Pesticide residues in food — 2015

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2015

Part II — Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food – 2015

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

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Geneva, 15–24 September 2015

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Abbreviations used

ABCB1	adenosine triphosphate–binding cassette subfamily B
ACH	adrenocortical hyperplasia
AChE	acetylcholinesterase
ACTH	adrenocorticotropic hormone
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AFC	antibody-forming cell; alveolar foam cells
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
ALT	alanine transaminase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ARfD	acute reference dose
ASAT	aspartate aminotransferase
ASMS	acetochlor *sec*-methyl sulfide
ASMSO	acetochlor *sec*-methyl sulfoxide
AST	aspartate transaminase
ATP	adenosine triphosphate
AUC	area under the concentration–time curve
AUC_{0-72}	area under the concentration–time curve from 0 to 72 hours
$AUC_{0-\infty}$	area under the concentration–time curve from time 0 to infinity
BMD	benchmark dose
BMDL	lower 95% confidence limit on the benchmark dose
BMDS	Benchmark Dose Software
BP	benzo(*a*)pyrene
BrdU	5-bromo-2′-deoxyuridine
BROD	benzoxyresorufin *O*-dealkylase
BSP	bromosulfophthalein
BUN	blood urea nitrogen
bw	body weight
CARC	Cancer Assessment Review Committee (USEPA)
CCPR	Codex Committee on Pesticide Residues
ChE	cholinesterase
CHO	Chinese hamster ovary
CI	confidence interval
C_{max}	maximum concentration
$C_{max/2}$	half the maximum concentration
CMEPA	2-chloro-*N*-(2-methyl-6-ethylphenyl) acetamide
CO	cardiac output
CPK	creatinine phosphokinase
CTL	control
CYP	cytochrome P450
DABQI	dialkylbenzoquinoneimine
DFA	difluoroacetic acid
DHA	dihydroartemisinin

DMF	*N*,*N*-dimethylformamide
DMO	dimethadione
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	dinitrochlorobenzene
ECG	electrocardiograph
ECOD	ethoxycoumarin *O*-deethylase
EEG	electroencephalogram
EMA	2-ethyl-6-methylaniline
EMIQ	3-ethyl,5-methylbenzoquinone-4-imine
eq	equivalents
Eq	equivalents
equiv	equivalents
ER	estrogen receptor
EROD	ethoxyresorufin *O*-deethylase
EU	European Union
F	female
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
FAO	Food and Agriculture Organization of the United Nations
FCA	Freund's Complete Adjuvant
Fd	found dead
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (USA)
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GD	gestation day
GGT	gamma-glutamyltranspeptidase
GLP	good laboratory practice
GSD	geometric standard deviation
GSH	glutathione
GST	glutathione *S*-transferase
HDT	highest dose tested
HEPA	2-hydroxyethyl phosphonic acid; 2-hydroxyethephon
HGPRT	hypoxanthine–guanine phosphoribosyltransferase
HH	hepatocyte hypertrophy
HLT	hypocellularity of lymphatic tissue
HMBA	hexamethylenebisacetamide
HPLC	high-performance liquid chromatography
HPP	hypocellularity of Peyer's patch
HPRT	hypoxanthine–guanine phosphoribosyltransferase
HRF	Human Cancer Relevance Framework
IC_{50}	median inhibitory concentration
IgM	immunoglobulin M
IL	interleukin (e.g. IL-3)
ILSI/RSI	International Life Sciences Institute/Risk Science Institute
IPCS	International Programme on Chemical Safety

ISO	International Organization for Standardization
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
IV	intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KCP	Kupffer cell pigmentation
K_i	dissociation constant
K_m	Michaelis-Menten constant
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LH	luteinizing hormone
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOQ	limit of quantification
LSC	liquid scintillation counting
M	male
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean cell volume
MDR1	multidrug resistance protein 1
M&K	Magnusson and Kligman
MMAD	mass median aerodynamic diameter
MMC	mitomycin C
MOA	mode of action
MRL	maximum residue limit
MRT_{tot}	mean total residence time
MS	mass spectroscopy/spectrometry
MTD	maximum tolerated dose
MW	molecular weight
N/A	not applicable
n.a.	not applicable
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NC	not calculable
ND	not detected; not determined
nd	not determined
NE	not examined
NMR	nuclear magnetic resonance
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NR	not reported
NZW	New Zealand White
OCT	ornithine carbamoyltransferase

OECD	Organisation for Economic Co-operation and Development
P	parental generation
P	probability
PBPK	physiologically based pharmacokinetic
pc	post-coitus; packed cells
PC	phagocytic cells
PCNA	proliferating cell nuclear antigen
PEG	polyethylene glycol
PND	postnatal day
p-OH-ASMSO	*para*-hydroxy acetochlor *sec*-amide methyl sulfoxide
p-OH-EMA	*para*-hydroxy-2-ethyl-6-methylaniline
ppb	parts per billion
PPIX	protoporphyrin IX
ppm	parts per million
PPO	protoporphyrinogen oxidase
PROD	pentoxyresorufin *O*-dealkylase
PWG	Pathology Working Group
QA	quality assurance
RBC	red blood cells
REL	rat erythroleukaemia
RITA	Registry of Industrial Toxicology Animal-data
ROHD	repeated oral high dose
ROLD	repeated oral low dose
RPMI	Roswell Park Memorial Institute
RR	relative risk
S9	9000 × *g* supernatant fraction from rat liver homogenate
SAR	structure–activity relationship
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
s-ESA	acetochlor *s*-ethanesulfonic acid
SOHD	single oral high dose
SOLD	single oral low dose
sRBC	sheep red blood cell
$t_{½}$	half-life
T_3	triiodothyronine
T_4	thyroxine
TDAR	T cell–dependent antibody response
t-ESA	acetochlor *t*-ethanesulfonic acid
TFNA	4-trifluoronicotinic acid
TFNA-AM	4-trifluoromethylnicotinamide
TFNA-OH	6-hydroxy-4-trifluoromethylnicotinic acid
TFNG	*N*-(4-trifluoronicotinoyl) glycine
TFNG-AM	*N*-(4-trifluoromethylnicotinoyl)glycinamide
Tk	terminal kill
TK	toxicokinetics; thymidine kinase
TLC	thin-layer chromatography
T_{max}	time to reach the maximum concentration

$T_{max/2}$	time to decline to half the maximum concentration
t-NCA	*tert*-norchloroacetochlor
t-OXA	acetochlor *t*-oxanilic acid
TRR	total radioactive residue
t-SAA	acetochlor *t*-sulfinylacetic acid
TSH	thyroid stimulating hormone
TTC	threshold of toxicological concern
U	uniformly labelled
UGT	uridine diphosphate-glucuronosyltransferase
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	ultraviolet
V_{max}	maximum velocity
VP	variegate porphyria
v/v	volume per volume
WBA	whole-body autoradiography
WBC	white blood cells
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

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 15–24 September 2015.

Seven of the substances evaluated by the WHO Core Assessment Group (acetochlor, cyazofamid, flonicamid, flumioxazin, flupyradifurone, lufenuron and quinclorac) were evaluated for the first time. Three compounds (abamectin, ethephon and penconazole) 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 223*. 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 2015, 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 2015 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.

Any comments or new information on the biological properties or toxicity of the compounds included in this volume should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Meeting on Pesticide Residues, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva, Switzerland.

TOXICOLOGICAL MONOGRAPHS
AND MONOGRAPH ADDENDA

ABAMECTIN

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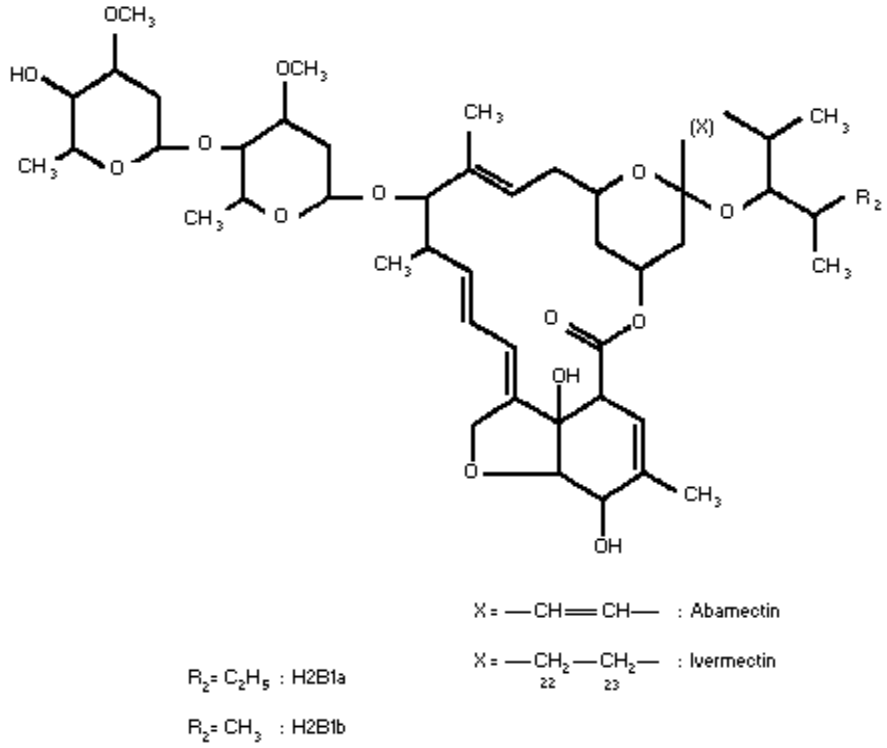
Explanation

Abamectin (Fig. 1) is the International Organization for Standardization (ISO)–approved common name for a mixture of the components avermectin B_{1a} (≥ 80%) [(2a*E*,4*E*,8*E*)-(5′*S*,6*S*,6′*R*,7*S*,11*R*,13*S*,15*S*,17a*R*,20*R*,20a*R*,20b*S*)-6′-[(*S*)-*sec*-butyl]-5′,6,6′,7,10,11,14,15,17a,20,20a,20b-dodecahydro-20,20b-dihydroxy-5′,6,8,19-tetramethyl-17-oxospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-pq][2,6]benzodioxacyclooctadecin-13,2′-[2*H*]pyran]-7-yl 2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl-α-L-arabino-hexopyranosyl)-3-*O*-methyl-α-L-arabino-hexopyranoside] and avermectin B_{1b} (≤ 20%) [(2a*E*,4*E*,8*E*)-(5′*S*,6*S*,6′*R*,7*S*,11*R*,13*S*,15*S*,17a*R*,20*R*,20a*R*,20b*S*)-5′,6,6′,7,10,11,14,15,17a,20,20a,20b-dodecahydro-20,20b-dihydroxy-6′-isopropyl-5′,6,8,19-tetramethyl-17-oxospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-pq][2,6]benzodioxacyclooctadecin-13,2′-[2*H*]pyran]-7-yl 2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl-α-L-arabino-hexopyranosyl)-3-*O*-methyl-α-L-arabino-hexopyranoside] (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 71751-41-2. Abamectin is a macrocyclic lactone product derived from the soil microorganism *Streptomyces avermitilis*. Because of the very similar biological and toxicological properties of the B_{1a} and B_{1b}

Fig. 1. Structural formulas of abamectin and ivermectin



Source: Annex 1, reference *91*

components, they can be considered to be equivalent. Abamectin is used as an insecticide and acaricide.

Abamectin was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1992, 1994, 1995 and 1997. In 1997, an acceptable daily intake (ADI) of 0–0.002 mg/kg body weight (bw) was established based on the no-observed-adverse-effect level (NOAEL) of 0.12 mg/kg bw per day for offspring toxicity in a two-generation reproductive toxicity study in rats, with the application of a reduced safety factor of 50 to account for the higher sensitivity of neonatal rats to abamectin, and the NOAEL of 0.24 mg/kg bw per day from a 1-year toxicity study in dogs, with the application of a safety factor of 100. The ADI was deemed to be appropriate for the sum of abamectin and its 8,9-*Z* isomer (a photodegraded product of abamectin), as the isomer was not expected to be of higher toxicity than abamectin. An acute reference dose (ARfD) was not established when abamectin was last evaluated by JMPR, as that evaluation pre-dated current JMPR guidance for the establishment of ARfDs for pesticides.

In 1996, the forty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established an ADI of 0–0.001 mg/kg bw for abamectin used as a veterinary drug, based on the NOAEL of 0.12 mg/kg bw per day in the study on reproductive toxicity in rats and application of a safety factor of 100.

Abamectin was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues (CCPR).

The majority of the toxicology studies were conducted with abamectin technical (also referred to as MK936 or avermectin B_1), which is a mixture of avermectin B_{1a} and avermectin B_{1b} (approximately 90:10). Some studies (mainly those requiring radiolabel) were conducted with either avermectin B_{1a} (also referred to as NOA422601) or avermectin B_{1b} (also referred to as NOA421704).

All critical studies were conducted in compliance with good laboratory practice (GLP), unless otherwise specified.

