA1537) and *E. coli* (WP2*uvrA*)	66.7–5000 µg/plate	+/−	RAB55-111 98.67%	Negative, precipitation observed from 3000 (TA1537) or 4000 µg/plate (all other strains) and above	Myhre (2014a)
Gene (point) mutation (HGPRT) assay	CHO cells	100–1500 µg/mL	+/−	RAB55-037 99.4%	Negative	Clarke & VanDyke (2012)
Chromosomal aberration	Human peripheral blood lymphocytes (4-h treatment, 22-h harvest, ±S9; 22-h treatment with immediate harvest, −S9)	5–2000 µg/mL (cytogenetic analysis at 100–950 µg/mL)	+/−	RAB55-104 97.04%	Negative, precipitation observed from 500 µg/mL onwards	Kellum (2015a)
Chromosomal aberration	Human peripheral blood lymphocytes (4-h treatment, 22-h harvest, ±S9; 22-h treatment with immediate harvest, −S9)	5–2000 µg/mL (cytogenetic analysis at 250–750 µg/mL)	+/−	RAB55-111 98.67%	Negative, precipitation observed from 500 µg/mL onwards	Kellum (2015b)
Chromosomal aberration	Human peripheral blood lymphocytes (4-h treatment, 20-h harvest, ±S9; 20-h treatment with immediate harvest, −S9 mix)	100–1500 µg/mL (250, 500 and 1500 µg/mL chosen for cytogenetic evaluation following 4-h exposures and 250, 500 and 800 µg/mL after 20-h exposure)	+/−	RAB55-064 98.5%	Positive for structural aberrations without activation at highest (precipitating) concentration; negative with activation; no evidence of aneuploidy	Roy (2013)

Type of study	Target	Concentration range tested	MA (±S9)	Batch no./ purity (%)	Result	Reference
Chromosomal aberration	Human lymphocytes (4-h treatment, 20-h harvest ± S9; 20-h treatment with immediate harvest –S9 mix)	250–3980 µg/mL (4-hr exposures with/without activation); 100–1500 µg/mL (20-h exposure); concentrations chosen for cytogenetics: see text below	+/–	RAB55-037 99.4%	Positive for structural aberrations at 2000 and 2800 µg/mL with activation, negative in the absence of S9 mix and for numerical aberrations at all	Roy & Jois (2015)

MA: metabolic activation; CHO: Chinese hamster ovary; S9: 9000 × *g* supernatant fraction from rat liver homogenate

No evidence of genotoxicity was obtained in four independent studies using different batches of triflumezopyrim in the bacterial reverse gene mutation (Ames) assay. With two of the batches, some precipitation was observed in *S. typhimurium* strains at higher concentrations (Myhre, 2014a, 2015a). However, precipitation was not observed in two other experiments suggesting that there were slight differences between the batches despite the purity being similar (Wagner & VanDyke, 2012, 2013).

There was also no evidence of genotoxicity in an in vitro mammalian cell gene mutation test (CHO/HGRPT assay) by Clarke & VanDyke (2012). An increase of mutants (10.4/10^6 clonable cells) was observed in the experiment with metabolic activation at one rather low concentration, but this was not reproducible when higher concentrations were tested, and this finding was considered to have occurred by chance (Table 19).

Table 19. Results of the HGPRT assay in CHO cells with triflumezopyrim [a]

Treatment	Average number of colonies	Mutants/10^6 clonable cells
Control (solvent)	0.8	4.3
Triflumezopyrim		
250 µg/mL	0	0
500 µg/mL	1.9	10.4
750 µg/mL	0.5	2.6
1000 µg/mL	0.4	2.4
1500 µg/mL	0.4	2.3
Benzo(a)pyrene, 4 µg/mL (positive control)	14.4	80.4

CHO: Chinese hamster ovary; HGPRT: hypoxanthine guanine phosphoribosyltransferase

[a] Two replicates combined, activation experiment

Source: Clarke & VanDyke (2012)

Four separate in vitro chromosomal aberration assays using the same four batches of triflumezopyrim as in the Ames tests were performed with partly contradictory results. The studies by Kellum (2015a,b) in human peripheral lymphocytes were unequivocal in providing no evidence of clastogenicity. In a similar study by Roy (2013), in contrast, a significant increase in structural aberrations was seen but these were confined to the experiments with treatment in the absence of metabolic activation (Table 20). At the concentrations at which positive findings were obtained, precipitation was observed. There was no evidence of clastogenicity in the experiment with activation, and no significant increase in numerical aberrations was noted in any group.

Table 20. Results of the chromosomal aberration assay in human peripheral blood lymphocytes

	Percentage of aberrant cells[a] per treatment					
	4 h treatment without activation		20 h treatment without activation		4 h treatment with activation	
Treatment (µg/mL)	Mitotic index (%)	aberrant cells (%)	Mitotic index (%)	aberrant cells (%)	Mitotic index (%)	aberrant cells (%)
Solvent control – DMSO	13.2	0	11.8	0	10.9	0
Triflumezopyrim						
250 µg/mL	13.1	0	10.9	0	10.1	0
500 µg/mL	12.1	0.5	8.9	0	9.4	1.0
800 µg/mL[b]	–	–	–	–	–	–
1500 µg/mL[b]	9.9	3.5*	5.5	17*	5.5	1.5
Positive control						
Mitomycin C, 0.6 µg/mL	6.6	24*	–	–	–	–
Mitomycin C, 0.3 µg/mL	–	–	5.9	26*	–	–
Cyclophosphamide, 5 µg/mL	–	–	–	–	4.9	22*

*: $P < 0.01$ (Fisher exact test)

[a] Mostly chromatid breaks, occurrence of gaps only excluded, two flasks combined.

[b] Visible precipitation,

Source: Roy (2013)

A positive result was also obtained by Roy & Jois (2015) in another study in human peripheral blood lymphocytes that had apparently already been performed in 2013. The highest concentrations used for cytogenetic analysis were 2000 µg/mL (four hours, activation), 2500 µg/mL (four hours, no activation) and 750 µg/mL (20 hours, no activation). This time, a significant increase in structural chromosomal aberrations was observed only in the presence of the metabolically activating S9 mix at the maximum concentration of 2000 µg/mL whereas no significant increases were seen without activation. Thus, the outcome was partly contradictory to the first positive study. Nonetheless, the positive result under activation conditions was confirmed in a repeat experiment at 2000 and 2800 µg/mL, whereas a slight increase at 2500 µg/mL was not significant. It must be emphasized, however, that all these increases were noted not only at concentrations with visible precipitation but also in the presence of substantial toxicity as evidenced by a lower mitotic index.

On balance, even though precipitation and cytotoxicity may have played a role, clastogenic potential of at least some batches of triflumezopyrim in vitro cannot be excluded.

(b) In vivo studies

Three micronucleus assays in mice and one in rats, conducted with four different batches of triflumezopyrim, were available.

Roy & Divi (2012) used batch RAB55-037 (purity 99.4%), which was the same batch that later proved positive in the in vitro chromosomal aberration assay by Roy & Jois (2015). Following a dose-finding pretest, the test substance (dissolved in 0.1% Tween 80 in 0.5% aqueous methylcellulose) was administered as a single gavage at dose levels of 500, 1000 or 2000 mg/kg bw to Crl:CD-1 mice (5 sex/dose level and termination time). A vehicle control group was also included as well as a positive control group receiving 40 mg/kg bw of cyclophosphamide monohydrate. Polychromatic erythrocytes from the bone marrow were collected from all groups at 24 hours post dosing and from the vehicle control and the high-dose group additionally at 48 hours post dosing; they were examined microscopically for the presence of micronuclei.

Neither mortality nor clinical signs were observed. No alterations in the ratio of polychromatic to normochromatic erythrocytes were observed, indicating that the test compound was non-toxic to the bone marrow. Moreover, based on the findings in a single-dose ADME study (Himmelstein, 2015, see section 1.1), it can be assumed that the compound reached the bone marrow. There was no increase in micronucleus incidence at any time point in the groups receiving triflumezopyrim whereas the positive test substance had the expected increase.

The potential clastogenicity of this batch seen in vitro (Roy & Jois, 2015)was not confirmed in this in vivo assay (Roy & Divi, 2012).

In a second, very similar micronucleus assay, batch RAB55-104 (purity 97.04%), with no evidence of clastogenicity in vitro, was applied by single gavage to male and female Crl:CD-1 mice at the same dose levels of 0, 500, 1000 or 2000 mg/kg bw. The study design, including the positive control group, was the same as in the previous micronucleus assay (Roy & Divi, 2012), the only exception being that the high-dose groups comprised seven male and seven female mice per time point of termination instead of five. The results were also the same as in the older study, with triflumezopyrim showing no evidence of genotoxicity in the micronucleus assay. However, the ratio of polychromatic to normochromatic erythrocytes was slightly but significantly depressed at 48 hours post dosing at the high-dose level in females, suggesting a certain impact of the test compound on the bone marrow and providing evidence that a sufficient amount of the test substance was distributed to this tissue, at least at this high dose (Myhre, 2014b).

In the third micronucleus assay, batch RAB55-111 (purity 98.67%, negative for clastogenicity in vitro) was tested in Crl:CD-1 (ICR) mice by means of a different method. Five or (at the maximum dose level) seven males and females per group received single oral doses of 0, 500, 1000 or 2000 mg/kg bw. The positive control groups received 30 mg/kg bw of cyclophosphamide. There were no mortality, no clinical signs and no effects on body weight.

In contrast to the previous studies, peripheral blood (and not the bone marrow) was examined. In all groups, blood samples were taken from the tail vein approximately 48 and 72 hours post dosing (in the positive control group only once at 48 hours). Analysis for micronuclei was conducted by flow cytometry. At least 20 000 reticulocytes were examined for the presence of micronuclei. The frequency of reticulocytes as immature cells in all RBCs was determined to give an indication of systemic or bone marrow toxicity.

Neither mortality, nor an increase in micronuclei, nor alterations in the percentage of reticulocytes were observed. Triflumezopyrim showed no evidence of genotoxicity in this micronucleus assay (Myhre, 2015b)

Another micronucleus assay was conducted by means of flow cytometrical evaluation of reticulocytes in peripheral blood. This assay was part of a subacute rat toxicity study using batch no. RAB55-014 (purity > 99%) (section 2.2a). The analysis was performed in male and female (5/sex) control and the high-dose rats receiving either the vehicle alone or the test compound by gavage for 14 days. The initial daily high dose of 1000 mg/kg bw was reduced to 600 mg/kg bw from day 5 onwards. In addition, another group (5/sex) were administered a single oral dose of triflumezopyrim at 2000 mg/kg bw two days prior to termination.

There was no evidence of a clastogenic effect in this study (Nabb, 2015a).