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Mice

Differences in pharmacokinetics between two subpopulations of the CF-1 mouse were investigated by administering a single oral (gavage) dose of 0.1 or 0.2 mg/kg bw of ^{3}H-labelled abamectin (purity 96.2%) or emamectin (purity 95.5%) or 0.2 mg/kg bw of ^{3}H-labelled ivermectin (93.5% ivermectin B_{1a}, 2.3% ivermectin B_{1b}), formulated in sesame oil, to groups of up to 44 female wild-type (natural expression of *mdr1a* P-glycoprotein, +/+) and 44 female mutant (deficient expression of *mdr1a* P-glycoprotein, −/−) female mice (weighing 18.9–32.2 g). Groups of mice were terminated at timed intervals during the study, and the concentrations of radioactivity in blood, plasma, brain and bile were measured. Urine and faeces were collected from selected animals to provide preliminary information on the routes and rates of excretion of radioactivity.

The presence of radioactivity in the tissues of wild-type and mutant female CF-1 mice indicated systemic exposure to ^{3}H-labelled abamectin, emamectin or ivermectin following a single oral (gavage) dose of each of these test substances. In all dose groups, it was apparent that exposure in mutant mice was greater than that in wild-type mice.

Following a single oral dose of abamectin at 0.2 mg/kg bw, mutant mice developed adverse clinical signs, and the study was terminated prematurely. There were no significant clinical signs observed in the wild-type mice. In animals administered abamectin at a reduced dose level of 0.1 mg/kg bw, the wild-type and mutant animals displayed no clinical abnormalities. In a similar experiment with emamectin (0.1 mg/kg bw) and ivermectin (0.2 mg/kg bw), no clinical abnormalities were observed in any animals, with one exception.

Within each dose group and genotype, the profiles of radioactivity in blood and plasma were similar. Regardless of dose, the maximum concentrations (C_{max}) were higher, distribution was prolonged and elimination was slower in mutant animals compared with wild-type animals. When normalized for dose, maximum concentrations of radioactivity in blood and plasma and areas under the concentration–time curve from 0 to 72 hours ($AUC_{0–72}$) were relatively similar across the dose groups within the wild-type mice, as were those for mutant mice, where reported. However, the blood and plasma profiles of all of the test substances investigated showed a shift in the pattern of absorption into the systemic circulation between 4 and 12 hours, which in some cases amounted to a secondary peak (Table 1).

Concentrations of test substances were 40–160 times higher in the brains of mutant mice than in those of wild-type mice. Concentrations of radioactivity in the brains of wild-type animals were typically lower than concentrations seen in the blood and plasma, whereas in mutant mice, concentrations were typically higher (Table 2).

Excretion profiles for all animals showed that the primary route of excretion was via faeces. Urinary elimination was minimal in wild-type animals but was up to 5 times higher in mutant animals, although still a minor route of elimination compared with faecal elimination. The presence of radioactivity in bile indicates that there is some biliary excretion of these test substances, although the extent of elimination via bile could not be determined.

In view of the above, it is concluded that following administration of a single oral dose of abamectin, emamectin or ivermectin to wild-type or mutant CF-1 mice, exposure was greater in mutant mice than in wild-type mice. Distribution and elimination also appeared to be slower, with concentrations in the brain tissue up to 160 times higher in mutant mice than in wild-type mice. The exposure profiles of the three test substances were broadly similar. Excretion profiles showed that the primary route of excretion was via faeces.

Table 1. Derived pharmacokinetic parameters for blood and plasma

Dose group	Animal type	C_{max} (µg equiv/g)	T_{max} (h)	$t_{½}$ (h)	$AUC_{0–72}$ (h·µg/g)	$AUC_{0–\infty}$ (h·µg/g)	Study duration (h)
Blood							
Abamectin 0.2 mg/kg bw	Wild-type	0.028	4	NR	0.51	0.52	0–96
	Mutant	NR	NR	NC	NC	NC	0–96[a]
Abamectin 0.1 mg/kg bw	Wild-type	0.010	4	NR	0.18	0.19	0–120
	Mutant	0.023	12	NR	0.66	0.73	0–120
Emamectin 0.1 mg/kg bw	Wild-type	0.013	8	18.6	0.22	0.23	0–96
	Mutant	0.019	12	37.6	0.77	0.89	0–96
Ivermectin 0.2 mg/kg bw	Wild-type	0.015	8	NR	0.29	0.29	0–72
	Mutant	0.030	8	NR	1.32	1.32	0–72
Plasma							
Abamectin 0.2 mg/kg bw	Wild-type	0.050	4	NR	0.93	0.95	0–96
	Mutant	NR	NR	NC	NC	NC	0–96[a]
Abamectin 0.1 mg/kg bw	Wild-type	NR	NR	NC	NC	NC	0–120
	Mutant	NR	NR	NC	NC	0.19	0–120
Emamectin 0.1 mg/kg bw	Wild-type[b]	0.026	8	19.5	0.43	0.43	0–96
	Mutant	0.034	12	NR	1.27	1.41	0–96
Ivermectin 0.2 mg/kg bw	Wild-type	0.032	8	NR	0.55	0.55	0–72
	Mutant	0.056	8	NR	2.40	2.41	0–72

$AUC_{0–72}$: area under the concentration–time curve from 0 to 72 hours; $AUC_{0–\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; NC: not calculable; NR: not reported; $t_{½}$: half-life; T_{max}: time to reach the maximum concentration

[a] Study terminated prematurely.

[b] Female 243 excluded.

Source: Gledhill (2008)

Table 2. Pharmacokinetic parameters in brains of mice

Dose group	Animal type	C_{max} (µg equiv/g)	T_{max} (h)	$t_{½}$ (h)	$AUC_{0–72}$ (h·µg/g)	$AUC_{0–\infty}$ (h·µg/g)	Study duration (h)
Abamectin 0.2 mg/kg bw	Wild-type	0.006	4	15.0	0.144	0.144[a]	0–96
	Mutant	NR	NR	NC	NC	NC	0–96[b]
Abamectin 0.1 mg/kg bw	Wild-type	0.002	4	NR	0.068	0.081	0–120
	Mutant	0.077	12	NR	2.532	2.760	0–120
Emamectin 0.1 mg/kg bw	Wild-type[c]	0.002	12	30.3	0.049	0.052	0–96
	Mutant	0.167	24	31.4	7.310	8.405	0–96
Ivermectin 0.2 mg/kg bw	Wild-type	0.006	0.5	NR	0.070	0.070	0–72
	Mutant	0.16	24	NR	7.095	7.095	0–72

$AUC_{0–72}$: area under the concentration–time curve from 0 to 72 hours; $AUC_{0–\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; NC: not calculable; NR: not reported; $t_{½}$: half-life; T_{max}: time to reach the maximum concentration

[a] AUC up to 72 hours only.

[b] Study terminated prematurely.

[c] Female 243 excluded.

Source: Gledhill (2008)

A 0.2 mg/kg bw dose of abamectin caused adverse clinical signs in mutant mice. In similar experiments with abamectin (0.1 mg/kg bw), emamectin (0.1 mg/kg bw) or ivermectin (0.2 mg/kg bw), the majority of animals displayed no adverse clinical signs.

The study was GLP compliant, and a quality assurance (QA) statement was attached **(**Gledhill, 2008).

Rats

Fifty-three rats of each sex (CRCD strain, body weights approximately 295 g [male] and 217 g [female]) were divided into four groups and treated orally, by gavage, with [5-^{3}H]avermectin B_{1a} (batch no. L-676,895-01S15, radiochemical purity 99.4%), [3,7,11,13,23-^{14}C]avermectin B_{1a} (batch no. L-676,895-29T01, radiochemical purity 99%) or unlabelled avermectin B_{1a} (batch no. L-676,895-00P76) dissolved in sesame oil, according to the schedule shown in Table 3. A further nine rats of each sex, treated with sesame oil alone, acted as controls.

Subgroups of three males and three females within each group were caged together, by sex, for the collection of urine and faeces. Urine and faeces were collected at 24-hour intervals until sacrifice. At necropsy, bone, brain, fat, gonads, heart, kidney, liver, gastrointestinal tract, lung, blood, muscle, spleen and residual carcass (less skin and tail) were collected. The same tissues from the animals within each subgroup were combined, and the total weights were determined. Blood and urine samples and 1:4 water homogenates of each tissue (0.5 mL) were weighed and combusted in a Packard 306 sample oxidizer, and the $^{3}H_2O$ and $^{14}CO_2$ were trapped and then counted for 10 minutes in a Packard scintillation spectrometer to determine total radioactivity.

All samples were combusted in duplicate, except for brain, lung, bone, spleen and heart, which were weighed and combusted directly.

The residue levels and depletion rates in the groups treated with tritiated avermectin B_{1a} were comparable with the values obtained from rats treated with ^{14}C-labelled avermectin B_{1a}. Therefore, it was concluded that tritium labelling at the 5-position of avermectin B_{1a} was stable.

Table 3. Treatment and sacrifice schedule

Group	No. of animals (M + F)	Test article	Specific activity (µCi/mg)[a]	Dose level × no. of doses	Sacrifice schedule
A	12 + 12	[^{3}H]Avermectin B_{1a}	350	1.42 mg/kg bw (M) × 1 1.38 mg/kg bw (F) × 1	3M + 3F at 1, 2, 4 and 7 days after dose
B	12 + 12	[^{3}H]Avermectin B_{1a}	350	0.142 mg/kg bw (M) × 1 0.138 mg/kg bw (F) × 1	3M + 3F at 1, 2, 4 and 7 days after dose
C	17 + 17	Avermectin B_{1a} [^{3}H]Avermectin B_{1a}	Unlabelled 350	0.142 mg/kg bw (M) × 15[b] 0.138 mg/kg bw (F) × 15[b]	3M + 3F at 1, 2, 4 and 7 days after last dose
D	12 + 12	[^{3}H]Avermectin B_{1a} [^{14}C]Avermectin B_{1a}	33 16.4	1.42 mg/kg bw (M) × 1 1.38 mg/kg bw (F) × 1	3M + 3F at 1, 2, 4 and 7 days after dose
E	3 + 3	Sesame oil	–	0.55 mL/rat (M) × 1 0.40 mL/rat (F) × 1	3 M + 3F 7 days after dose
F	6 + 6	Sesame oil	–	0.55 mL/rat per day (M) × 15 0.40 mL/rat per day (F) × 15	3M + 3F 7 days after last dose

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

[a] 1 µCi = 37 kBq.

[b] Fourteen daily doses of unlabelled material followed by a single dose of [^{3}H]avermectin B_{1a}.

Source: Alvaro et al. (1984)

The data reported for dose accountability were calculated on the basis of the total dose given to each group and are therefore average values from animals sacrificed 1, 2, 4 and 7 days after dosing. The total percentage of the dose accounted for in the analysed samples for each group ranged from 85.1% to 95.1% for tritium and from 94.5% to 94.9% for ^{14}C. Similarly, the excretion data reported were calculated on the basis of the total dose given to each group, whereas the daily elimination of the dose should have been calculated for each subgroup. Therefore, the excretion data in percentage of the dose for each subgroup sacrificed at intervals up to 7 days after dosing were recalculated based on the reported values of the daily elimination of the dose in micrograms. The total rate of excretion was very similar for both sexes and was independent of the dose level and treatment regimen. Excretion was moderately rapid, with 80–101% of the dose excreted within 96 hours. The route of excretion was independent of the dose level and sex. The dose was almost completely excreted, almost entirely via the faeces (86.8–104% of the dose). Urinary excretion was extremely low, accounting for 0.5–1.4% of the dose.

Mean residue levels in sampled tissues from male and female rats were highest in all tissues 24 hours after treatment and were dose dependent, as the residues in the high-dose groups (1.38/1.42 mg/kg bw) were approximately 10-fold higher than those in the low-dose groups (0.138/0.142 mg/kg bw). In contrast, the depletion rates were independent of dose level, as the tissue half-lives were comparable between the dose levels. There were no substantive differences in the depletion half-lives among the tissues. Most of the calculated half-lives were within the range 1.2 ± 0.3 days, although brain and testes showed greater variability. The highest residue levels at 24 hours in tissues, excluding the gastrointestinal tract, occurred in fat (2.89–5.30 and 0.16–0.32 parts per million [ppm] equivalents for the high and low dose levels, respectively). The brain contained the lowest residues (0.07–0.13 and 0.01 ppm equivalents for the high and low dose levels, respectively). In most instances, residue levels in the kidney were higher than the levels in the liver. Pretreatment for 14 days with unlabelled avermectin B_{1a} at a dose of 0.138/0.142 mg/kg bw had no effect on either the residue levels or depletion rates, compared with a single administration at the same dose level. The tissue residue levels in female rats were generally higher than the corresponding residue levels in male rats.

In view of the above, it can be concluded that avermectin B_{1a} is absorbed from the gastrointestinal tract and is distributed throughout all major tissues and organs sampled. It is rapidly eliminated from the body, almost exclusively in the faeces, and there is no evidence for tissue accumulation on repeated administration. With the exception of dose dependence for tissue residue levels, the toxicokinetic/ pharmacokinetic profile is not influenced by sex, dose level or treatment regimen.