No in vivo assays with the batch RAB55-064, which proved positive for chromosomal aberrations in vitro (Roy, 2013), were available.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproduction study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered in the diet to male and female Crl:CD®(SD) rats (30 rats/sex and dose in both the F_0/P and F_1 generations). Dietary concentrations were 0, 100, 500, 1500 and 3000 ppm. These dietary concentrations were adjusted during the lactation phase and for F_1 premating phase through postnatal day 42 to 0, 60, 300, 900 and 1800 ppm to maintain the approximate mean daily intakes observed during the premating and gestation phases. Based on substance intake in premating and gestation phases in both generations, these dietary concentrations approximated mean daily doses of 0, 7, 35, 105 and 210 mg/kg bw per day. The F_0 generation rats were bred within their treatment groups to produce F_1 litters after 71 days on test, and the F_1 rats were bred within their respective treatment groups to produce F_2 litters at least 71 days after weaning. In other words, there was one litter per generation. F_1 and F_2 litters were culled to four pups/sex per litter on postnatal day 4. On postnatal day 21, offspring in the F_1 litters were selected (one rat/sex per litter, when possible) to become F_1 adults and parents of an F_2 generation. The surviving F_2 pups were killed at weaning. End-points under evaluation included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, clinical pathology, reproductive indices, litter and pup parameters (including landmarks of sexual maturation), estrous cycle, sperm parameters, ovarian follicles, organ weights, as well as gross and microscopic pathology.

There were no test substance-related deaths or clinical signs of toxicity in parental animals in either generation. Adverse, test substance-related changes were observed at the two highest dose levels of 1500 and 3000 ppm. These comprised reductions in body weight, body weight gain and feed consumption, which often achieved statistical significance. Feed efficiency was also sometimes compromised.

No test substance-related gross lesions were observed at necropsy. Statistically significant liver weight increases were observed in females at the highest dose level. Kidney weight increases were also noted at 1500 ppm (Table 21). There were no test substance-related microscopic findings.

There were no effects on reproductive functions and performance up to the highest dose level of 3000 ppm. Mating behaviour, fertility, gestation length, parturition, litter size, pup and litter weight, sex ratio in pups, pup survival and lactation were not altered. Furthermore, estrous cycle and sperm parameters were not affected.

Table 21. Organ weight data in female rats in the two-generation reproduction study with triflumezopyrim

	Measure per dose level of triflumezopyrim				
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**1500 ppm**	**3000 ppm**
Liver weight					
F_0(P) females					
Absolute weight (g)	14.36	14.20	13.91	14.07	14.76
Relative weight (%)	4.31	4.30	4.31	4.41	4.67*
F_1 females:					
Absolute weight (g)	14.50	14.95	14.69	14.97	15.89*
Relative weight (%)	4.27	4.35	4.37	4.37	4.79*
Kidney weight					
F_0(P) females:					
Absolute weight (g)	2.26	2.27	2.24	2.30	2.41*
Relative weight (%)	0.68	0.69	0.70	0.72*	0.76*
F_1 females:					
Absolute weight (g)	2.42	2.40	2.42	2.58*	2.52
Relative weight (%)	0.71	0.70	0.72	0.75*	0.76*

ppm: parts per million; *: $P < 0.05$; *Source*: Munley (2015)

However, body weight gain in the pups in the highest dose group was diminished from day 4 onwards in both generations (Table 22). A two-day increase in the average time to preputial separation in F_1 pups was considered secondary to these body weight reductions. No offspring toxicity was seen at lower dose levels.

The NOAEL for parental toxicity was 500 ppm (equivalent to 35 mg/kg bw per day) based on decreased body weight and nutritional parameters at 1500 ppm (equivalent to 105 mg/kg bw per day).

The NOAEL for offspring toxicity was 1500 ppm (equivalent to 105 mg/kg bw per day) based on reductions in body weight in F_1 pups at 3000 ppm (equivalent to 210 mg/kg bw per day) during the lactation period and a (most likely secondary) delay in preputial separation in F_1 pups at 3000 ppm.

The NOAEL for reproductive toxicity was 3000 ppm (equivalent to 210 mg/kg bw per day), the highest concentration tested.

No evidence of endocrine disrupting properties was observed (Munley, 2015).

Table 22. Offspring effects in the two-generation reproduction study

	Measure per dose level of triflumezopyrim									
	0 ppm		100 ppm		500 ppm		1500 ppm		3000 ppm	
Parameter	**Mean**	***N***	**Mean**	***N***	**Mean**	***N***	**Mean**	***N***	**Mean**	***N***
F_0 (P)										
No. of viable pups/litter	12.2	26	13.4	26	13.1	27	12.3	27	12.8	29
% Day 1–4 survival	98.9	26	95.3	26	99.5	27	99.3	27	98.9	29
% Day 4–21 survival	91.8	26	99.5	25	99.5	27	99.5	27	100.0*	0
Pup weight/litter (g)										
At birth	6.6	26	6.7	26	6.9	27	6.8	27	6.3	29
Day 4	10.9	26	10.9	25	10.9	27	10.9	27	10.1*	29
Day 7	17.9	26	17.9	25	17.8	27	17.6	27	16.4*	29
Day 14	36.3	24	35.7	25	35.2	27	33.8*	27	32.1*	29
Day 21	55.4	24	55.8	25	55.2	27	54.1	27	49.2*	29
Balanopreputial separation (day)	44.4	30	44.7	30	44.1	30	45.2	29	46.4*	30
Vaginal patency (day)	32.5	29	32.5	30	32.1	30	32.7	30	33.3	30
F_1										
No. of viable pups/litter	13.7	27	13.0	28	12.9	28	13.3	24	13.5	29
% Day 1–4 survival	98.1	27	99.7	28	99.3	28	97.3	24	97.1	29
% Day 4–21 survival	99.5	27	99.6	28	100.0	28	99.5	24	98.3	29
Pup weight/litter (g)										
At birth	6.5	27	6.7	28	6.8	28	6.7	24	6.2	29
Day 4	10.3	27	10.5	28	11.1	28	10.2	24	9.3*	29
Day 7	17.0	27	17.2	28	18.0	28	16.3	24	14.4*	29
Day 14	35.8	27	35.6	28	36.6	28	33.5	24	30.2*	29
Day 21	56.5	27	56.9	28	58.2	28	52.7	24	45.8*	29

N: number of pregnant dams; no.: number; ppm: parts per million; *: $P < 0.05$ (Dunnett test, two-sided)

Source: Munley (2015)

(b) Developmental toxicity

Rat

In a developmental toxicity study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered by gavage to timemated Crl:CD(SD) female rats (20/dose group) on gestation days 6 through 20. The test substance was applied in 0.5% methylcellulose with 0.1% Tween 80 at daily doses of 0, 25, 50, 100 and 200 mg/kg bw per day. The dose volume was 10 mL/kg bw. Dams were euthanized on gestation day 21. Parameters evaluated in dams were body weight, body weight gain (absolute and corrected, that is, adjusted for the products of conception), feed consumption, survival, clinical signs, reproductive outcomes and gross pathology. Parameters evaluated in fetuses were incidences of external, visceral, head and skeletal malformations and variations.

There were no unscheduled deaths, clinical signs or abortions, but maternal toxicity was observed at the highest dose level as indicated by significant reductions in overall weight gain (~15% as compared to the control group over the treatment period) and feed consumption even though the mean absolute body weight was only slightly reduced. A very high decrease in body weight gain, by up to 77%, was noted on the first two days of dosing: mean body weight gain in high-dose dams on days 6 through 8 was 2.1 g as compared to 9.1 g in the control group.

In contrast, no adverse effects on number and weight of fetuses or the sex ratio were noted.

There was no increases in external, visceral or skeletal malformations, but two skeletal variations were statistically significantly increased (Table 23), albeit with no dose response.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on effects on body weight (gain) and feed consumption parameters at 200 mg/kg bw per day.

For developmental effects, the sponsor suggested that the NOAEL was 200 mg/kg bw per day, based on a lack of adverse effects at any dose. However, the significant increase in skeletal variations suggests developmental delay at the two highest dose levels. Even though short ribs were not accompanied by vertebral findings and can be expected to resolve at a later stage of development (as is typical for variations), the litter incidence in this study was 6 times higher than in the control group. Incomplete ossification of parietal skull was seen in seven fetuses in four litters. Both these figures are precisely at the upper limit of the historical control range, based on 19 studies performed between 2003 and 2013 in the same laboratory, that is, they will exceed the mean by far. Thus, the NOAEL for embryo/fetal toxicity was 50 mg/kg bw per day.

There was no evidence of teratogenicity since there was no increase in the incidence of any malformations. In addition, the observed variations are not likely to have resulted from a single exposure and, thus, are not suitable to derive the acute reference dose (ARfD) (Lewis, 2013).

Table 23. Fetal findings in the developmental toxicity study in rats

	Number of fetuses (litters) with the finding				
Parameter	**0 mg/kg bw per day**	**25 mg/kg bw per day**	**50 mg/kg bw per day**	**100 mg/kg bw per day**	**200 mg/kg bw per day**
Fetuses (litters) examined (external)	245 (19)	245 (20)	237 (20)	241 (20)	240 (20)
Fetuses (litters) examined (head/visceral)	124 (19)	124 (20)	119 (20)	122 (20)	119 (20)
Fetuses (litters) examined (skeletal–head)	121 (19)	121 (20)	118 (20)	119 (20)	121 (19)
Fetuses (litters) examined (skeletal–body)	245 (19)	245 (20)	237 (20)	241 (20)	240 (20)
Malformations					
Anus, atresia	–	–	–	1 (1)	–
Limb, bowed	–	–	–	1 (1)	–
Tail, tread-like	–	–	–	1 (1)	–
Brain, hydrocephaly	–	–	2 (1)	–	–

(*Continued on next page*)

Parameter	Number of fetuses (litters) with the finding				
	0 mg/kg bw per day	25 mg/kg bw per day	50 mg/kg bw per day	100 mg/kg bw per day	200 mg/kg bw per day
Lung lobe, absent	1 (1)	–	–	–	–
General, situs inversus	2 (2)	–	–	–	–
Lumbar arch, absent	–	–	–	1 (1)	–
Thoracic arch, absent	–	–	–	1 (1)	–
Thoracic arch, malpositioned	–	–	–	1 (1)	–
Lumbar centrum, absent	–	–	–	1 (1)	–
Thoracic centrum, absent	–	–	–	1 (1)	–
Rib, absent	–	–	–	1 (1)	–
Rib, fused	–	–	2 (1)	–	–
Variations					
Hyoid skull, unossified	–	1 (1)	–	1 (1)	–
Zygomatic skull, incomplete ossification	–	1 (1)	–	–	–
Interparietal skull, incomplete ossification	–	–	–	–	2 (2)
Supraoccipital skull, incomplete ossification	–	1 (1)	1 (1)	–	3 (2)
Parietal skull, incomplete ossification	–	1 (1)	1 (1)	–	7* (4)
Lumbar centrum, bipartite ossification	–	–	3 (1)	–	–
Thoracic centrum, bipartite ossification	7 (4)	4 (3)	11 (4)	7 (5)	7 (3)
Rib, short	1 (1)	1 (1)	1 (1)	7* (6)	9* (6)
Rib, cervical rib	1 (1)	–	–	1 (1)	3 (2)
Rib, wavy	–	–	1 (1)	–	–
Rib, short supernumerary	3 (3)	–	6 (4)	2 (2)	1 (1)
Sternebrae, unossified	–	–	–	–	1 (1)
Sternebrae, misaligned	–	–	1 (1)	–	2 (1)
Sternebrae, bipartite ossification	–	–	–	–	1 (1)
Sternebrae, incomplete ossification	–	–	–	–	1 (1)

bw: body weight; no.: number; *: $P < 0.05$; *Source*: Lewis (2013)

Rabbit

In a developmental toxicity study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered by gavage to time-mated New Zealand White [Hra:(NZW)SPF] female rabbits (22/group) on gestation days 7 to 28. The test substance was dissolved in 0.5% methylcellulose with 0.1% Tween 80 and applied at daily dose levels of 0, 50, 100, 250 or 500 mg/kg bw per day. The dose volume was 10 mL/kg bw. Parameters evaluated in does were survival, clinical signs, body weight, body weight gain (absolute and corrected, i.e. adjusted for the products of conception), feed consumption, haematology, reproductive outcomes, gravid uterine weights and gross pathology. Parameters evaluated in fetuses were body weight, fetal sex ratios, number of live and dead fetuses, fetal resorptions and incidences of external, visceral, head and skeletal malformations and variations.

One female at 500 mg/kg bw per day was euthanized in extremis on gestation day 26 due to body weight loss and markedly lower feed consumption; decreased defecation was also noted. All other females survived to the scheduled necropsy. However, lower mean body weight gains were noted in the highest dose group even though they were not associated with reduced mean body weight. Lower mean

feed consumption and decreased defecation were also observed in that group. In addition, haematological effects were confined to this high-dose group, consisting of lower mean RBC and eosinophil counts, slightly lower haematocrit and haemoglobin levels, but higher mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) levels and reticulocyte counts. The effects on RBC parameters were associated with higher mean spleen weights (absolute and relative to brain weight). There were no test substance-related macroscopic findings observed at any dose level. No test substance-related effects on body weight or feed consumption/feed conversion parameters, haematology or mean organ weights (including gravid uterine weight) were observed at 250 mg/kg bw or lower dose levels. Intrauterine fetal growth and survival were not affected by test substance administration at any dose level. Sex ratio was not skewed. No increase in fetal malformations or developmental variations was noted at any dose level and those that occurred were considered spontaneous in origin.

The NOAEL for maternal toxicity was 250 mg/kg bw per day based on clinical findings (decreased defecation), lower mean body weight gain and feed consumption, and haematological effects in line with what was seen in short-term studies in other species, at 500 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Charlap, 2013).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, triflumezopyrim (batch no. RAB55-037, purity 99.4%), dissolved in 0.5% methylcellulose with 0.1% Tween 80, was administered by gavage to male and female Crl:CD(SD) rats (12/sex per group) at doses of 0, 100, 500 or 2000 mg/kg bw. The dose volume was 10 mL/kg bw. A neurobehavioural test battery, consisting of FOB and motor activity assessments, was conducted on all rats prior to dosing, two hours after dosing (day 0) and on days 7 and 14. Other parameters evaluated included body weight, body weight gain, feed consumption, clinical signs and gross pathology. On day 16, 6 rats/sex per dose group were perfused in situ with a fixative. A microscopic neuropathological evaluation of the peripheral and central nervous systems and selected muscle tissues from the control and high-dose rats was conducted.

There were no unscheduled deaths or no clinical signs indicative of systemic toxicity. However, body weight losses were noted on the first day following dosing in males and in females at the two highest doses. These findings (which were not dose related in females) were associated with a lower feed intake in all these groups. In mid- and high-dose males, overall body weight gain was lower over the first seven days with the difference to the control group being statistically significant at the maximum dose level. This period was followed by a phase (days 7–14) in which, in contrast, the body weight gain was markedly higher at these dose levels. During this second week, feed consumption in males was significantly increased in all treated groups. In females, overall body weight gain and feed intake were not affected, leaving aside the immediate effect of dosing.

Most likely as a secondary effect to the initial body weight losses and lower body weight gain, some changes in neurobehavioural parameters and motor activity were observed on the day of dosing (but not at later time points) at 500 and 2000 mg/kg bw. On day 0, there was a significant increase in forelimb grip strength observed in females administered 2000 mg/kg bw. A reduction in the mean number of rearing movements was noted in the same female group on the same day. Overall motor activity (number and duration of movements) was decreased in males and females at 500 and 2000 mg/kg bw. Males and females at 2000 mg/kg bw also had a statistically significant higher incidence compared to control of high posture while in the open field. In addition, reductions in body temperature were noted in males and females administered 500 and 2000 mg/kg bw. The strongest difference was noted in high-dose females, with a mean of 33.0 °C at the highest dose level as compared to 34.6 °C in the control group.

There were no test substance-related gross or microscopic neuropathological findings.

The NOAEL was 100 mg/kg bw for males and females based on transient body weight losses and reductions in body weight gains, feed consumption, body temperature and motor activity that occurred in males and females on the day of dosing at 500 and 2000 mg/kg bw (Carpenter, 2013c).

No separate short-term neurotoxicity study was performed. However, neurotoxicological parameters were investigated in a 90-day oral feeding study in rats (see section 2.2a). No functional or pathological changes that could be indicative of neurotoxicity were observed in this study. Thus, the NOAEL for neurotoxicity was 6000 ppm (274 and 316 mg/kg bw per day in males and females, respectively), the highest tested dose tested (Papagiannis, 2013a).

(b) Immunotoxicity

In a 28-day immunotoxicity feeding study, triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to five groups of female Crl:CD(SD) rats (10/group) at dietary concentrations of 0, 100, 500, 2000 and 6000 ppm (equivalent to 0, 8.8, 41, 166 and 474 mg/kg bw per day). Cyclophosphamide (25 mg/kg bw per day) was administered to a sixth group as a positive control. Prior to termination, the immune system was stimulated by injecting sheep red blood cells (sRBCs) on day 24. Blood samples were collected from each rat on day 29. The serum samples were assayed for their concentrations of sRBCspecific immunoglobulin M (IgM) antibodies to provide a quantitative assessment of humoral immune response. Other parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, gross pathology and selected organ weights.

Although there were some systemic effects on body weight, body weight gain and feed efficiency at the two highest dose levels (which was in line with the outcome of the short-term toxicity studies), no evidence of immunotoxicity was obtained because absolute and relative spleen and thymus weights or the humoral immune response were not altered.

The NOAEL for immunotoxicity was 474 mg/kg bw per day, the highest dose tested (Hoban, 2015).

(c) Mechanistic studies

A number of mechanistic studies were performed to investigate mainly the potential of triflumezopyrim to cause endocrine disruption and to provide mechanistic explanations for the tumours observed at high-dose levels in the long-term studies in rats and mice.

Tests for endocrine activity and investigations on the mechanism behind uterine tumours

While the reproductive and developmental studies did not suggest that triflumezopyrim has a potential to cause adverse effects by interaction with the endocrine system, there was an increase in likely hormone-dependent uterine tumours in high-dose females as well as an increase in testicular hyperplasia in high-dose males in the long-term study in rats (Papagiannis, 2015c). In addition, a higher mean uterus weight was noted in a 90-day feeding study in rats (Ford, 2015). These findings justify a need for further studies.

Triflumezopyrim (batch no. RAB55-037, purity 99.4%) was tested in a number of receptor binding assays with dopamine (produced by human recombinant CHO or CHO-K1 cells) and melatonin (isolated from chicken brain or obtained from human recombinant CHO-K1 cells). Methods employed in this non-standard study were adapted from the scientific literature to maximize reliability and reproducibility. Results were quantified by radioligand binding and evaluated for either stimulation or inhibition. No increases or decreases by 50% or more were observed. According to the criteria for assumption of an effect used in this study, there was no evidence of a positive response (Hsing, 2015).

The ability of triflumezopyrim (batch no. RAB55-004, purity > 99%) to bind to testosterone or estrogen receptors was evaluated by means of competitive in vitro binding assays. In this study, it was investigated if and to what extent the test substance would compete with [^{3}H]methyltrienolone in binding to the human testosterone receptor in LnCAP cells and with 17-β-estradiol in binding to the human estrogen receptor in MCF-7 cells. Triflumezopyrim was evaluated at eight concentrations between 1.0×10^{-8} and 1.0×10^{-4} mol/L. Radioinert methyltrienolone, a known testosterone receptor agonist, and radioinert 17-β-estradiol, an estrogen receptor agonist, were used as positive controls and to verify test system performance; these positive controls gave the expected strong binding responses. In contrast, triflumezopyrim did not competitively bind to the testosterone or estrogen receptors when tested up to the maximum concentration of 1.0×10^{4} mol/L (Nabb, 2015b).

Triflumezopyrim (batch no. RAB55-037, purity 99.4%) was evaluated in a uterotrophic assay for its potential to act as an estrogen receptor agonist. Another objective of the study was to investigate a possible impact of the test substance on prolactin levels in serum. Three groups of young adult ovariectomized Crl:CD(SD) rats (15/group) were dosed by gavage with 0, 300 or 500 mg/kg bw per day of the test substance for six consecutive days. The animals were killed approximately 2–4 hours after the last administered dose. Two separate ovariectomized positive control groups received either 0.1 mg/kg bw per day of the estrogen receptor agonist 17α-ethynyl estradiol or 20 mg/kg bw per day of the dopamine (D2) receptor agonist bromocriptine mesylate. Body weights and clinical observations were recorded daily and feed consumption was recorded on days 1 and 6. Vaginal cytology was evaluated daily to assess the potential of the test substance to induce cytological changes consistent with those observed with the 17α-ethynyl estradiol positive control. At necropsy, uterine weights were determined in order to assess the ability of the test substance to induce uterine growth. Blood was collected at termination from all animals for analysis of serum prolactin concentrations.

There were no unscheduled deaths, and clinical signs were confined to eyelid ptosis in all rats following administration of the positive control substance bromocriptine mesylate. Yellow-stained bedding was observed in the cages of all rats administered 300 and 500 mg/kg bw per day of triflumezopyrim on days 2–6 and 1–6, respectively, most likely reflecting the excretion of the test article. Over the treatment period, rats at 300 and 500 mg/kg bw per day lost an average of 3.7 and 15.9 g body weight, respectively (representing 1.3% and 5.7% of initial body weight, respectively), compared to a gain of 17.2 g in the negative control group. In consequence, the mean final body weight in rats administered 300 and 500 mg/kg bw per day triflumezopyrim at termination was statistically significantly decreased by approximately 7% and 11% compared to the negative control group values, respectively. Mean daily feed consumption at 300 and 500 mg/kg bw per day was 34% and 54% lower than in the negative control group, respectively. Mean daily feed efficiency was also compromised in the treated groups. Adverse effects on body weight (gain), feed consumption and efficiency were also observed with the two positive control substances.

All animals receiving triflumezoyprim remained in diestrous for the duration of the study. At necropsy, no gross observations were noted. No effects on uterine weight were observed demonstrating that the test substance was negative in the uterotrophic assay. However, following administration of the test substance at both dose levels, serum prolactin levels at termination were reduced by 32% and 60%, respectively, although only the decrease at 500 mg/kg bw per day was statistically significant (Table 24).