The study was not GLP compliant, and no QA statement was attached (Alvaro et al., 1984).

In another study, [23-^{14}C]avermectin B_{1a} was administered as a single oral dose at two dose levels – i.e. 0.5 mg/kg bw (low dose) and 5 mg/kg bw (high dose) – to several groups of male and female rats (Hanlbm: WIST (SPF)). Biliary excretion was investigated after single oral administration at the low dose level in bile duct–cannulated male and female rats. Additionally, [23-^{14}C]avermectin B_{1a} was administered intravenously at the low dose level to male rats in order to obtain additional insight into the routes of excretion.

At the time of dosing with radiolabelled avermectin B_{1a}, the animals of groups B1, D1, F1–F4 and L1 had a body weight of approximately 200 g, corresponding to 7 and 11 weeks of age for males and females, respectively. For the biliary excretion investigation (groups G1 and G2), the animals had a body weight of approximately 250 g and 210 g, corresponding to 9 and 12 weeks of age for males and females, respectively.

For administration, [23-^{14}C]avermectin B_{1a} was dissolved in a 3:2 volume per volume (v/v) mixture of polyethylene glycol 200/ethanol at different concentrations: intravenous, about 1 mg/mL; and oral, 0.2 mg/mL and 2 mg/mL for the low and high dose levels, respectively. For the oral administration, each animal received 0.5 mL of the administration solution by stomach tube. The

intravenous administration was performed by injection into the tail vein with a volume of 0.1 mL each.

Groups B1/D1. After single oral administration of [23-^{14}C]avermectin B_{1a}, the urine (0–168 hours), faeces (0–168 hours), expired air (0–48 hours) and serial blood (0–72 hours) were individually collected, and the radioactivity was determined by liquid scintillation counting. Expired air was collected only from animals of the high-dose group (D1). Seven days after dosing, the animals were killed by exsanguination after anaesthesia with carbon dioxide. The terminal blood was collected; whole blood was separated into plasma and red blood cells by centrifugation. The tissues and organs were removed by dissection and taken for radiometry.

Groups G1/G2. [23-^{14}C]Avermectin B_{1a} was administered to bile duct–cannulated male and female rats. During the experimental period, artificial bile fluid was infused into the duodenum of the animals. Urine (0–48 hours), faeces (0–48 hours) and bile fluid (0–48 hours) were individually collected, and the radioactivity was determined by liquid scintillation counting. Two days after dosing, the animals were killed, and the gastrointestinal tract, including contents, and the remaining carcass were collected for radiometry.

Groups F1–F4. At four different time points after administration, three animals each were killed by exsanguination, and terminal blood was collected. Plasma was separated from whole blood by centrifugation. The time points selected were based on the blood kinetics as determined in groups B1 and D1. After sacrifice, the tissues and organs were removed by dissection and analysed for residual radioactivity.

Group L1. [23-^{14}C]Avermectin B_{1a} was administered intravenously to male rats. Urine and faeces were collected and analysed for radioactivity. At two time points – i.e. 6 hours (t_1) and 24 hours (t_2) – after administration, two animals were sacrificed. One animal was immediately frozen as a whole; the frozen carcass was embedded in gelatine solution and frozen as a block. Whole-body sections were taken at different body levels, and the radioactivity in the tissues, organs and areas of the gastrointestinal tract was determined by radioluminography.

From the second animal of each sampling time point, the gastrointestinal tract was removed, and the loops of the intestinal tract were carefully separated without damaging the intestinal tract. Thereafter, the gastrointestinal tract was placed in a straight line on a framed specimen stage, which was submerged in a mixture of solid carbon dioxide and hexane at approximately −80 °C. The gastrointestinal tract was embedded in gelatine solution and frozen as a block. Sections were taken at different levels, and the radioactivity in the contents and wall of the gastrointestinal tract was determined by radioluminography.

The details of this experiment are summarized in Table 4.

The test substance was stable in the administration solution, as indicated by a radiochemical purity greater than 96% (determined by thin-layer chromatography) at the time points of dosing for each group.

After a single oral administration, the blood concentration–time curves demonstrated a moderately rapid absorption from the gastrointestinal tract into the systemic circulation at both dose levels, independent of sex. The maximum concentration (C_{max}) in blood at both dose levels and in both sexes was reached within 4–8 hours after administration, accounting for about 0.06 ppm and 0.6 ppm [23-^{14}C]avermectin B_{1a} equivalents for the low and high doses, respectively. After reaching the maximum concentration, residues in blood depleted within 15–27 hours to half of the maximum concentration. The area under the concentration–time curve (AUC) values were similar between the sexes – i.e. 1.2 h·µg/g and 1.3 h·µg/g at the low dose and 17 h·µg/g and 18 h·µg/g at the high dose for males and females, respectively. Maximum blood concentrations and AUC values increased in a proportional fashion, with the dose level indicating a dose-independent systemic bioavailability (Table 5).

Table 4. Summary of dosing and sampling regimen for each group treated with [23-^{14}C]avermectin B_{1a}

Group	Number of each sex	Dose and route	Sampling regimen
B1	4 males 4 females	Low dose oral	Collection of urine and faeces at different time points, blood kinetics 0–48 h, and various tissues 7 days after administration
D1	4 males 4 females	High dose oral	Collection of urine, faeces and expired air at different time points, blood kinetics 0–72 h, and various tissues 7 days after administration
F1	12 males	Low dose oral	Collection of various tissues at different time points, i.e. 6, 24, 48 and 72 h after administration
F2	12 females	Low dose oral	Collection of various tissues at different time points, i.e. 6, 24, 48 and 72 h after administration
F3	12 males	High dose oral	Collection of various tissues at different time points, i.e. 8, 24, 48 and 72 h after administration
F4	12 females	High dose oral	Collection of various tissues at different time points, i.e. 8, 24, 48 and 72 h after administration
G1	6 males	Low dose oral	Collection of urine, bile and faeces at different time points up to 48 h after administration and gastrointestinal tract and remaining carcass after sacrifice
G2	6 females	Low dose oral	Collection of urine, bile and faeces at different time points up to 48 h after administration and gastrointestinal tract and remaining carcass after sacrifice
L1	4 males	Low dose intravenous	Collection of urine and faeces, whole-body radioluminography and radioluminography of the gastrointestinal tract at two different time points, i.e. 6 and 24 h after administration

Source: Hassler (2001)

Table 5. Blood kinetic parameters of [23-^{14}C]avermectin B_{1a} after single oral administration

	Group B1: 0.5 mg/kg bw		Group D1: 5 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**
C_{max} (ppm)	0.057	0.049	0.616	0.522
T_{max} (h)	4	8	8	8
$T_{max/2}$ (h)	19	24	26	35
AUC (h·µg/g)	1.2	1.3	17.0	18.0

bw: body weight; C_{max}: maximum concentration in blood; ppm: parts per million; T_{max}: time to reach C_{max}; $T_{max/2}$: time to decline to half C_{max}
Source: Hassler (2001)

The apparent extent of absorption, based on the amount excreted via the kidneys and the bile, was determined to be 3–5% of the dose. However, the real extent of absorption is significantly higher based on consideration of the tissue residue data and comparison of urinary excretion following oral or intravenous administration. The total tissue residues determined at the time point of the maximum concentration – i.e. 6 and 8 hours after administration – accounted for about 50% of the dose for both dose levels. The comparison of urinary excretion after oral and intravenous administration results in a ratio that is equivalent to the biovailability of a test substance. A bioavailability factor of 0.86 was calculated for abamectin, which justifies the assumption of almost complete oral absorption.

The orally administered doses were excreted moderately rapidly. The major part of the dose was excreted via the faeces – i.e. more than 92% of the dose at both dose levels and for both sexes within 7 days after administration. Only very small amounts were renally excreted, accounting for about 1%, essentially independent of sex and dose level. At the low dose level, low amounts of the absorbed dose – i.e. 5% in males and 3% in females – were also eliminated via bile and ultimately excreted with the faeces. The rate of excretion was slightly slower in females than in males. Whereas males excreted 81% and 73% of the dose within 48 hours after administration of the low and high doses, respectively, females showed an excretion of 69% and 48% of the low and high doses, respectively, within the same period. The excretion of the absorbed test substance via expired air was insignificant. Only 0.01% of the dose was determined in the expired air within 48 hours after administration of the high dose.

The rates and routes of excretion were essentially independent of the dose level, the route of administration and the sex of the animals.

The non-biliary excretion into the gastrointestinal tract was investigated after intravenous administration of [23-^{14}C]avermectin B_{1a} to male rats at the low dose of 0.5 mg/kg bw. At two different time points – i.e. 6 and 24 hours after administration – the gastrointestinal tract of one animal was removed after sacrifice, and the concentration of radioactivity was determined in different parts of the gastrointestinal tract by radioluminography. Six hours after intravenous administration, the contents of the stomach revealed, as expected, only a low concentration of radioactivity (0.016 ppm [23-^{14}C]avermectin B_{1a} equivalents), whereas the secretory epithelium of the stomach wall revealed a concentration of 0.380 ppm equivalents. The intestinal wall from the duodenum to the rectum showed concentrations of radioactivity in the range of 0.250–0.800 ppm [23-^{14}C]avermectin B_{1a} equivalents, indicating an excretion of [23-^{14}C]avermectin B_{1a} and/or its metabolites into the intestinal tract. The contents of the gastrointestinal tract revealed increasing concentrations from the duodenum to the ileum, accounting for 1–4 ppm equivalents. Thereafter, the concentration of radioactivity in the contents decreased from the colon (1.9 ppm equivalents) through to the rectum (0.04 ppm equivalents). The concentration in the rectum at the last position was significantly lower than the corresponding concentration in the wall of the rectum. Twenty-four hours after intravenous administration, the concentration in the intestinal wall decreased to levels in the range of 0.15–0.40 ppm equivalents. The contents of the gastrointestinal tract showed increasing concentrations from 0.5 ppm equivalents in the duodenum up to 3.2 ppm equivalents at the last determined position in the rectum. The high concentration of radioactivity in the stomach contents 24 hours after administration may be due to oral uptake of excreted faeces (i.e. sacrophagy of rodents).

After single oral administration of the low dose, all selected tissues and organs achieved the highest residue levels 6 hours after administration, with the exception of fat, where the residues reached the maximum level 24 hours after administration. The highest residues were found in adrenals, fat, liver and pancreas, in the range 0.67–1.05 ppm [23-^{14}C]avermectin B_{1a} equivalents. All other selected tissues and organs showed maximum residue levels between 0.1 and 0.6 ppm equivalents, except for testes (0.04 ppm equivalents) and brain (0.01 ppm equivalents). The maximum residue levels for males and females were similar.

After reaching their maximum concentrations, the tissue residues depleted following monophasic first-order kinetics, with half-lives in the range of 12–17 hours for males and 13–33 hours for females. Within 7 days after administration, the tissue residue levels were low. Based on the slowest depletion rate, the highest residue levels were determined in fat, accounting for 0.065 and 0.073 ppm [23-^{14}C]avermectin B_{1a} equivalents in males and females, respectively.

After administration of the high dose, the residues were about 10 times higher, corresponding to the 10-fold higher dose level. The profile of the maximum levels was essentially the same as that determined at the low dose. The highest residue levels were determined in fat, accounting for 11.3 and 13.5 ppm equivalents for males and females, respectively, followed by adrenals (10.3 and 10.9 ppm equivalents, respectively), liver (9.4 and 10.6 ppm equivalents, respectively) and pancreas (6.3 and 7.6 ppm equivalents, respectively). The calculated depletion half-lives, based on monophasic first-order kinetics, ranged from 12 to 20 hours in males and from 20 to 35 hours in females. The residues

declined within 7 days after administration to levels not exceeding 0.1 ppm [23-^{14}C]avermectin B_{1a} equivalents, except in fat, where residue levels of 0.95 ppm equivalents in males and 1.15 ppm equivalents in females were determined.

Approximately 50% of the administered dose was totally recovered in selected tissues and organs 6 and 8 hours after administration of the low and high doses, respectively. The absorbed test substance and/or its metabolites were widely distributed within the body. After reaching the maximum residue concentration, the residues depleted to approximately 1% and 2% of the dose within 168 hours in males and females, respectively.

The residue levels in tissues and organs determined by whole-body radioluminography 6 and 24 hours after the single intravenous administration at the low dose confirm the wide and nonspecific distribution of the radioactivity.

The concentrations of radioactivity in the contents of the small intestine, appendix and large intestine were 1.8, 2.8 and 3.0 ppm [23-^{14}C]avermectin B_{1a} equivalents, respectively. The presence of radioactivity in the contents of the intestinal tract after intravenous administration, especially at a high concentration, confirms the assumption of a non-biliary excretion of absorbed avermectin B_{1a} into the intestinal tract and ultimately excretion in the faeces.

Comparison of the tissue residue levels after intravenous administration with those in rats that were given a similar oral dose shows that the residue levels were similar at the corresponding time points, which indicates almost complete absorption after oral administration.