Table 24. Serum prolactin levels following administration of triflumezopyrim or the positive control substances to ovariectomized female rats

Mean prolactin levles ± SD (ng/mL) [a]				
Vehicle control	**Triflumezopyrim, 300 mg/kg bw/day**	**Triflumezopyrim, 300 mg/kg bw/day**	**17α-ethynyl estradiol, 0.1 mg/kg bw/day**	**Bromocriptine mesylate, 20 mg/kg bw/day**
21.0 ± 15.9	14.3 ± 10.7	8.3 ± 8.0*	38.2 ± 22.5	0.4 ± 0.5*

bw: body weight; SD: standard deviation; *: $P < 0.05$ (Dunnett non-parametric test, two-sided) *Source*: Kanefsky (2015)

[a] Fifteen rats/group.

The two positive control substances gave the expected responses.

All 15 rats administered 17α-ethynyl estradiol showed cytological markers indicative of either proestrous or estrous on day 4. At necropsy, 12/15 rats had uterine fluid within the uterus lumen and increased uterine weights. Absolute uterine wet weight and blotted weight were increased to 198% and 162% of the negative control, respectively. The relative organ weight was also higher. Serum prolactin levels appeared higher compared to the negative control group, but the difference was not statistically significant and the standard deviation was very high.

In contrast, all animals administered bromocriptine mesylate remained in diestrous for the duration of the study. At necropsy, no gross observations and no effects on uterine weight were noted. Serum prolactin levels were decreased by 98% compared to the negative control. It may be concluded that triflumezopyrim acted in this test in a way similar to the dopamine (D2) receptor agonist even though the decrease in prolactin was less pronounced. There was no evidence of an estrogen-like mode of action of triflumezopyrim (Kanefsky, 2015).

A mechanistic study was performed in (intact) adult female Crl:CD(SD) rats to evaluate whether alterations in progesterone to estrogen ratio, secondary to alterations in serum prolactin concentrations, might explain the induction of the uterine tumours in the two-year combined chronic toxicity/carcinogenicity feeding study in rats (Papagiannis, 2015c).

For this purpose, young adult females (50/group; ~100 days old at study start, non-pregnant and nulliparous) were fed either a control diet or a diet containing 8000 ppm triflumezopyrim (batch no. RAB55-037, purity 99.4%) for approximately 90 days. For the treated group, a mean daily intake of 389 mg/kg bw triflumezopyrim was calculated. A third group ($n = 50$) was administered the positive control substance bromocriptine mesylate (in 0.1% Tween 80 in 0.5% methylcellulose prepared deionized water) by gavage at a dose of 10 mg/kg bw daily. For dosing, this dopamine receptor agonist was dissolved in 0.1% Tween 80 in 0.5% methylcellulose prepared deionized water. The animals were monitored daily for clinical signs of toxicity and underwent a more detailed clinical examination once a week. Body weight and feed consumption were determined at least weekly throughout the study. Blood was collected by sublingual blood collection after two weeks and one month, and serum was prepared for hormonal assessment (prolactin, estradiol and progesterone) by means of commercially available radioimmunoassay kits. Vaginal lavage samples were taken immediately after blood collection in order to correlate serum hormone concentrations with the estrous status of the animals. At scheduled kill, surviving rats were necropsied. Liver, uterus, ovary and pituitary were weighed.

There were no unscheduled deaths in the treated animals. Some clinical signs of toxicity, beginning on day 6 or 7, were attributed to test substance administration. These signs comprised dehydration, ruffled fur and hunched or high posture. In the positive control group, three rats were found dead or were euthanized for humane reasons, but the causes of these deaths were unknown.

In the triflumezopyrim group, statistically significant decreases in mean body weights were first noted on day 8 and continued throughout treatment. Mean final body weights were 15.1% lower than in the vehicle control group at necropsy. Feed consumption and feed efficiency were clearly impaired. Similar but less pronounced effects on body weight and feed efficiency were observed in the positive control group (Table 25).

Changes in absolute and relative organ weight in both the triflumezopyrim-treated and the positive control groups reflect the lower mean body weights (in particular with regard to the liver). Nevertheless, they may be indicative of an effect of both triflumezopyrim and bromocriptine mesylate on the uterus since an increase was noted even though the effect was not significant. The positive control substance also had an effect on the pituitary (decrease) and on the ovary weight (increase). An overview of organ weight findings is given in Table 25.

Table 25. Body and organ weight data following administration of triflumezopyrim or bromocriptine mesylate

	Measure (mean ± SD)		
Parameter	**Negative control group**	**Triflumezopyrim**	**Bromocriptine mesylate [a]**
Terminal body weight (g)	319.1 ± 20.7	270.9 ± 16.8*	309.1 ± 18.3*
Absolute liver weight (g)	10.021 ± 0.859	10.295 ± 1.189	9.881 ± 0.882
Relative liver weight (%)	3.144 ± 0.229	3.797 ± 0.329*	3.196 ± 0.203
Absolute pituitary weight (mg)	17 ± 5	14 ± 3[#]	13 ± 3[#]
Relative pituitary weight (%)	0.005 ± 0.001	0.005 ± 0.001	0.004 ± 0.001[#]
Absolute uterus weight (g)	0.828 ± 0.347	0.790 ± 0.329	1.006 ± 0.630
Relative uterus weight (%)	0.260 ± 0.109	0.292 ± 0.122	0.327 ± 0.213
Absolute ovaries weight (mg)	142 ± 52	121 ± 20[#]	168 ± 33[#]
Relative ovaries weight (%)	0.044 ± 0.015	0.045 ± 0.007	0.055 ± 0.011[#]

SD: standard deviation; *: $P < 0.05$ (Dunnett test, two-sided); [#]: $P < 0.05$ (Dunnett non-parametric test, two-sided)

[a] Dopamine receptor agonist positive control.

Source: O'Connor (2016)

There were no gross pathological findings in any group that could be attributed to treatment. However, there was an apparent shift in the stages of the estrous cycle in the group treated with triflumezopyrim. In fact, there was a marked increase in the number of females in diestrous and a reduction in the number of rats in estrous. Such a change was not apparent in the group receiving the positive control substance.

In the group receiving the dietary triflumezopyrim treatment, estradiol, progesterone and prolactin were significantly reduced after two weeks and after one month (Table 26).

Table 26. Hormonal changes in intact female rats following administration of triflumezopyrim or bromocriptine mesylate

	Hormone measures (mean ± SD) [a]		
Determination time	**Prolactin (ng/mL)**	**Estradiol (pg/mL)**	**Progesterone (ng/mL)**
Fourteen days			
Negative control	55.9 ± 65.3	102 ± 31.6	10.7 ± 9.87
Triflumezopyrim	4.67 ± 9.68*	67.5 ± 17.1*	4.42 ± 5.14*
Bromocriptine mesylate	28.5 ± 37.5	78.1 ± 23.4*	10.0 ± 6.92
One month			
Negative control	54.0 ± 51.7	111 ± 32.5	15.4 ± 11.9
Triflumezopyrim	26.1 ± 33.9*	84.9 ± 27.9*	9.4 ± 8.98*
Bromocriptine mesylate	33.1 ± 25.6	94.9 ± 30.1*	13.8 ± 8.03

SD: standard deviation; *: $P < 0.05$ (Dunnett non-parametric test, two-sided)

[a] Mean of all animals/group, regardless of stage of estrous cycle.

Source: O'Connor (2016)

Since the decrease of progesterone was more pronounced than that of estradiol, the progesterone to estradiol ratio was skewed to the estrogen side. The positive control substance had a similar effect on estrogen and prolactin whereas progesterone levels were not affected. However, there is considerable uncertainty in this data as indicated by the large standard deviations; this is partly due to different estrous cycle stages. Furthermore, the reduction in concentration of all three hormones, but in particular prolactin, in the group receiving triflumezopyrim were much more pronounced after 14 days of treatment than after 1 month, casting doubts on hormonal levels in the two-year Papagiannis (2015c) study. Ageing processes are also not reflected by this data. Nonetheless, it may be concluded that the endocrine milieu in the female rat is altered by dietary administration of triflumezopyrim at least at high doses.

Mechanistic study on possible mode of action for liver tumour formation in the mouse

A mechanistic study on liver toxicity was conducted in male Crl:CD (ICR) mice because a significant increase in liver adenoma had been observed in male mice of this strain in an 18-month feeding study by Papagiannis (2015d). Triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to mice (30 animals/dose level) via their diets for up to 29 days. Interim kills of 10 animals/group took place on days 3 and 8, following two and seven days of exposure, respectively. Nominal dietary concentrations were 200, 800, 2500 and 7000 ppm (corresponding to 35, 149, 420 and 1210 mg/kg bw per day, respectively, for the day 3 subset; 35, 151, 450 and 1275 mg/kg bw per day for the day 8 subset; 32, 133, 411 and 1273 mg/kg bw per day for the entire study period, i.e. the day 29 subset, respectively). A negative control group of the same size was also included, as well as a positive control group receiving 1000 ppm of phenobarbital salt.

Parameters evaluated during the in-life phase included body weight, body weight gain, feed consumption and clinical signs. At termination, liver and duodenum were grossly examined. Liver weights were determined. Liver sections taken from all animals at necropsy were examined for microscopic pathology (using haematoxylin and eosin staining), cell proliferation (Ki67 labelling) and induction of CYP450 enzyme activities. Gene expression for CYP enzymes was evaluated in livers from the control, 7000 ppm group and positive control groups from the day 3 and day 8 subsets.

All animals in all subsets survived to scheduled kill. Absolute and relative liver weights were increased at the two highest dose levels at all time points, as well as in the phenobarbital group. In the same groups, minimal (and in the positive control group also mild or moderate) centrilobular hypertrophy of hepatocytes was observed. Other histological findings, that is, an increase in mitotic figures and individual cell necrosis, were confined to the phenobarbital group and not observed with triflumezopyrim. However, staining with Ki67 revealed evidence of hepatocellular proliferation on day 8 in the group receiving 7000 ppm of the test substance and in the phenobarbital group at all time points.

Dietary exposure to triflumezopyrim at 7000 ppm was associated with changes in enzyme activity and gene expression similar to those seen with phenobarbital and consistent with constitutive androstane receptor (CAR) activation. There were increases in the activities of CYP2B and in the gene expression for CYP2B10 but also in CYP1A activity and CYP1A1 gene expression. Effects on CYP3A and CYP4A were less pronounced and there was always consistency between enzyme activity and gene expression. Total CYP content was increased in the 7000 ppm group only on day 8 but to a slightly lower extent than with phenobarbital. Effects at 2500 ppm, if any, were minimal. There were no biologically relevant effects at 200 or 800 ppm (Mukerji, 2015a).

Mechanistic studies on possible mode of action and human relevance of lung tumours in the mouse

A study was conducted to evaluate potential cell proliferation in lungs of female mice following short-term exposure (≤7 days) to triflumezopyrim. Triflumezopyrim (batch no. RAB55-037, purity 99.4%) was administered to female Crl:CD1 (ICR) mice (20/concentration) at concentrations of 0, 200, 2500 or 7000 ppm. A positive control substance, isoniazid, was administered at 1300 ppm. Ten female mice per group were killed at each time point, that is, on day 4 and day 8 (equivalent to three days or seven days of dietary exposure, respectively, due to initiation of exposure on day 1). The overall mean daily intake of triflumezopyrim at 200, 2500 and 7000 ppm was calculated as 39, 462 and 1235 mg/kg bw per day for the day 4 subset, and as 44, 533 and 1581 mg/kg bw per day for the day 8 subset, respectively. The overall mean daily intake of 1300 ppm isoniazid corresponded to 247 and 273 mg/kg bw per day for the day 4 and day 8 subsets, respectively. Parameters evaluated during the in-life phase included body weight, body weight gain, feed consumption and clinical signs. Animals were administered 5-bromo-2′-deoxyuridine (BrdU) using surgically implanted Alzet® osmotic pumps, starting near the day of study initiation and continuing until termination. Lung sections from all animals were examined microscopically for both histopathology (using haematoxylin and eosin staining) and cellular proliferation assessment (BrdU labelling).

Short-term dietary exposure of female mice to triflumezopyrim at concentrations of up to 7000 ppm did not result in any test substance-related microscopic findings in the lung or any effects on cellular proliferation of terminal bronchiolar epithelium. In addition, there were no toxicologically significant effects observed on any in-life parameter evaluated during the study. Mice administered the positive control substance, in contrast, displayed a greater proliferative response of bronchiolar epithelium within terminal bronchioles compared to control mice at both the day 4 and day 8 examination time points, thereby confirming the adequacy of the methods used in the study to detect a proliferation response (Mukerji, 2015b).

The potential differential metabolism of triflumezopyrim (batch no. RAB55-037, purity 99.4%) in mouse and human lung tissue was assessed. FemalevCD-1 (ICR) mouse and human lung microsomes (from a female donor) were incubated with triflumezopyrim in the presence of a nicotinamide adenine dinucleotide (reduced) (NADH)-regenerating system, and samples were collected over 180 minutes. Inhibitors specific to Cyp2e1 (4-methyl pyrazole) and Cyp2f2 (5-phenyl-1-pentyne) were added to the lung microsome assay to evaluate the specificity of both P450 enzymes in the metabolism of triflumezopyrim.

Triflumezopyrim was not metabolized by female mouse or female human lung microsomes with or without inhibitors. No activation by Cyp2f2 was observed. (Cyp2f2 is a prominent P450 in mouse lung that metabolizes a number of primary lung toxicants.) The in vitro clearance rate of triflumezopyrim in female mouse and female human microsomes could not be calculated and, therefore, a comparison between species could not be obtained (Nabb, 2015c).

(d) Toxicity of metabolites

No toxicological studies with metabolites of triflumezopyrim have been performed. The proposed definition of residue for triflumezopyrim in rice is parent only. In rice grain plus hull, the major portion of the residue was parent. Low levels of two metabolites, IN-Y2186 and IN-RPD47, were detected in some residue samples, but mean residue levels for both were below the limit of quantitation (< 0.01 mg/kg). Accordingly, human exposure is extremely low. In addition, the sponsor provided information on the use of quantitative structure–activity relationship [(Q)SAR] models to assess structural alerts for mutagenicity for IN-Y2186 and IN-RPD47, in comparison to triflumezopyrim, using the following software packages: OECD QSAR Toolbox v3.4 and ADMET Predictor v7.2 (Ames, chromosomal aberration); Discovery Studio v4.1TOPKAT (Ames); Leadscope v4.4 (Ames, chromosomal aberration, hypoxanthine guanine phosphoribosyltransferase [HGPRT] mutation, mouse lymphoma, micronucleus); and OASIS TIMES v2.27.19 (Ames, chromosomal aberration, comet, clastogenicity, transgenic rodent and micronucleus). The functional group profiles of IN-Y2186 and IN-RPD47 were essentially covered by that of triflumezopyrim which itself was concluded as being not genotoxic (section 2.4). The universal consensus from all the models indicates a lack of mutagenic potential for both metabolites. Thus, the absence of toxicological studies is not considered a data gap.

3. Observations in humans

Triflumezopyrim is a newly developed active ingredient that has not yet been manufactured on an industrial scale for commercial use. Accordingly, only a limited number of workers have been involved with the manufacture of this compound to date. The sponsor provided the information that no health problems have been reported in employees who were involved in the handling, testing or manufacturing of triflumezopyrim. No related information was available from the open literature.

Comments

Biochemical aspects

Following oral administration of a single dose of ^{14}C-radiolabelled triflumezopyrim at 10 mg/kg bw to rats, the compound was rapidly absorbed. Absorption accounted for 80–90% of the administered dose. Absorption was lower (60–70%) when a single high dose of 200 mg/kg bw was administered. The absorbed substance was rapidly and widely distributed throughout the body, with highest concentrations found in liver and kidneys. Relatively high levels were also found in skin and bone marrow in male rats and in the gastrointestinal tract and its contents in females, although absolute levels were low within 24 hours. There were no other marked sex differences in kinetics or metabolism, and dose and position of radiolabel also had little effect. Excretion was biphasic, rapid and nearly complete within 48 hours, mainly via urine and faeces. There was evidence of significant biliary excretion, followed by enterohepatic circulation. However, potential for accumulation was low.

Triflumezopyrim was extensively metabolized by hydroxylation, oxidation, hydrolysis, decarboxylation and rearrangement reactions at various positions in the molecule, resulting in a number of metabolites. Hydroxylation of the trifluoromethyl phenyl ring formed IN-R6U70, found in rat faeces (14–27% of the administered dose) and urine (0.7–2.2% of the administered dose). Most of the minor metabolites were present in urine and faeces at concentrations of less than 1%. Up to 41% of unchanged triflumezopyrim was excreted in urine and 18% in faeces. The pattern of ADME was very similar when radiolabelled triflumezopyrim (10 mg/kg bw) was administered once daily for 14 consecutive days to female rats except that only six metabolites could be identified (Himmelstein, 2015, 2016).

Toxicological data

The acute toxicity of triflumezopyrim was studied in rats after oral administration ($LD_{50} \geq 4930$ mg/kg bw; Carpenter, 2013a; Merrill, 2014a; Fallers, 2014), dermal administration ($LD_{50} \geq 5000$ mg/kg bw; Lowe, 2012a) and inhalation ($LC_{50} \geq 5$ mg/L; Lowe, 2012b). Triflumezopyrim was not irritating to the skin of rabbits (Lowe, 2012c; Merrill, 2014b). Eye irritation studies in rabbits found no (Merrill, 2014c) or only very mild effects (Lowe, 2012d). There was no evidence of skin sensitization in the guinea pig (Lowe, 2012e; Merrill, 2014d).

Oral short-term toxicity studies (mostly feeding studies) were performed in mice, rats and dogs. In all three species, the liver was a target organ and adverse effects on the haematopoietic system were also observed. Some additional findings observed in only one or two studies might reflect the use of different batches.

In a 28-day study in the mouse, the dietary dose levels were 0, 200, 800, 2500 or 7000 ppm (equal to 0, 34, 129, 416 and 1100 mg/kg bw for males and 0, 41, 161, 504 and 1340 mg/kg bw for females, respectively). The NOAEL was 2500 ppm (equal to 416 mg/kg bw per day) based on reduced haematocrit and haemoglobin, increased reticulocyte counts and extramedullary haematopoiesis in the spleen. The effects on the liver (increased weight, hepatocellular hypertrophy and stimulation of microsomal UDPGT activity and a number of CYP enzymes) were considered adaptive rather than adverse, reflecting the mode of action behind most of the findings in the rodent liver (Anand, 2015).

In the 90-day feeding study in mice, dietary doses of 0, 200, 800, 2500 or 7000 ppm (equal to 0, 31, 125, 417 and 1130 mg/kg bw per day for males and 0, 44, 177, 476 and 1530 mg/kg bw per day for females, respectively) were administered. The NOAEL was 2500 ppm (equal to 417 mg/kg bw per day) based on adrenal hyperplasia at 7000 ppm (equal to 1130 mg/kg bw per day) in males, suggesting a possible additional effect of triflumezopyrim at the high dose, albeit of equivocal toxicological significance. In addition, findings at the two highest dose levels confirmed the adaptive liver effects seen in the 28-day study but not the haematological findings (Papagiannis, 2015a).

In a 14-day oral gavage study that also examined toxicokinetics and genotoxicity, rats were administered 0, 25, 300 or 1000 mg/kg bw per day with the high dose reduced to 600 mg/kg bw per day on day 5. The NOAEL was 25 mg/kg bw per day based on reductions in T_3 and/or T_4 levels at the high and mid dose level, which may be related to the effects on the liver (increased organ weight, hypertrophy of hepatocytes and periportal vacuolation). Toxicokinetic parameters such as C_{max}, T_{max} and AUC confirmed the results of the ADME studies. There was no evidence of genotoxicity (i.e. clastogenicity) as no micronucleus induction in reticulocytes was seen (Nabb, 2015a).

Three 28- or 90-day feeding studies in rats were performed. In the 28-day study, the dietary dose levels were 0, 200, 800, 4000 and 20 000 ppm (equal to 0, 17, 65, 309 or 653 mg/kg bw per day for males and 0, 16, 64, 317 and 627 mg/kg bw per day for females, respectively) (Anand, 2013). In the 90-day studies, the dietary dose levels were 0, 100, 400, 1500 or 6000 ppm (equal to 0, 4.5, 18, 70 and 274 mg/kg bw per day for males and 0, 6, 23, 83 and 316 mg/kg bw per day for females, respectively, in the Papagiannis (2013a) study and 0, 4.2, 17, 63.9 and 257.1 mg/kg bw for males and 0, 5.1, 20.4, 74.2 and 278 mg/kg bw for females, respectively, in the Ford (2015) study). Decreases in body weight and body weight gain and in feed consumption and efficiency were consistently seen in these studies. Induction of microsomal liver enzymes (certain CYPs and UDPGT) was observed, supporting the mode of action for liver weight increases and liver cell hypertrophy (Anand, 2013). Haematological changes were observed in the 28-day (Anand, 2013) and the more recent 90-day study (Ford, 2015). The absence of an effect on RBCs in the earlier 90-day study (Papagiannis, 2013a) might be because the changes were relatively modest and occurred only at the highest dose. The same considerations apply to the increased uterine weight observed at the highest dose only in the Ford (2015) study.

Although a lower NOAEL of 17 mg/kg bw per day (based on slight reductions in body weight gain and feed consumption in males only at the LOAEL of 65 mg/kg bw per day) was identified in the 28-day study, the nearly identical NOAELs of 64 and 70 mg/kg bw per day in the two 90-day studies (Papagiannis, 2013a; Ford, 2015) conducted using a higher number of animals appear more robust.

Special investigations of neurotoxicity in the Papagiannis (2013a) study did not reveal any neurotoxic potential.

In a 90-day study in dogs, triflumezopyrim was administered at dietary doses of 0, 100, 400, 1000 or 4000 ppm (equal to 0, 3, 12.2, 26.6 and 115 mg/kg bw per day for males and 0, 2.7, 12.2, 26.9 and 131.0 mg/kg bw per day for females, respectively), the NOAEL was 400 ppm (equal to 12.2 mg/kg bw per day) based on lower body weight gain and feed consumption, decreased thymus weights and thymus lymphoid depletion at 1000 ppm (equal to 26.6 mg/kg bw per day). Haematological effects such as extramedullary haematopoiesis in the liver or decreases in RBC parameters were confined to the highest dose (Papagiannis, 2013c).