It can be concluded that after oral administration to rats, the majority of avermectin B_{1a} was almost completely absorbed and then eliminated predominantly by non-biliary excretion into the gastrointestinal tract before excretion with the faeces. The rates and mechanisms of oral absorption and subsequent excretion were independent of the dose level and the sex; however, the depletion of tissue residues was more rapid in males than in females, by approximately 2-fold.

The study was GLP compliant, and a QA statement was attached (Hassler, 2001).

In another study, single oral doses of [23-^{14}C]avermectin B_{1b} were administered at two dose levels – i.e. 0.5 mg/kg bw (low dose) and 5 mg/kg bw (high dose) – to two groups of male and female rats (Hanlbm: WIST (SPF)) weighing approximately 200 g, corresponding to 7 and 9 weeks of age for the males and females, respectively.

Avermectin B_{1b} was dissolved in a 4:2 v/v mixture of polyethylene glycol 200/ethanol at a concentration of 0.2 mg/mL and 2 mg/mL for the low and high dose levels, respectively. Each animal received 0.5 mL of the administration solution by stomach tube. Specimen collections were made from the individual animals at the following time intervals/time points after administration:

- *Urine*: 0–6, 6–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 hours;
- *Faeces*: 0–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 hours;
- *Serial blood*: 0.5, 1, 2, 4, 8, 12, 24 and 48 hours.

Seven days after dosing, the animals were killed. The terminal blood was collected; whole blood was separated into plasma and red blood cells by centrifugation. The tissues and organs, including the remaining carcass, were taken for examination of residual radioactivity.

Urine and faeces pools according to sex and animal group were prepared by mixing equal aliquots of the collected samples from each animal.

Radioactivity in all samples was measured by liquid scintillation counting. The metabolite pattern in urine and faeces was determined using thin-layer chromatography. Metabolic profiles in these excreta samples were analysed by high-performance liquid chromatography (HPLC).

After a single oral administration, the blood concentration–time curves for both sexes demonstrate a moderately rapid absorption from the gastrointestinal tract into the systemic circulation at both dose levels.

Table 6. Blood kinetic parameters of [23-^{14}C]avermectin B_{1b} after a single oral administration to rats

	Group B1: 0.5 mg/kg bw		Group D1: 5 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**
C_{max} (ppm)	0.044	0.048	0.49	0.42
T_{max} (h)	8	4	4	8
$T_{max/2}$ (h)	9	13	14	21
AUC (h·µg/g)	0.72	0.86	9.0	11.5

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration in blood; ppm: parts per million; T_{max}: time to reach C_{max}; $T_{max/2}$: time to decline to half C_{max}
Source: Briswalter (2003a)

The parameters of the kinetics of [23-^{14}C]avermectin B_{1b} in blood are summarized in Table 6.

After a single oral administration at the low and high dose levels – i.e. 0.5 and 5 mg/kg bw (groups B1 and D1) – the apparent absorption of avermectin B_{1b}, based on the radioactivity determined in urine and tissue residues, accounted for only 6–7% of the dose. Very low levels (4–5% of the administered dose) were determined in the urine over the experimental period of 168 hours. However, comparison with avermectin B_{1a} (Hassler, 2001) indicates that the actual extent of absorption is significantly higher.

The rates and routes of excretion were essentially independent of the dose level and the sex of the animals.

At the low dose level, only 5% and 4% of the dose were renally excreted within 168 hours after administration to male and female rats, respectively. The majority of the dose was excreted via the faeces, accounting for 94% and 91% of the dose in male and female rats, respectively. Female rats showed a slightly slower excretion compared with male rats. Whereas male rats excreted 94% of the dose within 48 hours of administration, female rats excreted 82% of the dose within the same period. This sex difference in the rate of excretion is comparable with that seen with avermectin B_{1a} in rats (Hassler, 2001). However, the residues in excised tissues and carcass 168 hours after administration accounted in total for only 2% of the dose for both male and female rats.

The rates and routes of excretion after single oral administration of the 10-fold higher high dose (5 mg/kg bw) were very similar to those following administration of the low dose. Approximately 4% of the dose was excreted with the urine in both sexes, and 89% and 92% of the dose were excreted with the faeces in male and female rats, respectively. Again, a slightly slower excretion was observed in female rats. Whereas male rats excreted 89% of the dose within 48 hours after administration, female rats excreted 70% of the dose within the same period.

After absorption, the test substance was widely distributed in the whole body of the animals. At the low dose (0.5 mg/kg bw), the tissue residue levels 7 days after oral administration were low. The highest residue levels were found in fat, accounting for 0.09 and 0.16 ppm [23-^{14}C]avermectin B_{1b} equivalents in male and female rats, respectively. All other tissues and organs showed residue levels below 0.04 ppm equivalents. After administration at the high dose (5 mg/kg bw), the residue levels were about 10 times higher, corresponding to the 10-fold higher dose. The tissue distribution was very similar to that observed at the low dose. The highest residue levels were found in fat, accounting for 1.4 and 1.6 ppm [23-^{14}C]avermectin B_{1b} equivalents in male and female rats, respectively. All other tissues and organs showed residue levels below 0.3 ppm equivalents.

It can be concluded that after oral administration to rats, [23-^{14}C]avermectin B_{1b} was almost completely absorbed and then eliminated predominantly by non-biliary excretion into the gastrointestinal tract and ultimately excreted with the faeces. The rates and mechanisms of absorption and excretion were independent of the dose level and the sex, with the exception of a slower rate of

excretion in females. The absorption, distribution, degradation and excretion of [23-^{14}C]avermectin B_{1b} and [23-^{14}C]avermectin B_{1a} were essentially similar.

The study was GLP compliant, and a QA statement was attached (Briswalter, 2003a).

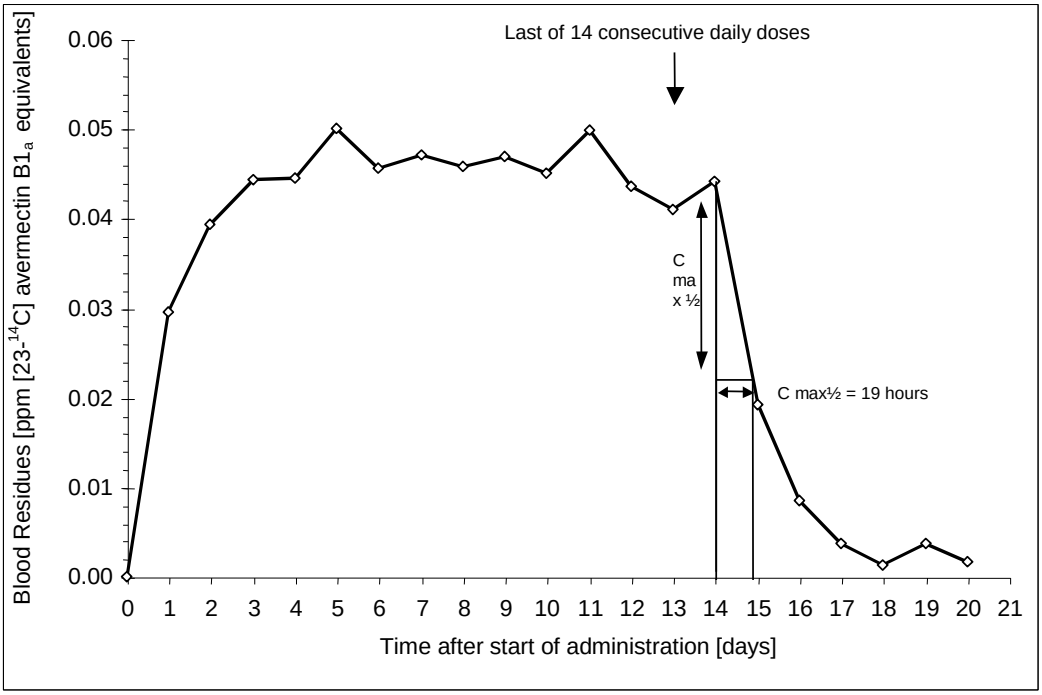
To examine the distribution and clearance of [23-^{14}C]avermectin B_{1a} after multiple dosing, [23-^{14}C]avermectin B_{1a} was orally administered to 16 female rats. The rats received up to 14 consecutive daily doses at a nominal dose of 0.5 mg/kg bw. Four animals each were assigned to subgroups (T1–T4), which were sacrificed at four different time points after the start of administration, and the avermectin B_{1a}–related residues were determined in tissues and organs. The excreted radioactivity was determined in urine and faeces of subgroup T4 at daily intervals. The dosing, sampling and sacrifice time points are summarized as follows:

- *Dosing*: days 0–13;
- *Sacrifice of subgroups*: T1 = day 1, T2 = day 7, T3 = day 14, T4 = day 20;
- *Serial blood sampling T4*: daily on days 1–20;
- *Excreta T4*: daily on days 0–20.

At the time of dosing with radiolabelled avermectin B_{1a}, the rats had a body weight of approximately 180 g and were about 9 weeks of age. The test substance avermectin B_{1a} was dissolved in a 3:2 v/v mixture of polyethylene glycol 200/ethanol at a concentration of about 0.2 mg/mL. Each animal received 0.5 mL of the administration solution by stomach tube. At the defined time points specified above, blood and excreta were collected from subgroup T4. At terminal sacrifice, the tissues and organs were taken for examination of residual radioactivity.

A plateau of blood concentration was reached 3 days after the start of dosing, at about 0.045 ppm avermectin B_{1a} equivalents. One day after the last of 14 consecutive daily doses, the blood residue levels declined rapidly, reaching half the maximum concentration within 19 hours (Fig. 2).

Fig. 2. Profile of [23-^{14}C]avermectin B_{1a} ppm equivalents in blood



Source: Hassler (2003)

During the dosing period – i.e. 14 consecutive daily doses – a steady state in terms of excretion was reached 2 days after the first administration. The majority of the administered test substance (96%) was excreted 7 days after the last of 14 consecutive daily doses. Less than 1% of the total dose remained in the tissues and organs at the end of the experimental period. The administered test substance was excreted almost exclusively with the faeces, accounting for 96% of the total dose. An insignificant amount (0.8% of the total dose) was excreted with the urine.

A plateau of the tissue residue levels was reached after 7 days of multiple dosing for all selected tissues and organs, except for thyroid, which showed an almost linear increase of the residue levels during the dosing period. The highest residue levels were found in fat, accounting for 2.6 ppm [23-^{14}C]avermectin B_{1a} equivalents, followed by adrenals (0.77 ppm equivalents), pancreas (0.66 ppm equivalents), liver (0.63 ppm equivalents) and thyroid (0.60 ppm equivalents). All other tissues and organs reached maximum levels below 0.5 ppm [23-^{14}C]avermectin B_{1a} equivalents. The calculated half-lives for the depletion of the residual radioactivity from tissues and organs, assuming monophasic first-order kinetics, were in the range of 1–3 days for all selected tissues and organs.

The residue levels in tissues and organs 7 days after the last of 14 consecutive daily doses revealed a similar distribution pattern and were only 2–4 times higher than the tissue residue levels 7 days after a single oral administration of [23-^{14}C]avermectin B_{1a} at the low dose (0.5 mg/kg bw), except for fat, which showed a 7 times higher residue level.

In summary, the rates and routes of excretion and the tissue distribution did not change upon multiple dosing compared with single dosing. With the exception of the thyroid, the tissue residues reached a plateau after 7 days of dosing, and for all tissues, the residues declined very rapidly after the dosing period.

The study was GLP compliant, and a QA statement was attached (Hassler, 2003).

1.2 Biotransformation

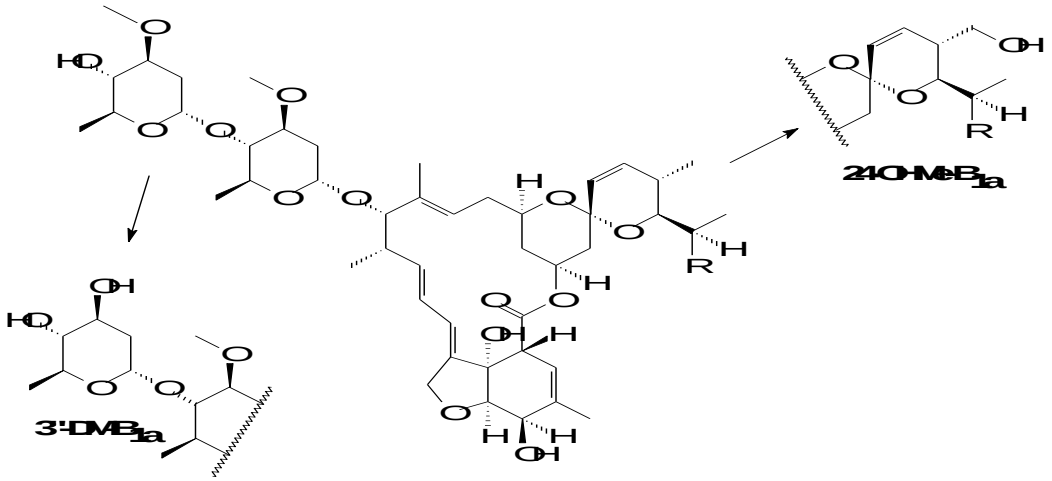
Selected tissue samples derived from the study by Alvaro et al. (1984) (described in section 1.1 above) were analysed for parent avermectin B_{1a}. Liver, kidney, muscle and fat samples were analysed by an indirect method, reverse isotope dilution assay. The residues were separated and purified by reversed-phase HPLC radioactivity profiling and quantified by calculation. Radioactivity in liquid samples was measured by liquid scintillation counting.

Metabolite structures were identified by nuclear magnetic resonance spectrometry and fast atom bombardment mass spectrometry. The isolated metabolites were identified as 24-hydroxymethyl-avermectin B_{1a} (24-OHMe-B_{1a}) and 3″-*O*-desmethyl-avermectin B_{1a} (3″-DM-B_{1a}) and were used as reference substances. Comparison of the HPLC profiles of tissue extracts from rats dosed with both ^{3}H- and ^{14}C-labelled avermectin B_{1a} demonstrated that the profiles were identical. All radioactivity peaks had a constant ^{3}H/^{14}C ratio and the same percentage of recovered radioactivity.

Parent avermectin B_{1a} as a percentage of the total residue was not dependent on dose level. The half-lives of avermectin B_{1a} in tissues of male rats (0.6–1.0 day) were slightly less than in tissues of female rats (0.9–1.0 day). The proportion of avermectin B_{1a} as a percentage of the total residue was similar for both ^{3}H- and ^{14}C-labelled avermectin B_{1a}. Avermectin B_{1a} as a percentage of total residue was lower in tissues from males than in tissues from females. Pretreatment with unlabelled avermectin B_{1a} for 14 days exerted a minimal or negligible effect on tissue residue levels. The residues in liver, kidney, muscle and fat were characterized by HPLC. In addition to unchanged avermectin B_{1a}, two metabolites were observed and subsequently identified. Identification was accomplished by comparison of the retention times of the metabolites and reference substances and also by co-chromatography with reference substances. The reference substances were isolated from rat liver microsome incubation of avermectin B_{1a}. The metabolites were identified as 24-OHMe-B_{1a} and 3″-DM-B_{1a}. The metabolic pathway is illustrated in Fig. 3.

Quantitative results for the metabolites were reported only for some treatment and sampling regimens. In females of the group treated with a single dose of 1.4 mg/kg bw and sacrificed 2 days

Fig. 3. Metabolic pathway of avermectin B_{1a} formed in rats in vivo



Source: Maynard, Wislocki & Lu (1986)

after treatment, 24-OHMe-B_{1a} and 3″-DM-B_{1a} accounted for 6% and 24%, respectively, of the total residue in liver and for 3% and 24%, respectively, in kidney. Unchanged avermectin B_{1a} accounted for 46% of the total residue in liver and 55% in kidney. The metabolic profile in muscle was similar to the profiles in liver and kidney, but no quantitative results were reported. The metabolic profile in fat was different from that in the other tissues. A non-polar fraction was detected that was assumed to consist of non-polar conjugates of 24-OHMe-B_{1a} and 3″-DM-B_{1a}. Other unknown minor metabolite fractions occurred on the chromatograms presented in the report, but no quantitative data were reported.

The use of a tritium label on avermectin B_{1a} is appropriate for animal metabolism studies. Unchanged parent avermectin B_{1a} as a proportion of total residues is not dose dependent but is slightly lower in male rats than in female rats. The tissue half-lives of avermectin B_{1a} are slightly lower in male rats than in female rats. Avermectin B_{1a} and/or its metabolites do not accumulate in liver, kidneys, muscle or fat on repeated administration of a low dose.

The study was not GLP compliant (Maynard, Wislocki & Lu, 1986).

In a multiple oral dosing study with [23-^{14}C]avermectin B_{1a} in female rats (Hassler, 2003), the metabolite pattern of urine and faeces investigated at three different time intervals during the dosing period – i.e. days 0–1, days 6–7 and days 13–14 – was essentially identical for all of the analysed time intervals. The major part of the administered test substance was excreted with the faeces as unchanged parent, accounting for about 40% of the daily dose.

From the study by Hassler (2001) (described in section 1.1 above), selected tissue samples were analysed to identify and quantify individual metabolites to derive the metabolic pathways of orally administered [23-^{14}C]avermectin B_{1a} at a dose of 0.5 or 5 mg/kg bw. Urine, faeces, bile, fat and muscle specimens from male and female rats generated in that study were separately pooled and used for analysis in the present study.

Urine, faeces and bile pools were prepared, according to sex and animal group, by mixing equal aliquots of the collected samples from each animal (0–168 hours or 0–48 hours for bile duct–cannulated animals). Fat and muscle pools were prepared by mixing the whole samples (remaining after determination of the total radioactive residues) from animals of subgroups at the designated time points (8, 24 and 48 hours). Radioactivity in all samples was measured by liquid scintillation counting, and the quantitative metabolite pattern was determined by thin-layer chromatography. Metabolites were isolated by chromatographic techniques, such as solid-phase extraction followed by

HPLC and thin-layer chromatography, and were identified by liquid chromatography–mass spectrometry, liquid chromatography–nuclear magnetic resonance and/or co-chromatography with authentic reference substances.

The metabolite pattern in urine, faeces and bile was complex but qualitatively independent of the sex and the dose level, with some quantitative variations. The following 11 metabolites were isolated:

- 3″-*O*-desmethyl-avermectin B_{1a} (3″DM[1]);
- 24a-hydroxy-avermectin B_{1a} (24aOH);
- 27-hydroxy-avermectin B_{1a} (27OH);
- 28-hydroxy-avermectin B_{1a} (28OH);
- 3″-*O*-desmethyl, 24a-hydroxy-avermectin B_{1a} (3″DM,24aOH);
- 3″-*O*-desmethyl, 27-hydroxy-avermectin B_{1a} (3″DM,27OH);
- 3″-*O*-desmethyl, 4a-hydroxy-avermectin B_{1a} (3″DM,4aOH);
- desoleandrosyl, 3′-*O*-desmethyl, 4a-hydroxy-avermectin B_{1a} (DO,3′DM,4aOH);
- 8a-oxo-avermectin B_{1a} (8aOxo) (code: NOA 448111);
- 8a-hydroxy-avermectin B_{1a} (8aOH) (code: NOA 448112);
- 4-hydroxy, 8a-oxo-avermectin B_{1a} (4OH,8aOxo) (code: NOA 457465).

Unchanged avermectin B_{1a} and the metabolites 3″DM, 24aOH, 27OH, 3″DM,24aOH and 3″DM,27OH represented the majority of the faecal radioactivity. Avermectin B_{1a} accounted for about 25–45% of the dose, and the individual metabolites 3″DM, 24aOH, 27OH, 3″DM,24aOH and 3″DM,27OH accounted for 3–27%. The minor metabolites 28OH, 3″DM,4aOH and DO,3′DM,4aOH each accounted for 0.4–2%. The soil metabolites 8aOxo (NOA 448111), 8aOH (NOA 448112) and 4OH,8aOxo (NOA 457465) were additionally detected in faeces, accounting for 0.3%, 0.4% and 0.08% of the dose, respectively.

The major faecal components, avermectin B_{1a} and the metabolite 3″DM, were not present in urine. The metabolites 24aOH, 27OH, 3″DM,24aOH and 3″DM,27OH represented the majority of the urinary radioactivity, each accounting for 0.02–0.4% of the dose. The metabolites 28OH and DO,3′DM,4aOH were detected in very small quantities, each less than 2% of the dose.

In bile, the major components detected were 3″DM,24aOH, 3″DM,27OH, 3″DM and avermectin B_{1a}, each accounting for 0.1–1.2% of the dose. The metabolites 27OH and DO,3′DM,4aOH were detected in very small quantities, less than 0.1% and less than 0.3% of the dose, respectively. Avermectin B_{1a} was the major component detected in the fat and muscle, accounting for 92% and 72% of fat or muscle radioactivity, respectively. Metabolite 3″DM accounted for 1.7% and 19% of the dose in the fat and muscle, respectively.

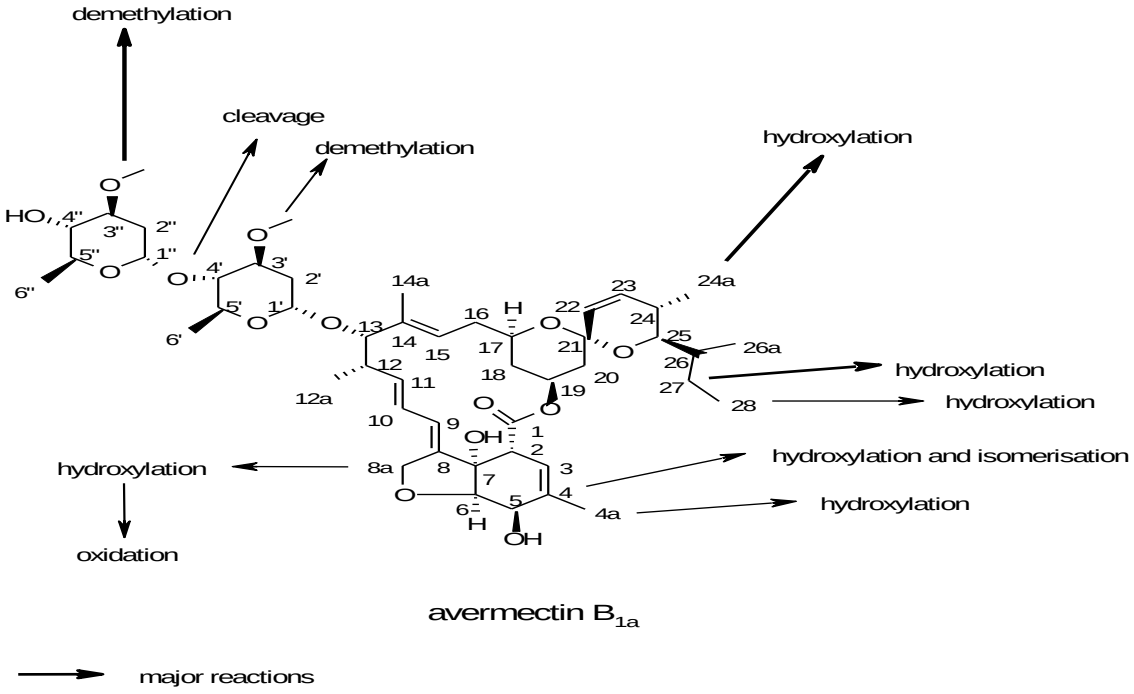
Based on the structures identified, the metabolism of avermectin B_{1a} in the rat proceeds predominantly via demethylation, hydroxylation, cleavage of the oleandrosyl ring and oxidation reactions (Fig. 4). The metabolites identified are the result of the following reaction steps:

- demethylation at the oleandrosyl ring, as in 3″DM;
- hydroxylation at different positions, as in 24aOH, 27OH, 28OH and 8aOH (NOA 448112); combined with demethylation, as in 3″DM,24aOH, 3″DM,27OH and 3″DM,4aOH;
- cleavage of the oleandrosyl ring, as in DO,3′DM,4aOH;
- oxidation of the 8a position, as in 8aOxo (NOA 448111) and 4OH,8aOxo (NOA 457465).

The metabolic pathways of avermectin B_{1a} in the rat are proposed in Fig. 5.

[1] Abbreviated from 3″-DM-B_{1a}, as shown in Fig. 3 and as used in the description of the study by Maynard, Wislocki & Lu (1986) above.

Fig. 4. Proposed reaction scheme for the metabolism of avermectin B_{1a} in rats



Source: Briswalter (2003b)

In another study (Briswalter, 2003a), the metabolite pattern in urine and faeces was complex but qualitatively independent of the sex and the dose level, with some quantitative variations. The metabolite pattern of avermectin B_{1b} was qualitatively similar to that obtained with avermectin B_{1a}, with some minor variations.

Unchanged avermectin B_{1b} accounted for 9–17% of the dose. The major metabolite fractions detected in excreta were fractions B7, B10 and B13, each accounting for 7–33% of the dose. It is considered that these fractions could correspond to fractions A7, A10 and A13 after administration of avermectin $B_{1a.}$ These metabolite fractions, identified as 3″DM,24aOH, 3″DM and 24aOH, respectively, were the major metabolites identified in the course of the metabolism study of avermectin B_{1a} in the rat.

Therefore, it can be concluded that the metabolic pathways of avermectin B_{1a} and avermectin B_{1b} in the rat are similar.

2. Toxicological studies

2.1 Acute toxicity

Studies of the acute toxicity of abamectin are summarized in Table 7, and acute dermal and eye irritation and skin sensitization studies with abamectin are summarized in Table 8.