In a one-year study in dogs, triflumezopyrim was administered at dietary doses of 0, 40, 100, 400 or 2000 ppm (equal to 0, 1.5, 3.3, 11.1 and 53.2 mg/kg bw per day for males and of 0, 1.2, 3.4, 10.8 and 55.9 mg/kg bw per day for females, respectively), the NOAEL was 2000 ppm (equal to 53.2 mg/kg bw per day), the highest dose tested (Papagiannis, 2015b). The reasons for these differences between the two dog studies are unknown but may be due to the use of different test batches.

In an 18-month study in mice (Papagiannis, 2015d), triflumezopyrim was administered at dietary doses of 0, 200, 800, 2500 or 7000 ppm (equal to 0, 20, 84, 248 and 727 mg/kg bw per day for males and 0, 22, 88, 283 and 810 mg/kg bw per day for females). The NOAEL was 800 ppm (equal to 84 mg/kg bw per day) based on a marked increase in liver weight (up to 16%) in both sexes and increased occurrence of centrilobular hypertrophy of hepatocytes in males at 2500 ppm (equal to 248 mg/kg bw per day). The progression of hypertrophy to tumours at the highest dose indicates that these liver effects in mice are potentially adverse. Moreover, an increase in extramedullary haematopoiesis in the spleen was observed in males at the two highest dose levels. In females, splenic haematopoiesis and spleen weights were significantly increased at the highest dose level. In addition, there was an increase in hepatic haematopoiesis in high-dose females.

A significant increase in liver adenomas was observed in males at the highest dose level. There is some evidence from the 28-day study in mice (Anand, 2015) and from a mechanistic study (Mukerji, 2015a) that this oncogenic effect might be related to CAR activation leading to increased gene expression, induction of hepatic CYP enzymes and hepatocellular hypertrophy. These events in rodents can be accompanied by cell proliferation and may eventually result in the development of liver tumours provided exposure is sufficiently high and long-lasting. Binding to and activation of CAR plays a crucial role in this mode of action, which is shared by phenobarbital. This mode of action is generally considered of low relevance to humans.

In female mice, there was an increase in the incidence of bronchoalveolar carcinoma and (combined) adenoma and carcinoma at the highest dose level, as indicated by a statistically significant positive trend. On the other hand, there were no significant differences in the pairwise comparison. No plausible mode of action has been described. However, mechanistic studies have shown that triflumezopyrim is not metabolized either by mouse or human lung microsomes (Nabb, 2015c) and that there was no increase in proliferation of the bronchoalveolar epithelium in female mouse lung following three or seven days of dietary administration of triflumezopyrim (Mukerji, 2015b). Although these results do not exclude the relevance of the oncogenic effect, it appears that any such response would require prolonged duration of exposure to a high dose.

In a two-year chronic toxicity and carcinogenicity study in rats (Papagiannis, 2015c), triflumezopyrim was administered at dietary concentrations of 0, 100, 500, 2000 or 8000 ppm (equal to 0, 3, 15.9, 70.6 and 283.8 mg/kg bw per day for males and 0, 3.2, 17.3, 73.8 and 395.9 mg/kg bw per day for females, respectively). Because of marked decreases in body weight and body weight gain and on feed consumption and efficiency, the MTD was clearly exceeded in males at the highest dose and in females at the two highest dose levels, with effects observed, in particular in females, even during the first year of the study. At termination, mean body weight of surviving females at 2000 ppm was more than 20% lower than that of the controls; in surviving females at 8000 ppm, mean body weight was nearly 43% lower than that of the controls. The NOAEL was 500 ppm (15.9 mg/kg bw per day) based on effects on body weight, feed intake and efficiency, increases in liver and uterus weights and non-neoplastic histopathological findings in liver, lungs, testes and uterus.

Although no carcinogenic effects were observed in male rats, there was a significant increase in the incidence of liver adenoma and in (benign) granular cell tumours and malignant uterine tumours (squamous cell carcinoma and endometrial adenocarcinoma) in high-dose females. (The

possible explanation for the liver tumours is the same as for mice.) For uterine tumours, an endocrine-mediated mechanism based on a reduction in prolactin and subsequent disturbances in estrogen and progesterone levels has been proposed. This mode of action is supported, in part, by mechanistic studies (Kanefsky, 2015; O'Connor, 2016) and by evidence from the open literature (Harleman et al., 2012; Yoshida, Inoue & Takahashi, 2015). However, the Meeting considered that the extremely reduced body weight in high-dose females was more likely to have contributed to uterine tumour development.

The Meeting concluded that triflumezopyrim showed some evidence of carcinogenicity in male (liver) and female mice (lungs). The increase in uterine and liver tumours occurred only at an excessively high dose in female rats. The findings in the rat were considered not relevant for human risk assessment at dietary exposure levels.

The Meeting noted that the trend tests for carcinogenic effects used by the sponsor in the long-term studies were performed by rank order, not by administered doses. There are arguments for and against both approaches.

Triflumezopyrim was tested for genotoxicity in an adequate range of studies, both in vitro and in vivo. There was limited evidence of genotoxicity in vitro but no evidence in vivo.

The Meeting concluded that triflumezopyrim is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and because tumours were observed only at very high doses, the Meeting concluded that triflumezopyrim is unlikely to pose a carcinogenic risk to humans at levels occurring in the diet.

In a two-generation study in rats, triflumezopyrim was administered at dietary doses of 0, 100, 500, 1500 or 3000 ppm (equivalent to 0, 7, 35, 105 and 210 mg/kg bw per day) (Munley, 2015). The NOAEL for parental toxicity was 500 ppm (equivalent to 35 mg/kg bw per day) based on decreased body weight, body weight gain and feed consumption in adult rats at 1500 ppm (equivalent to 105 mg/kg bw per day). The NOAEL for offspring toxicity was 1500 ppm (equivalent to 105 mg/kg bw per day) based on decreased body weight gain and subsequent delay in preputial separation at 3000 ppm (equivalent to 210 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equivalent to 210 mg/kg bw), the highest dose tested.

In a developmental toxicity study in rats, triflumezopyrim was administered by gavage at dose levels of 0, 25, 50, 100 and 200 mg/kg bw per day on gestation days 6 through 20. The NOAEL for maternal toxicity was 100 mg/kg bw per day based on reduced body weight gain and feed consumption, which started soon after commencement of treatment, at 200 mg/kg bw per day. The increase in skeletal variations indicative of developmental delay was seen in fetuses at 100 and 200 mg/kg bw per day, but this was not considered an acute effect, and the NOAEL for embryo/fetal toxicity was 50 mg/kg bw per day (Lewis, 2013).

In a developmental toxicity study in rabbits, triflumezopyrim was administered at doses of 0, 50, 100, 250 or 500 mg/kg bw per day. The NOAEL for maternal toxicity was 250 mg/kg bw per day based on lower body weight gain, feed intake and reduced defecation and haematological effects. The NOAEL for embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested, based on lack of effects up to the highest dose of 500 mg/kg bw per day, when maternal toxicity was already apparent (Charlap, 2013).

The Meeting concluded that triflumezopyrim is not teratogenic.

In an acute neurotoxicity study in rats, the NOAEL was 100 mg/kg bw based on reductions in body temperature and motor activity at 500 mg/kg bw and above. These findings were, however, accompanied by or secondary to transient body weight losses. There were no neuropathological findings (Carpenter, 2013c). There was no evidence of neurotoxicity in a 90-day rat study that included FOB observations up to 274 mg/kg bw per day, the highest dose tested (Papagiannis, 2013a).

The Meeting concluded that triflumezopyrim is not neurotoxic.

In an immunotoxicity study in which female rats received dietary doses of 0, 100, 500, 2000 or 6000 ppm for 28 days (equivalent to 0, 8.8, 41, 166 and 474 mg/kg bw per day), no evidence of immunotoxicity was observed (Hoban, 2015).

The Meeting concluded that triflumezopyrim is not immunotoxic.

Toxicological data on metabolites and/or degradates

No data were available. Computational analysis of the plant metabolites IN-Y2186 and IN-RPD47 did not identify any structural alerts for genotoxicity and indicated that their functional group profiles were covered by that of triflumezopyrim, which was considered unlikely to be genotoxic in vivo.

Human data

No health problems have been reported in the small number of employees who have been involved in the handling, testing or manufacturing of triflumezopyrim.

The Meeting concluded that the existing database on triflumezopyrim 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 triflumezopyrim of 0–0.2 mg/kg bw on the basis of a NOAEL of 15.9 mg/kg bw per day for effects on body weight and body weight gain, feed consumption and efficiency, increased liver and uterus weights and non-neoplastic histopathological findings in liver, lungs, testes and uterus in the long-term study in rats. A safety factor of 100 was applied. A slightly lower NOAEL (12.2 mg/kg bw per day) obtained in the 90-day dog study was based on effects on body weight and effects secondary to this such as lymphoid depletion in the thymus; however, these effects were not confirmed in a one-year study in dogs. Hence, the NOAEL in the rat carcinogenicity study was considered a more robust basis on which to establish the ADI.

The upper bound of the ADI provides a margin of exposure of at least 3600 relative to the dose level at which liver adenomas were increased in male mice (727 mg/kg bw per day). The margin was at least 4000 relative to the dose level resulting in an increased incidence of bronchoalveolar tumours in female mice (810 mg/kg bw per day).

The Meeting established an ARfD of 1 mg/kg bw on the basis of the NOAEL of 100 mg/kg bw in the acute neurotoxicity study in rats. A safety factor of 100 was applied. The same NOAEL was obtained for maternal toxicity in the developmental study in rats, which is also a suitable basis on which to establish an ARfD. Although a lower NOAEL was identified for embryo/fetal toxicity in the same developmental toxicity study (increased skeletal variations likely indicative of delayed development), these findings are unlikely to result from a single exposure and, therefore, are not an appropriate basis for the ARfD.

Levels relevant to risk assessment of triflumezopyrim

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity [a]	Toxicity	800 ppm, equal to 84 mg/kg bw per day	2500 ppm, equal to 248 mg/kg bw per day
		Carcinogenicity	2500 ppm, equal to 248 mg/kg bw per day	7000 ppm, equal to 727 mg/kg bw per day
Rat	Acute neurotoxicity study [b]	Neurotoxicity	100 mg/kg bw	500 mg/kg bw
	Two-year studies of toxicity and carcinogenicity [a]	Toxicity	500 ppm, equal to 15.9 mg/kg bw per day	2000 ppm, equal to 70.6 mg/kg bw per day (males)
		Carcinogenicity	2000 ppm, equal to 73.8 mg/kg bw per day (females)	8000 ppm, equal to 396 mg/kg bw per day [d]
	Two-generation study of reproductive toxicity [a]	Reproductive toxicity	3000 ppm, equivalent to 210 mg/kg bw per day [c]	–
		Parental toxicity	500 ppm, equivalent to 35 mg/kg bw per day	1500 ppm, equivalent to 105 mg/kg bw per day
		Offspring toxicity	1500 ppm, equivalent to 105 mg/kg bw per day	3000 ppm, equivalent to 210 mg/kg bw per day
	Developmental toxicity study [b]	Maternal toxicity	100 mg/kg bw per day	200 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	100 mg/kg bw per day
Rabbit	Developmental toxicity study [b]	Maternal toxicity	250 mg/kg bw per day	500 mg/kg bw per day
		Embryo and fetal toxicity	500 mg/kg bw per day [c]	–
Dog	Thirteen-week study of toxicity	Toxicity	400 ppm, equal to 12.2 mg/kg bw per day	1000 ppm, equal to 26.6 mg/kg bw per day
	One-year study of toxicity [a]	Toxicity	2000 ppm, equal to 53.2 mg/kg bw per day [c]	–

[a] Dietary administration.