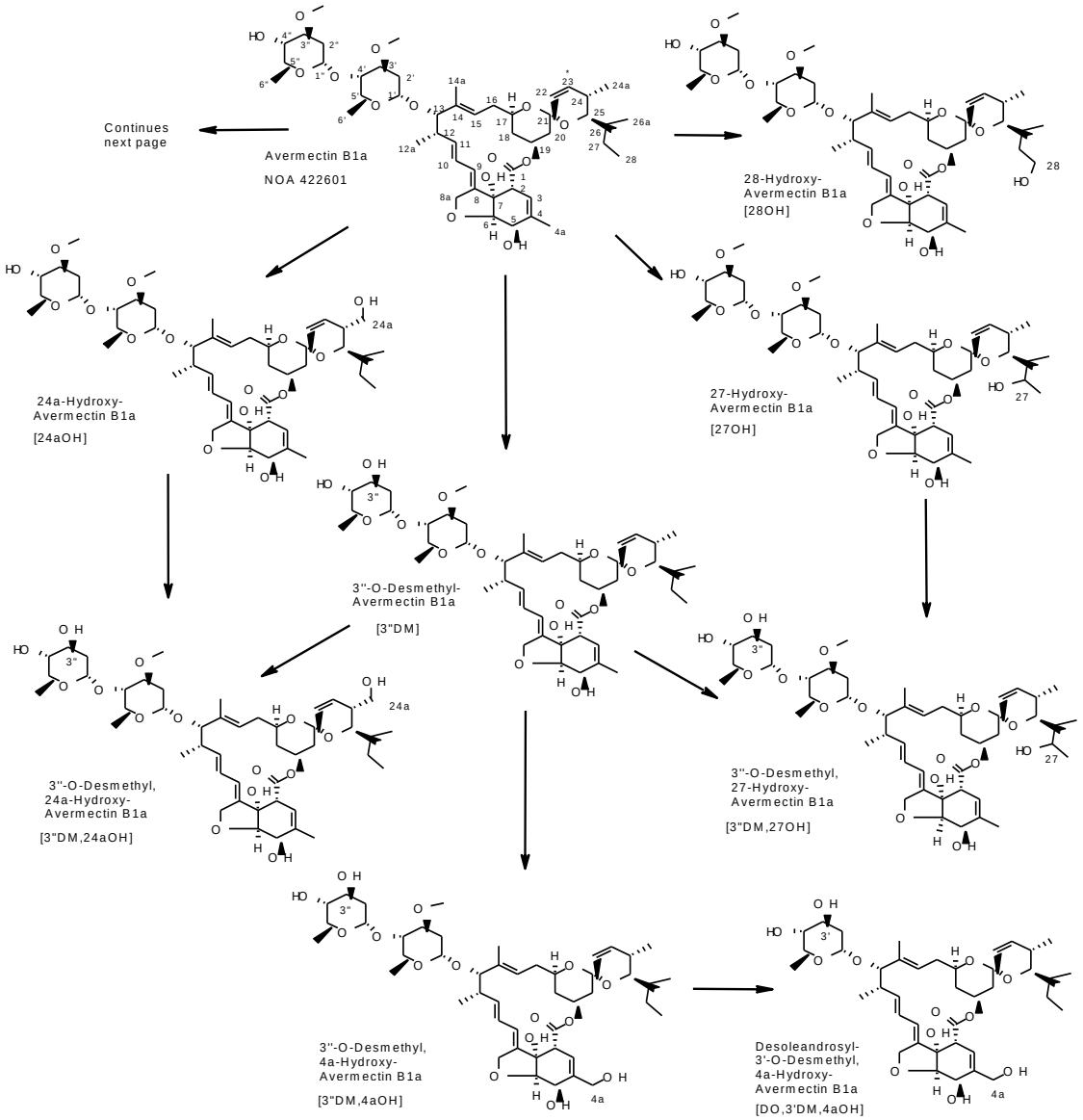
2.2 Short-term studies of toxicity

(a) Oral administration

Rats

In a dose range–finding study, groups of 10 male and 10 female rats (CRCD strain) approximately 3 weeks of age (weighing 37–59 g) were treated orally with abamectin technical (MK-0936, batch no. L-676,863-00V50, purity 94.0%) for 8 weeks by admixture in the diet at a constant

Fig. 5. Proposed pathways for the metabolism of avermectin B_{1a} in rats



Source: Briswalter (2003b)

concentration of 0, 5, 20, 40 or 60 ppm. The 20 ppm dietary concentration was increased to 25 ppm at the start of week 7. Severe toxicity and excessive mortality in the groups treated at 60 and 40 ppm necessitated termination of the surviving animals on days 5 and 15, respectively. Two additional groups of animals, treated at 10 and 15 ppm, were placed on test in week 5 and were treated for 4 weeks only. Mean achieved dose levels were 0.5, 1.2, 1.9, 2.2 and 5.0 mg/kg bw per day for 5, 10, 15, 20/25 and 40 ppm, respectively. A dose range could not be calculated for the 60 ppm group. Samples of all diets prepared in weeks 3 and 7 were analysed for test substance concentration. Further diets were prepared, sampled and analysed for homogeneity and stability. The animals were observed daily for mortality and clinical signs of toxicity, and feed consumption was measured for a 6-day period each week. Body weights were recorded pretest and weekly thereafter. All survivors were killed and discarded without necropsy after 4 weeks (10 and 15 ppm) or 8 weeks (0, 5 and 20/25 ppm) of treatment.

Table 7. Summary of acute toxicity studies with abamectin

Species	Strain	Sex	Route	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	Crl:CD[SD] BR	M + F	Oral[a]	89.3	M: 232 mg/kg bw[b] F: 214 mg/kg bw Combined: 221 mg/kg bw	Glaza (2001)
Rat	CRCD	M + F	Oral[c]	91.4	M: 8.7 mg/kg bw F: 12.8 mg/kg bw[d]	Robertson (1981a)
Rat	RccHan:WIST	M + F	Dermal	89.3	M: > 2 000 mg/kg bw F: > 700 and < 2 000 mg/kg bw	Sieber (2011)
Rabbit	New Zealand White	M + F	Dermal	94	> 2 000 mg/kg bw	Gordon (1984a)
Rat	Crl:HanWis G1x:BRL) BR	M + F	Inhalation (4 h)	96.7	< 0.21 mg/L (MMAD 4.2 µm)	Ruddock (2001a)
Rat	Alpk:AP_fSD	M + F	Inhalation (4 h)	88.3	> 0.034 and < 0.21 mg/L[e] (MMAD: 2.11–2.29 µm)	Noakes (2003)

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male; MMAD: mass median aerodynamic diameter

[a] Vehicle: water.

[b] Clinical signs of toxicity for both males and females at a dose of 275 mg/kg bw included hypoactivity, staggered gait, dyspnoea, red stained face, wet area around the mouth, mydriasis, lacrimation, wet urogenital area, thin appearance, hunched posture, cold to the touch, prostration and tremors. The three surviving animals had fully recovered by day 8. All surviving animals of this group exhibited body weight gain throughout the study. Clinical signs of toxicity in both males and females in the 500 mg/kg bw dose group included hypoactivity, staggered gait, wet/brown area around the mouth, lacrimation, possible bronchial congestion, thin appearance, hunched posture, hypersensitivity, dyspnoea, cold to the touch, prostration, tonic convulsions and tremors. The surviving female returned to a normal appearance by day 6 after treatment and exhibited body weight gain during the study. At necropsy, the findings consisted of ocular, nasal and/or oral discharges of variable consistency and perianal/perineum staining. All other findings were considered incidental and not test material related. There were no visible lesions in any of the animals that survived to study termination.

[c] Vehicle: sesame oil.

[d] Clinical signs of toxicity comprising ataxia and whole-body tremors occurred in all dose groups (6.67, 10, 15, 22.5 and 33.75 mg/kg bw) within 3 hours of treatment. The symptoms persisted until day 6 in all male groups, but females treated at 6.67 mg/kg bw were essentially of normal appearance on day 2. Females treated at higher dose levels continued to show symptoms until day 4. Deaths occurred in all groups except for females treated at 6.67 mg/kg bw. Death occurred from 3 hours to 6 days and from 5 hours to 5 days post-treatment in males and females, respectively. The body weight gain of survivors was unaffected by treatment at all dose levels. There were no treatment-related gross lesions at necropsy, and all gross and histological lesions were considered to be incidental to treatment.

[e] Clinical signs characteristic of mild respiratory tract irritation were seen immediately after day 1 for all animals exposed to 0.051 mg/L and for four females at 0.034 mg/L. Changes indicative of mild to moderate systemic toxicity were also apparent following exposure at 0.051 mg/L, although a full recovery from all clinical signs was apparent for all surviving animals by day 15.

Table 8. Summary of acute dermal and eye irritation and skin sensitization studies with abamectin

Species	Strain	Sex	End-points	Purity (%)	Result	Reference
Rabbit	New Zealand White	M + F	Skin irritation	94	Not irritating	Robertson (1981b)
Rabbit	New Zealand White	M + F	Eye irritation	89.3	Not irritating	Glaza (2000)
Guinea-pig	Crl:[HA]BR	F	Skin sensitization	96.7	Not sensitizing (M&K)	Ruddock (2001b)

F: female; M: male; M&K: Magnusson and Kligman

Sixteen animals treated at 60 ppm died on day 3 or 4 of the test, and the remaining animals were terminated on day 5. Ten animals treated at 40 ppm died on days 3–5, and the remaining animals were terminated on day 15. One female rat treated at 15 ppm was killed in a moribund condition on day 16. As the animal had an enlarged and misshapen cranium, its condition is considered to be due to a congenital abnormality, hydrocephalus, rather than to treatment with abamectin. All other animals survived the designated treatment periods. Treatment-related clinical signs comprising tremors, decreased activity, red-coloured discharge in the oronasal region and staining of the urogenital area first occurred on day 2 in animals treated at 40 and 60 ppm. Slight tremors and/or urogenital staining also occurred in some animals treated at 15 and 20 ppm. Tremors persisted in some animals treated at 40 ppm until sacrifice on day 15, but animals treated at 15 and 20 ppm showed improving conditions after week 1 and, with the exception of some animals with unkempt fur and urogenital area staining, were of normal appearance. Treatment-related clinical signs at 15 and 20 ppm were no longer apparent after week 3. Animals treated at 5 and 10 ppm did not show clinical signs of toxicity, other than one female at 5 ppm that displayed slight tremors on day 2 only. As tremors did not occur at 10 ppm, the transient slight tremors in one female at 5 ppm are considered to be of equivocal toxicological significance.

Treatment at 40 ppm elicited an approximate 52% reduction in body weight gain in both sexes during the 2 weeks of treatment prior to sacrifice. Treatment at 15 and 20/25 ppm elicited transient growth retardation, 9.4–17.7% and 15.2–18.7%, respectively, in both sexes during the first 2 weeks of treatment only. Body weight gain was unaffected by treatment at 5 and 10 ppm. The feed consumption of both sexes treated at 40 ppm was reduced by 24.9–47.0% during the 2 weeks of treatment prior to sacrifice, but was unaffected by treatment at dietary concentrations up to 20/25 ppm. No gross or histological investigations were performed.

In view of the above, the NOAEL in rats of both sexes was 10 ppm abamectin technical (equivalent to 1.2 mg/kg bw per day), based on the occurrence of typical clinical signs and reduction in body weight gain at 15 ppm (equivalent to 1.9 mg/kg bw per day) and higher. This represented the NOAEL after 4 weeks of dosing; however, based on the lack of progression of changes observed at 20/25 ppm (equivalent to 2.2 mg/kg bw per day) between weeks 4 and 8, this NOAEL is also considered to be applicable after 8 weeks.

The study was not GLP compliant (Gordon, 1984b).

Dogs

In a 12-week dose range–finding study, five groups of two male and two female Beagle dogs (30–31 weeks old, weighing 7.8–12.0 kg) were treated continuously for 12 weeks with abamectin technical (MK-0936, batch no. L-676,863-00V50, purity 94%) by admixture in the diet at variable concentrations designed to provide dose levels of 0, 0.25, 0.5, 1.0 and 4.0 mg/kg bw per day. Treatment of the highest-dose group was discontinued for 9 days on day 20 and reinstated at a dose level of 2.0 mg/kg bw per day from day 29. Owing to persistent adverse reactions to treatment, the group was killed and discarded without necropsy on day 36. All animals were examined 7 days/week, with less detailed observations recorded on weekends and holidays. Body weights were recorded twice a week, and feed consumption was measured daily. A qualitative evaluation of the pupil response to light was performed daily on weekdays. Necropsy and postmortem examinations were not performed at the end of the study.

No deaths occurred at any dose level, but the animals treated at 4.0/2.0 mg/kg bw per day were killed and discarded on day 36. During treatment at 4.0 mg/kg bw per day, feed consumption was markedly reduced, generally to less than 25% of the ration offered. When the dose level was reduced to 2.0 mg/kg bw per day, feed consumption remained reduced by approximately 50%. In one female dog, rapid respiration, disorientation, tremors, weakness and slight uncoordination occurred on 1–3 occasions during treatment at 4.0 mg/kg bw per day. These signs disappeared on suspension of dosing and did not reappear on commencement of treatment at 2.0 mg/kg bw per day. Prior to sacrifice, the animals either lost weight or did not gain weight.

Absence of pupil constriction in response to light (i.e. mydriasis) occurred, starting in week 1, in the animals treated at 1.0 and 4.0 mg/kg bw per day. The effect also occurred in the latter group when the dose level was reduced to 2.0 mg/kg bw per day. The observation was not consistent but occurred 1–5 times/week in individual animals. Absence of pupil constriction did not occur in animals treated at 0.25 or 0.5 mg/kg bw per day.

The body weight gains of the groups treated at 0.25, 0.5 and 1.0 mg/kg bw per day were unaffected by treatment. The feed consumption of these groups was not reported.

A very steep dose–response curve is evident for orally administered abamectin in the dog, and the maximum tolerated dose (MTD) was clearly exceeded at dose levels of 2.0 mg/kg bw per day and higher. Therefore, the MTD is 1.0 mg/kg bw per day. The NOAEL was identified as 0.5 mg/kg bw per day, based on the occurrence of mydriasis starting in week 1 of treatment at 1.0 mg/kg bw per day. Markedly reduced feed consumption, body weight loss and clinical signs of intoxication were observed at 2.0 mg/kg bw per day and above.

The study was not GLP compliant (Gordon, 1984c).

In an 18-week study, four groups of three male and three female Beagle dogs (26–42 weeks old, weighing 7.5–12.5 kg) were treated daily with avermectin B_{1a} (batch nos 9603-10-13-1, P14 and P17, purities not specified) by oral gavage as a solution in sesame oil at a dose of 0.25, 0.5, 2.0 or 8.0 mg/kg bw per day. Another two groups of dogs, treated with either tap water or sesame oil, served as control groups.

Treatment of the groups with 8.0 and 2.0 mg/kg bw per day was discontinued after one and three doses, respectively, and the survivors were maintained untreated until study termination. All animals were examined 5 days/week, with less detailed observations recorded on weekends and holidays. Body weights were recorded twice a week, and rectal temperatures were recorded daily from day 87. Ophthalmological examinations were performed pretest and in weeks 3, 8, 11 and 16. A qualitative evaluation of the pupil response to light was performed daily for the first 3 weeks and 5 days/week thereafter. Haematology and serum biochemistry determinations were performed on all animals (fasted) pretest and in weeks 1 (8.0 mg/kg bw per day only), 4, 8, 13 and 17. Additional haematological investigations were performed in weeks 13 and 15 on one animal treated at 0.25 mg/kg bw per day. Urine analysis was performed in weeks 4, 8, 13 and 18.

Electrocardiographic (ECG) recordings were performed on all animals pretest and in weeks 2, 3, 8, 12 and 17. ECG recordings were also performed on day 1 for animals treated at 8.0 mg/kg bw per day. All animals, decedents and fasted survivors, were examined postmortem, and tissues were preserved. Organ weights were recorded for animals surviving to termination. Major organs and tissues and all gross lesions from animals in all treated and control groups were processed to sections stained with haematoxylin and eosin and examined microscopically. Frozen liver samples were also stained with Oil Red O to detect the presence of neutral lipid. Frozen kidney samples from the 0.25 mg/kg bw per day group and the sesame oil control group were stained with Oil Red O.

There were no deaths or treatment-related clinical signs in dogs treated at 0.25 mg/kg bw per day. One dog died in the 0.5 mg/kg bw per day dose group after 18 doses. Three dogs in the 2.0 mg/kg bw per day group died after the third dose, and three dogs died within 24 hours of receiving the first dose in the 8.0 mg/kg bw per day group. Signs of toxicity at 0.5, 2.0 and 8.0 mg/kg bw per day included whole-body muscular tremors, ataxia, mydriasis and ptyalism. In addition, at 2.0 and 8.0 mg/kg bw per day, tonic convulsions and emesis were observed. Dosing of the 2.0 and 8.0 mg/kg bw per day groups was discontinued after the third and first doses, respectively, owing to the severity of clinical signs.

Body weight gain was unaffected by treatment at 0.25 mg/kg bw per day, but body weight loss occurred in some animals treated at 0.5 or 2.0 mg/kg bw per day. Survivors at the higher dose levels showed weight gains comparable with those of the controls after the cessation of treatment, with the exception of one animal treated at 8.0 mg/kg bw per day. There were no treatment-related

changes in haematology and serum chemistry profiles at dose levels up to 2.0 mg/kg bw per day. Treatment-related effects at 8.0 mg/kg bw per day were confined to the samples taken 4 hours after the first dose.

Red and white blood cell counts, haemoglobin concentrations and haematocrit values were slightly elevated 4 hours after the first dose at 8.0 mg/kg bw per day. Serum glucose concentrations at 8.0 mg/kg bw per day were elevated in four animals, and protein levels were slightly raised in two animals, but these are considered to be sequelae of stress. Urine analysis profiles were unaffected by treatment at all dose levels. No treatment-related ocular defects were apparent at any dose level. The pattern of ECG traces was unaffected by treatment up to 2.0 mg/kg bw per day. In week 1, animals treated at 8.0 mg/kg bw per day showed bradycardia (range 32–109 beats/minute) and an increased QT interval (range 0.24–0.28 second). The effects were greatest in the animals that subsequently died. Subsequent ECG traces from all dose groups showed no abnormalities. Individual ECG data were not presented in the report.

An addendum to this study on supplemental histology was provided. Treatment-related histopathological lesions were confined to decedent animals at all dose levels. Consistent changes were confined to the hepatobiliary system in all but one decedent. Diffuse vacuolation of hepatocytes occurred in three, two and one animal treated at 8.0, 2.0 and 0.5 mg/kg bw per day, respectively. Oil Red O staining did not reveal neutral lipid in the vacuoles. Minimal to slight oedema of the gallbladder, primarily in the subserosal connective tissue, occurred in two animals treated at 2.0 mg/kg bw per day and two animals at 8.0 mg/kg bw per day. The female treated at 2.0 mg/kg bw per day that died on day 49 following perforation of the jejunum and peritonitis had severe, multifocal necrotizing enteritis. As no other animals showed gastrointestinal lesions, either gross or microscopic, it is considered that the lesion was not treatment related. There were no treatment-related organ weight changes or histopathological alterations in surviving animals at any dose level.

The above information suggests a very steep dose–response curve for orally administered avermectin B_{1a} in the dog, and the MTD was clearly exceeded at dose levels above 0.5 mg/kg bw per day. A NOAEL of 0.25 mg/kg bw per day was identified in both sexes, based on mortality, clinical signs of toxicity, reduced body weight gain or body weight loss, and histopathological changes in the liver at 0.5 mg/kg bw per day. Signs of toxicity were observed starting on day 7 at 0.5 mg/kg bw per day, on day 3 at 2.0 mg/kg bw per day and on day 1 (within 3 hours after dosing) at 8.0 mg/kg bw per day.

The study was not GLP compliant. However, the supplemental histology study was GLP compliant (Allen & Robertson, 1976; Allen, 1982).

In a 53-week dietary study, four groups of six male and six female Beagle dogs (20–24 weeks old, body weight range 4.9–8.7 kg) were treated orally with abamectin technical (MK-0936, batch no. L- 676,863-00V54, purity > 89% by liquid chromatography) by dietary admixture at variable concentrations calculated to provide dose levels of 0, 0.25, 0.5 and 1.0 mg/kg bw per day. The dose levels were selected on the basis of a 12-week study in dogs in which 0.5 mg/kg bw per day was found to be the NOAEL (Gordon, 1984c). Clinical signs were recorded daily, and the eyes were checked for direct light-induced pupil constriction on weekdays. Body weights were recorded weekly, and feed consumption was usually measured 4 times/week. Opthalmoscopic examinations were performed pretest and in weeks 12, 25, 40 and 52. Haematology, serum biochemistry and urine analyses were performed pretest and in weeks 4, 8, 12, 26, 38 and 52. All animals, decedents and survivors, were subjected to necropsy, postmortem examination, organ weight recording (not decedents) and extensive tissue preservation.

All tissues from all animals were processed to sections stained with haematoxylin and eosin and examined microscopically without knowledge of the treatment group. Frozen sections of liver and kidney were stained with Oil Red O to indicate the presence of neutral lipids.

Deaths were confined to the highest dose level, 1.0 mg/kg bw per day. Two male animals were killed in a moribund condition, one in week 33 and another in week 38. One animal showed

Table 9. Incidence of absent or decreased pupil reactivity to direct light

	0 mg/kg bw per day	**0.25 mg/kg bw per day**	**0.5 mg/kg bw per day**	**1.0 mg/kg bw per day**
No. of observations of absent or decreased pupil reactivity to direct light	0	4	101 (3%)	422 (15%)
No. of dogs in which observation seen	–	Two times in each of two animals	In all but one animal	At least once in all animals treated

bw: body weight
Source: Gordon (1984d)

weight loss and lethargy, and the other showed excessive salivation, unsteadiness and lateral recumbence prior to sacrifice. A third male dog died in week 38 without clinical signs of toxicity. The feed consumption pattern of this animal indicated an ingested dose of 1.3–1.5 mg/kg bw per day for several days before death. Treatment-related clinical signs in surviving animals at 1.0 mg/kg bw per day were confined to absent or decreased pupil reactivity to direct light (Table 9). The observation was made 422 times at 1.0 mg/kg bw per day (occurrence rate approximately 15%) and 101 times at 0.5 mg/kg bw per day (occurrence rate approximately 3%). It also occurred 4 times at 0.25 mg/kg bw per day (twice in each of two animals). The effect occurred at least once in all animals treated at 1.0 mg/kg bw per day and in all but one animal at 0.5 mg/kg bw per day, but the presence of the effect in individuals was inconsistent. As the observation is highly subjective, was not performed blind and occurred inconsistently at 0.5 mg/kg bw per day, the significance of the very low incidence at 0.25 mg/kg bw per day is considered equivocal.

Body weight gain and feed consumption were unaffected by treatment at 0.25 and 0.5 mg/kg bw per day. The overall mean body weight gain of the group treated at 1.0 mg/kg bw per day was 29.1% lower than that of concurrent controls, and feed consumption was markedly reduced. Concentrations of abamectin technical in diet in the high-dose group appeared to be unpalatable. There were no treatment-related ophthalmoscopic findings at any dose level.

There were no haematological changes at any dose level in surviving animals. However, one male treated at 1.0 mg/kg bw per day and killed in week 38 showed slight leukocytosis, predominantly neutrophilia, and slightly elevated packed cell volume just prior to sacrifice. Treatment-related serum biochemistry effects occurred at 1.0 mg/kg bw per day only. Serum urea and total protein concentrations were slightly reduced throughout the treatment period. The changes in serum biochemistry are considered to be physiological sequelae to markedly reduced feed consumption. No effects on urine analysis profiles occurred at any dose level. There were no treatment-related organ weight changes or gross or histopathological alterations at any dose level in all animals, including three male decedents treated at 1.0 mg/kg bw per day.

A NOAEL for abamectin technical of 0.25 mg/kg bw per day was identified in the dog, based on mydriasis at 0.5 mg/kg bw per day. Signs of toxicity were observed starting in the first week at 0.5 and 1.0 mg/kg bw per day.

The study was GLP compliant (Gordon, 1984d).

Clinical signs of neurotoxicity in dogs, including their time of first appearance, in the above three studies are compared in Table 10.

Table 10. Clinical signs observed in dog studies

Study description	0.25 mg/kg bw per day	0.5 mg/kg bw per day	1.0 mg/kg bw per day	2.0 mg/kg bw per day	4.0 mg/kg bw per day	8.0 mg/kg bw per day
12-week dose range–finding study (feeding) (0.25, 0.5, 1.0, 4.0/2.0 mg/kg bw per day) (2 M/2 F)	Mydriasis was never noted	Mydriasis was never noted	Mydriasis was noted starting in week 1, 1–5 times/week	Mydriasis was noted; feed consumption and body weight were markedly reduced; dogs were sacrificed on day 36	Mydriasis was noted starting in week 1, 1–5 times/week; tremor, rapid respiration and weakness were seen; dosing was ceased on day 20 (marked decrease in feed consumption and body weight) and reduced to 2.0 mg/kg bw per day on day 29	–
18-week toxicity study (gavage) (0.25, 0.5, 2.0 and 8.0 mg/kg bw per day) (3 M/3 F)	Mydriasis was never noted	Mydriasis was seen on treatment day 7 in one dog; in week 2, mydriasis was seen in 4/6 dogs; whole-body tremor, ataxia and ptyalism were seen in one dog at week 2 and persisted until the end of the study	–	Mydriasis, ataxia, ptyalism and anorexia were seen in all dogs by day 3 of dosing; after the third dose, three dogs died with signs of convulsion or tremors prior to death; dosing was ceased after the third dose	–	Ptyalism, emesis, ataxia and whole-body tremors were seen within 3 h after first dose, and three dogs died after first dose; mydriasis was seen 24 h after dosing in surviving dogs; dosing was ceased after the first dose
53-week toxicity study (feeding) (0.25, 0.5 and 1.0 mg/kg bw per day) (6 M/6 F)	Mydriasis was noted in week 10 in two dogs, but not before or after that time; not treatment related	Mydriasis was noted in week 1 in one dog (1×); by week 6, five dogs had mydriasis	Mydriasis was noted in week 1 in one dog (2×); by week 3, four dogs had mydriasis; by week 11, 11 dogs had mydriasis; by week 38, three males died	–	–	–

bw: body weight; F: females; M: males
Source: Allen & Robertson (1976); Allen (1982); Gordon (1984c,d)

(b) Exposure by inhalation

Rats

In a 5-day preliminary inhalation toxicity study, groups of two male and two female HsdBrlHan: WIST rats were exposed nose-only to abamectin technical (milled) at 0 (control), 1, 4, 10 or 25 µg/L for a 6-hour period daily, for up to 5 consecutive days. Clinical observations were made daily throughout the study. Body weights were measured daily, and feed consumption was measured continuously throughout the study. At the end of the scheduled period, the animals were killed and subjected to a full postmortem examination. Selected organs were weighed, and specified tissues were taken for subsequent histopathological examination. Abamectin technical is non-volatile (vapour pressure $< 3.7 \times 10^{-6}$ Pa), and it exists in physical form as a solid "wet cake".