[b] Gavage administration.

[c] Highest dose tested in study.

[d] MTD was clearly exceeded at this dose level, effects not relevant for human risk assessment at dietary exposure levels.

Estimate of acceptable daily intake (ADI)

0–0.2 mg/kg bw

Estimate of acute reference dose (ARfD)

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 triflumezopyrim

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Very rapid and > 80% at low dose (10 mg/kg bw); lower (60–70%) at high dose (200 mg/kg bw)
Dermal absorption	No data
Distribution	Rapid and extensive; highest concentrations in liver, kidney, skin, bone marrow and gastrointestinal tract
Potential for accumulation	Low
Rate and extent of excretion	Rapid and nearly complete via urine (40–48%) and faeces (43–53%) with a significant contribution of biliary elimination (up to ~30%) within 48 hours
Metabolism in animals	Variety of pathways (hydroxylation, hydrolysis, oxidation, decarboxylation, significant elimination also in chemically unchanged form; up to 41% in urine and 18% in faeces)
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	> 4930 mg/kg bw
Rat, LD_{50}, dermal	> 5000 mg/kg bw
Rat, LC_{50}, inhalation	> 5 mg/L (air, 4 hour nose-only exposure to aerosol)
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Mildly irritating in one study, non-irritating in another
Guinea pig, dermal sensitization	Non-sensitizing (Magnusson–Kligman)
Short-term studies of toxicity	
Target/critical effect	Decrease in body weight and feed consumption/efficiency; anaemia; decrease in thymus weight; lymphoid depletion
Lowest relevant oral NOAEL	12.2 mg/kg bw per day (90 day, dog)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (28 day, rat, highest dose tested)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decrease in body weight; liver, lung, uterus
Lowest relevant NOAEL	Rat: 15.9 mg/kg bw per day (500 ppm)
Carcinogenicity	Carcinogenic in mice (liver adenomas, bronchoalveolar tumours) at high doses [a]; effects in rats (liver adenomas, uterine tumours) confined to a dose clearly exceeding the MTD and, therefore, not relevant for human risk assessment
Genotoxicity	Unlikely to be genotoxic in vivo [a]
Reproductive toxicity	
Target/critical effect	No effects on reproduction
Lowest relevant parental NOAEL	35 mg/kg bw per day
Lowest relevant offspring NOAEL	105 mg/kg bw per day
Lowest relevant reproductive NOAEL	210 mg/kg bw per day (highest dose tested)

Developmental toxicity	
Target/critical effect	Skeletal variations and developmental delay
Lowest relevant maternal NOAEL	100 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	50 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	100 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	274 mg/kg bw per day (rat, 90 days)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No evidence of immunotoxicity
Studies on toxicologically relevant metabolites	No relevant metabolites identified
Human data	No reports of health effects in those involved in its manufacture or handling

[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	Two-year, rat	100
ARfD	1 mg/kg bw	Acute neurotoxicity, rat; developmental toxicity, rat	100

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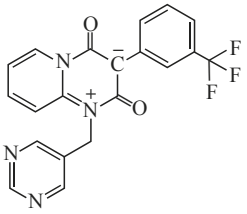
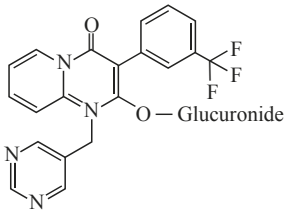
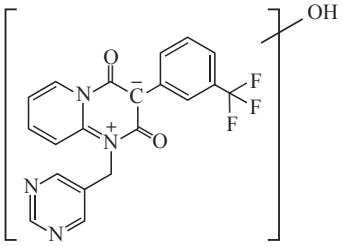
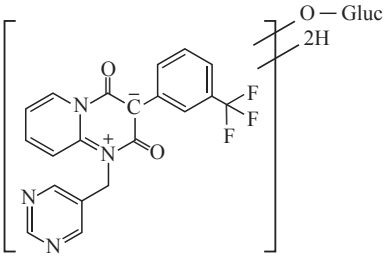
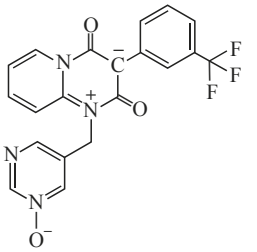
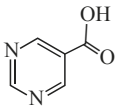
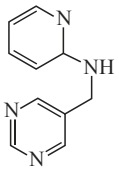
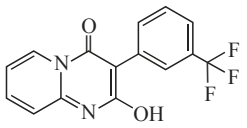
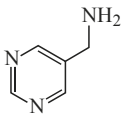
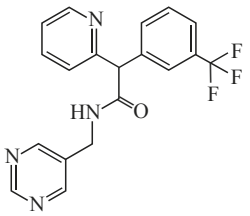
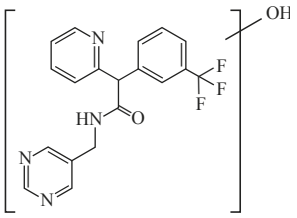
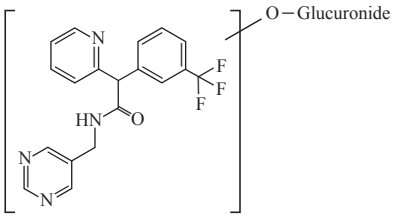
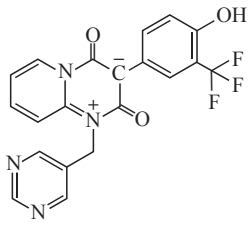
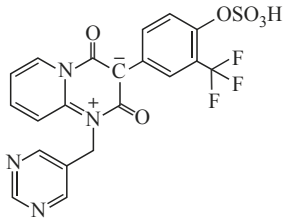
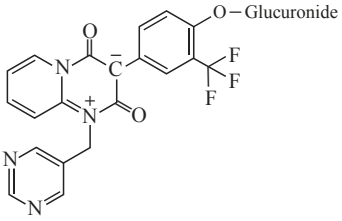
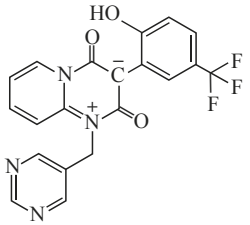
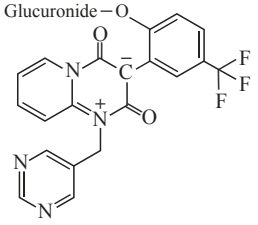
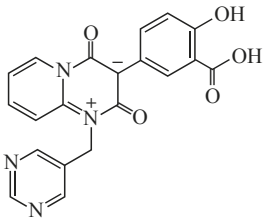
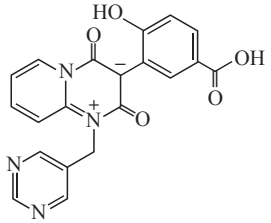
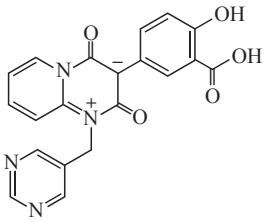
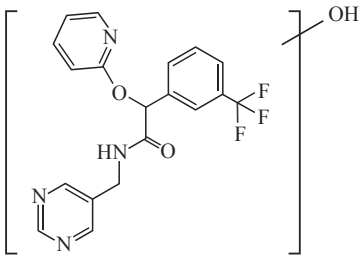
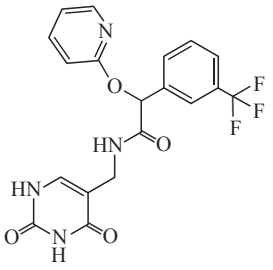
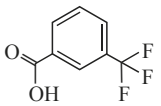
Appendix 1

Chemical names and code numbers for triflumezopyrim and its degradation products

Code number (synonyms)	Description	Compound found in:	Structure
DPX-RAB55 Common name: Triflumezopyrim Parent, active substance	CAS name: 2,4-dioxo-1-(5-pyrimidinylmethyl)-3-[3-(trifluoromethyl)phenyl]-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt IUPAC name 2,4-dioxo-1-(pyrimidin-5-ylmethyl)-3-[3-(trifluoromethyl)phenyl]-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-1-ium-3-ide CAS No.: 1263133-33-0 Structural formula: $C_{20}H_{13}F_3N_4O_2$ Molecular weight: 398	Soil (aerobic, anaerobic, photolysis) Water sediment (hydrolysis, high temperature hydrolysis, aqueous photolysis, water sediment systems, outdoor water sediment, aerobic flooded soil Crops: rice Rotational crops: wheat Livestock: hen, goat Rat	
RAB55 glucuronide (glucuronic acid conjugate of DPX-RAB55)	CAS name: 2-(hexopyranuronosyloxy)-4-oxo-1-(5-pyrimidinylmethyl)-3-[3-(trifluoromethyl)phenyl]-4*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{26}H_{21}F_3N_4O_8$ Molecular weight: 574	Rat	
OH-RAB55 (4 isomers) (hydroxylated RAB55)	CAS name: Not available CAS No.: Not available Structural formula: $C_{20}H_{13}F_3N_4O_3$ Molecular weight: 414	Rat	
hydrated RAB55 glucuronide	CAS name: Not available CAS No.: Not available Structural formula: $C_{26}H_{23}F_3N_4O_9$ Molecular weight: 592	Rat	
IN-R3Z91 (*N*-oxide)	CAS name: 3,4-dihydro-1-[(1-oxido-5-pyrimidinyl)methyl]-2,4-dioxo-3-[3-(trifluoromethyl) phenyl]-2*H*-pyrido[1,2-*a*] pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{13}F_3N_4O_3$ Molecular weight: 414	Crops: rice Rotational crops: wheat Livestock: hen, goat Rat	

Code number (synonyms)	Description	Compound found in:	Structure
IN-RPA16	CAS name: 5-pyrimidinecarboxylic acid CAS No.: 4595-61-3 Structural formula: $C_5H_4N_2O_2$ Molecular weight: 124	Soil (aerobic soil) Water sediment (flooded soil, outdoor water sediment) Crops: rice Livestock: goat	
IN-RPA19	CAS name: *N*-[(5-pyrimidinyl) methyl]-2-pyridinamine CAS No.: Not available Structural formula: $C_{10}H_{10}N_4$ Molecular weight: 186	Soil (soil photolysis) Water (outdoor water sediment) Crops: rice Rotational crops: wheat Rat	
IN-RPD47	CAS name: 2-hydroxy-3-[3-(trifluoromethyl)phenyl]-4*H*-pyrido[1,2-*a*]pyrimidin-4-one CAS No.: 1262986-78-6 Structural formula: $C_{15}H_9F_3N_2O_2$ Molecular weight: 306	Soil (aerobic soil, anaerobic soil) Water sediment (flooded soil, outdoor water sediment) Crops: rice Rotational crops: wheat Livestock: hen, goat Rat	
IN-RUA92	CAS name: 5-pyrimidinylmethanamine CAS No.: 25198-95-2 Structural formula: $C_5H_7N_3$ Molecular weight: 109	Rat	
IN-RUB93	CAS name: *N*-(5-pyrimidinylmethyl)-α-[(3-(trifluoromethyl)phenyl]-2-pyridineacetamide CAS No.: Not available Structural formula: $C_{19}H_{15}F_3N_4O$ Molecular weight: 372	Water (aqueous photolysis, outdoor water sediment) Crops: rice Rat	
OH-RUB93 (hydroxylated RUB93)	CAS name: Not available CAS No.: Not available Structural formula: $C_{19}H_{15}F_3N_4O_2$ Molecular weight: 388	Rat	
RUB93 *O*-glucuronide (2 isomers)	CAS name: Not available CAS No.: Not available Structural formula: $C_{25}H_{23}F_3N_4O_8$ Molecular weight: 564	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
IN-R6U70	CAS name: 3,4-dihydro-3-[4-hydroxy-3-(trifluoromethyl) phenyl]-2,4-dioxo-1-(5-pyrimidinylmethyl)-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{13}F_3N_4O_3$ Molecular weight: 414	Crops: rice Rotational crops: wheat Livestock: hen, goat Rat	
R6U70 sulfate (sulfate conjugate of IN-R6U70)	CAS name: 3,4-dihydro-2,4-dioxo-1-(5-pyrimidinylmethyl)-3-[4-(sulfooxy)-3-(trifluoromethyl) phenyl]-2*H*-pyrido[1,2-*a*] pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{13}F_3N_4O_6S$ Molecular weight: 494	Livestock: goat Rat	
R6U70 glucuronide (glucuronic acid conjugate of IN-R6U70)	CAS name: 3-[4-(hexopyranuronosyloxy)-3-(trifluoromethyl) phenyl]-3,4-dihydro-2,4-dioxo-1-(5-pyrimidinylmethyl)-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{26}H_{21}F_3N_4O_9$ Molecular weight: 590	Livestock: goat Rat	
IN-R6U71	CAS name: 2-hydroxy-3-[2-hydroxy-5-(trifluoromethyl) phenyl]-4-oxo-1-(5-pyrimidinylmethyl)-4*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{13}F_3N_4O_3$ Molecular weight: 414	Crops: rice Rat	
R6U71 glucuronide (glucuronic acid conjugate of INR6U71)	CAS name: 3-[2-(hexopyranuronosyloxy)-5-(trifluoromethyl) phenyl]-3,4-dihydro-2,4-dioxo-1-(5-pyrimidinylmethyl)-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{26}H_{21}F_3N_4O_9$ Molecular weight: 590	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
IN-R6U72 (hydroxy acid)	CAS name: 3-[3-carboxy-4-hydroxyphenyl]-3,4-dihydro-2,4-dioxo-1-(5-pyrimidinylmethyl)-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{14}N_4O_5$ Molecular weight: 390	Crops: rice Livestock: goat Rat	
IN-R6U73 (hydroxy acid)	CAS name: 3-[5-carboxy-2-hydroxyphenyl]-3,4-dihydro-2,4-dioxo-1-(5-pyrimidinylmethyl)-2*H*-pyrido[1,2-*a*]pyrimidinium inner salt CAS No.: Not available Structural formula: $C_{20}H_{14}N_4O_5$ Molecular weight: 390	Crops: rice Livestock: goat	
IN-SBV06	CAS name: α-[(2-pyridinyl) oxy]-*N*-(5-pyrimidinylmethyl)-3-(trifluoromethyl) benzeneacetamide CAS No.: Not available Structural formula: $C_{19}H_{15}F_3N_4O_2$ Molecular weight: 388	Soil (aerobic soil) Water (outdoor water sediment) Crops: rice Rotational crops: wheat Livestock: hen Rat	
OH-SBV06 (hydroxylated SBV06)	CAS name: Not available CAS No.: Not available Structural formula: $C_{19}H_{15}F_3N_4O_3$ Molecular weight: 404	Rat	
IN-SBY68	CAS name: α-[(2-pyridinyl) oxy]-*N*-[(1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl) methyl]-3-(trifluoromethyl) benzeneacetamide CAS No.: Not available Structural formula: $C_{19}H_{15}F_3N_4O_4$ Molecular weight: 420	Soil (aerobic soil, anaerobic soil)	
IN-Y2186	CAS name: 3-(trifluoromethyl)-benzoic acid CAS Number: 454-92-2 Structural formula: $C_8H_5F_3O_2$ Molecular weight: 190	Soil (soil photolysis) Crops: rice Rotational crops: wheat Rat	

Appendix 2

Overview of the use of different batches of triflumezopyrim in the toxicological studies and information on differences in study outcomes when more than one batch was used

Batch	Purity	Studies/Study types
RAB55-037	99.4%	ADME; acute oral; acute dermal; acute inhalation; dermal irritation; ocular irritation; dermal sensitization; subacute dermal, rat (28 day); subchronic oral, dog (90 day); long-term, mouse, rat; two-generation, rat; developmental toxicity, rat, rabbit; acute neurotoxicity, rat; immunotoxicity, rat; genotoxicity (Ames, HGPRT); chromosomal aberration in vitro; micronucleus assay; mechanistic studies
RAB55-104	97.04%	Acute oral; skin irritation; eye irritation; skin sensitization; subchronic oral, rat (90 day); genotoxicity (Ames); chromosomal aberration in vitro; micronucleus assay
RAB55-028	99%	Subacute oral, rat and mouse (28 day)
RAB55-031	98.8%	Subchronic oral, mouse and rat (90 day), dog (28 day)
RAB55-014	>99%	Subacute gavage, rat (14 day) with a micronucleus assay and toxicokinetic investigations
RAB55-064	98.5%	Subchronic oral, dog (1 year); genotoxicity (Ames); chromosomal aberration in vitro
RAB55-111	98.67%	Genotoxicity (Ames); chromosomal aberration in vitro; micronucleus assay

Summary of deviations in study results noted when different batches of triflumezopyrim were used to investigate the same end-point or effect

- **Eye irritation in rabbits**
  Very mild eye irritation (transient corneal opacity and chemosis) in the rabbit with batch **037** in the study by Lowe (2012d), which was not seen with batch **104** in the study by Merill (2014c).
- **Effects on red blood cells (RBCs) in mice**
  Haematological changes suggestive of weak anaemia in the 28-day feeding study by Anand (2015), using batch **028**, at 7000 ppm. No such findings in the 90-day study by Papagiannis (2015a), using batch **031**, at the same dose. Evidence of haematological effects (extramedullary haematopoiesis) seen in the 18-month study by Papagiannis (2015d) with batch **037**; however, the haematological parameters themselves were not measured.
- **Subchronic toxicity in rats (90-day studies)**
  In a feeding study by Papagiannis (2013a), in which batch **031** was used, the only effects at the highest dose level of 6000 ppm were a lower body weight gain and reduced feed consumption in both sexes. It was noted that, as in the mouse, batch **031** did not cause haematological findings. In the similar study by Ford (2015) with batch **104**, the effects on nutritional parameters at 6000 ppm were confirmed but, in addition, uterine weight was clearly increased and haematological parameters were affected.
- **Subchronic toxicity in dogs (90-day and one-year studies)**
  In the 90-day study by Papagiannis (2013c) with batch **037**, the LOAEL was 1000 ppm (approximately 26.6 mg/kg bw per day) at which effects on body weight, RBCs and thymus (lymphoid depletion) were apparent. In the 1-year study of the same author (Papagiannis, 2015b) but with batch **064**, however, no effects at all were observed up to the highest dose level of 2000 ppm (approximately 53 mg/kg bw per day).
- **Precipitation in the Ames test**
  In a total of four Ames tests with approximately the same concentrations, precipitation was observed at high doses of 3000 or 4000 µg/plate, at least in one strain, with the batches **104** (Myhre 2015a) and **111** (Myhre 2014a) but not with the batches **037** and **064** (Wagner & VanDyke, 2012, 2013).

- **Chromosomal aberration in vitro**
  In two of four chromosomal aberration tests in human lymphocytes, positive results were obtained with batches **037** (Roy & Jois, 2015) and **064** (Roy, 2013) but not with the batches **104** and **111** (Kellum, 2015a, b). Precipitation was observed with all apart from **037**.

It is worth noting that batch **037** proved negative in the mouse bone marrow micronucleus assay in vivo (Roy & Divi, 2012). It was also this batch that was used in most toxicological studies and the ADME and mechanistic studies. The long-term study in rats on which the ADI was based, as well as the developmental toxicity and the acute neurotoxicity study in rats from which the ARfD was derived, were performed with the same batch. Batch **037** was also used in the 90-day study in dogs which gave the lowest NOAEL of all toxicological studies. All end-points except the short-term toxicity in rodents were covered by valid studies in which batch **037** was applied. Thus, it would be most reassuring if the specification of triflumezopyrim that is intended for marketing was identical to that one of batch **037**.

ANNEX 1

Reports and other documents resulting from previous Joint Meetings of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

1. Principles governing consumer safety in relation to pesticide residues. Report of a meeting of a WHO Expert Committee on Pesticide Residues held jointly with the FAO Panel of Experts on the Use of Pesticides in Agriculture. FAO Plant Production and Protection Division Report, No. PL/1961/11; WHO Technical Report Series, No. 240, 1962.

2. Evaluation of the toxicity of pesticide residues in food. Report of a Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1963/13; WHO/Food Add./23,. 1964.

3. Evaluation of the toxicity of pesticide residues in food. Report of the Second Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1965/10; WHO/Food Add./26.65,. 1965.

4. Evaluation of the toxicity of pesticide residues in food. FAO Meeting Report, No. PL/1965/10/1; WHO/Food Add./27.65,. 1965.

5. Evaluation of the hazards to consumers resulting from the use of fumigants in the protection of food. FAO Meeting Report, No. PL/1965/10/2; WHO/Food Add./28.65,. 1965.

6. Pesticide residues in food. Joint report of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 73; WHO Technical Report Series, No. 370,. 1967.

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19. 1972 Evaluations of some pesticide residues in food. AGP:1972/M/9/1; WHO Pesticide Residue Series, No. 2, 1973.

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This volume contains toxicological monographs that were prepared by the 2017 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Geneva on 12–21 September 2017.

The monographs in this volume summarize the safety data on 13 pesticides that could leave residues in food commodities. These pesticides are bicyclopyrone, chlormequat, cyclaniliprole, fenazaqun, fenpyrazamine, fenpyroximate, fosetyl-aluminium, isoprothiolane, natamycin, oxamyl, propylene oxide, thiophanate-methyl and triflumezopyrim. The data summarized in the toxicological monographs served as the basis for the acceptable daily intakes and acute reference doses that were established by the Meeting.

This volume and previous volumes of JMPR toxicological evaluations, many of which were published in the FAO Plant Production and Protection Paper series, contain information that is useful to companies that produce pesticides, government regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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