The test atmospheres generated (1, 4, 10 and 25 µg/L) were acceptable with regard to stability and physical characteristics.

At 25 µg/L, two females were killed on day 2 (one before the exposure period on that day, the other after 4.5 hours of exposure), and one male was killed on day 3 (before the exposure period on that day), all as a result of adverse clinical signs (including reduced splay reflex and stability, tremors, shaking, irregular breathing, decreased visual placement, tail erection, hunched posture, piloerection, decreased activity, stains around the mouth and nose), body weight loss and markedly reduced feed consumption. There were no further exposures at this concentration. The surviving male was allowed to recover between days 3 and 6. At 10 µg/L, one female was killed after 2 consecutive exposure days as a result of adverse clinical signs, body weight loss and reduced feed consumption. The other female lost weight, had reduced feed consumption and exhibited some adverse clinical signs. Males at this level showed some clinical signs, but had normal growth and feed consumption. The three remaining animals in this group continued to be exposed as scheduled until termination. Concentrations of 25 and 10 µg/L were, as a result of the findings, considered to be too high for a subsequent repeated-exposure study. At 4 µg/L, reduced splay reflex was seen in one female between days 4 and 6 and in one male on day 5. At 1 µg/L, growth of males was slightly lower than in controls, and one female had reduced splay reflex between days 5 and 6. There were no treatment-related microscopic findings, at any of the dose levels tested, in animals exposed for 5 consecutive days.

In view of the above findings, no exposure concentrations higher than 4 µg/L were recommended for the subsequent 30-day inhalation study.

The study was GLP compliant, and a QA statement was attached (Pinto, 2006a).

In the main study, groups of 10 male and 10 female Han Wistar (HsdBrlHan:WIST) rats were exposed nose-only for 6 hours/day to a target abamectin technical (milled) concentration of 0 (control), 0.1, 0.5 or 2.5 µg/L for 5 days/week over a 30-day study period.

Detailed clinical observations were made weekly. Body weights were measured weekly, and feed consumption was measured continuously throughout the study. Eyes were examined pre-study and pre-termination. A functional observational battery and an assessment of locomotor activity were performed in week 4. On day 30, the surviving animals were killed and fully examined postmortem. Cardiac blood samples were taken for clinical pathology from all animals, selected organs were weighed and specified tissues were taken for subsequent histopathological examination.

The test atmospheres generated were acceptable with regard to general stability and physical characteristics. Concentrations were close to target concentrations, and particle sizes were within the required range.

One female exposed to 2.5 µg/L was found prostrate, shaking and gasping with a swollen head on day 2, prior to the start of the exposure period. This animal was sent for necropsy. One female exposed to 2.5 µg/L was ungroomed, with stains around the mouth, hunched posture and piloerection on day 15 (week 3) only. Two females at this exposure concentration had abnormal respiratory noise in week 5.

Body weight for females exposed to 2.5 µg/L was lower than control values on days 15 and 22 (maximum difference 5%). This difference was predominantly due to one female that had poor growth during this period.

Locomotor activity was statistically significantly lower than in controls for females exposed to 2.5 µg/L, both overall (1–50 minutes) and during minutes 36–40. Activity was generally lower during the latter part of the assessment period for these animals.

Plasma alanine aminotransferase activity was statistically significantly lower than in controls for males exposed to 0.5 and 2.5 µg/L. Spleen weight was statistically significantly higher than control values for males exposed to 2.5 µg/L. Owing to the small magnitude of the changes and in the absence of any histopathology, these findings are considered to be of no toxicological significance. There were no treatment-related macroscopic or microscopic findings at any exposure concentration. No abnormalities were seen in males at any exposure concentration or in females exposed to 0.1 or 0.5 µg/L.

The study was GLP compliant, and a QA statement was attached (Pinto, 2006b).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 74 male and 74 female 6-week-old mice (Crl:CD-1 (ICR)BR strain, body weight range 15.5–33.2 g) were administered abamectin (MK-0936, batch no. L-676,863-00V54, initial purity 91.1%, purity determined to be 89.0% weight per weight [w/w] in week 34) orally for 93 weeks, by admixture in the diet, at concentrations adjusted to provide dose levels of 0 (control I), 0 (control II), 2.0, 4.0 and 8.0 mg/kg bw per day. All female groups started on study were killed and discarded shortly after the initiation of treatment because of the presence of tremors at all dose levels within 24 hours and deaths at 4.0 and 8.0 mg/kg bw per day. New groups of females were started on test, at the same dose levels, approximately 1 month later. Treatment of the male group with 8.0 mg/kg bw per day was discontinued after 89 weeks when survival had reached 40%. The stability and homogeneity of abamectin in the diet were confirmed prior to initiation of treatment, and achieved dietary concentrations were assayed for each dose group on 6- to 8-week pooled samples throughout the study.

Twelve animals of each sex per group were killed for blood sampling in week 25/26, and a further 12 animals of each sex per group in week 52. Mortality checks and clinical observations were made daily, and detailed physical examinations, including palpation, weekly. Body weights were recorded pretest and weekly thereafter. Feed consumption was measured weekly in 12 animals of each sex per group for 6 days/week. An ophthalmological examination of all animals was conducted pretest and in the control and high dose group animals in weeks 51/53 and 91. Haematological and serum chemistry investigations were performed in weeks 25/26 and 52 in 12 animals of each sex per group, in all moribund animals after week 69, and pre-termination for all surviving animals. All animals killed after 26 and 52 weeks for blood sampling were subjected to necropsy, gross postmortem examination and tissue preservation only. All decedent and surviving animals scheduled for sacrifice after 93 weeks were subjected to detailed necropsy and postmortem examination. Organ weights were recorded for all animals that survived to scheduled sacrifice. Tissue/organ samples were preserved for all animals that died or were killed during the study, and all animals killed at the end of the study were preserved. Microscopic examination of tissues, gross lesions and palpated masses was performed on all decedents and survivors from all groups scheduled to be killed after 93 weeks.

Mortality and body weight data (93-week groups only) were statistically analysed by the Mantel-Haenszel trend test. Tumour incidences were analysed by the extended Mantel-Haenszel procedure implementing adjustments for variables.

Treatment-related tremors occurred in several females from all the original treatment groups after 1 day. Seven and three females treated at 8.0 and 4.0 mg/kg bw per day, respectively, died on day 2. Treatment was withdrawn, the affected animals were replaced and treatment was recommenced

1 week later. Treatment-related tremors recurred in some females from all groups. Therefore, all female mice were terminated, and new groups of females were restarted on study 4 weeks later. Following restart of the study, only two females at 8.0 mg/kg bw per day showed sporadic tremors, one in week 89 and the other in week 91. Treatment-related tremors did not occur at lower dose levels, although one female treated at 4.0 mg/kg bw per day showed circling and tremors 1 day prior to death in week 49. As the animal was subsequently diagnosed with metastatic lymphoma of the pituitary gland, the tremors are considered to be secondary to the tumour. Treatment-related tremors did not occur in males at any dose level. Transient tremors, considered not to be treatment related, occurred 1 day before sacrifice, in a moribund control II male and two moribund males treated at 8.0 mg/kg bw per day.

A treatment-related increase in mortality occurred in males treated at 8.0 mg/kg bw per day ($P = 0.012$), and treatment of the group was discontinued after 89 weeks. There was no effect on male survival at lower dose levels or in any of the female groups ($P > 0.05$). Male mortality incidences after 93 weeks of treatment were 48%, 48% and 68%, in order of ascending dose level, compared with an incidence of 50% in both male control groups. Female mortality incidences after 93 weeks of treatment were 40%, 40% and 32%, in order of ascending dose level, compared with incidences of 28% and 32% in female control groups. Treatment-related effects on weight gain were confined to the female group treated at 8.0 mg/kg bw per day. At this dose level, there was an overall reduced weight gain of 21.4% compared with the mean weight gain of the control groups. There was no effect on feed consumption at any dose level. No treatment-related ocular defects occurred, and there were no treatment-related changes in the haematological and serum biochemistry profiles at any dose level. Several individual animals in all treated and control groups showed isolated, abnormal haematological and serum biochemistry parameters; in general, these could be related to specific clinical observations or are common geriatric or morbid changes in mice.

At necropsy, there were no treatment-related effects either on organ weights or on the nature and distribution of gross lesions. Statistical analysis of the incidence of tumour-bearing animals for each tumour site showed that there were no statistically significant positive or negative trends in incidence for any of the tumour sites in either sex. There were no treatment-related non-neoplastic histopathological findings at any dose level in either sex. An increased incidence of dermatitis occurred in males treated at 4.0 and 8.0 mg/kg bw per day. There were 3/49, 6/50 and 11/50 occurrences in male groups, in order of ascending dose level, compared with control incidences of 2/50 and 3/50. The increased incidences at 4.0 and 8.0 mg/kg bw per day are unlikely to be treatment induced, as dermatitis related to fighting among male cage-mates is common. The incidences of dermatitis in female groups were 0/50, 0/49 and 1/50, compared with control female incidences of 0/50 and 1/50. All other histopathological findings were evenly distributed between the control and treated groups and were of the type and severity that generally occur in the strain of mouse used.

In view of the above, the NOAEL for abamectin technical in mice was identified as 4.0 mg/kg bw per day in both sexes, based on increased mortality in males and reduced body weight gain in females at 8.0 mg/kg bw per day. There was no increase in tumour incidence in mice in this study.

The study was GLP compliant, and a QA statement was attached (Gordon, 1985a).

Rats

Groups of 65 male and 65 female 5-week-old Sprague-Dawley-derived rats (Crl:CD(SD)BR strain, body weight range 93–191 g) were administered abamectin technical (batch no. L-676,863-00V54, initial purity 91.1%, purity determined to be 89% w/w in week 51) orally for 104 weeks, by admixture in the diet, at concentrations adjusted to provide dose levels of 0 (control I), 0 (control II), 0.75, 1.5 and 2.0 mg/kg bw per day. The highest dose level was increased to 2.5 mg/kg bw per day for weeks 11 and 12, followed by 1 day off-dose.

Fifteen animals of each sex per group were designated for interim sacrifice at week 53, and 50 animals of each sex per group were sacrificed after 104 weeks of treatment for the oncogenicity study. Mortality checks and clinical observations were made daily, and detailed physical

examinations, including palpation, weekly. Body weights were recorded pretest and weekly thereafter. Feed consumption was measured weekly in 12 animals of each sex per group for 5 or 6 days/week. The eyes of all animals were examined pretest and of control and high dose group animals in weeks 26, 52/53, 76 and 102/103. Haematological, serum chemistry and urine analysis investigations were performed at weeks 12, 25, 38 and 51 in 10 animals of each sex per group designated for sacrifice after 52 weeks, in week 78 on 10 animals of each sex per group from the main study animals, and pre-terminally in all survivors. All decedent and surviving animals were subjected to detailed necropsy and postmortem examination. Organ weights were recorded for all animals that survived to scheduled sacrifice. Tissue/organ samples from all animals sacrificed at 52 weeks, those that died or were killed during the study and all animals killed at the end of the study were preserved. Microscopic examination of tissues was performed on all control (I) and high-dose animals killed at 52 weeks, all decedents from all groups scheduled to be killed after 52 weeks, and all decedents and survivors from all groups scheduled to be killed after 104 weeks. Mortality and body weight data (104-week groups only) were statistically analysed by the Mantel-Haenszel trend test, and tumour incidences by the extended Mantel-Haenszel procedure implementing adjustments for variables.

Treatment-related clinical signs comprising whole-body tremors and unthrifty appearance were confined to the group treated at 2.0 mg/kg bw per day and, in all but one instance, first occurred in week 12 after the dose level was increased to 2.5 mg/kg bw per day. The clinical signs persisted intermittently until termination. Tremors were also noted in a further two females at 2.0 mg/kg bw per day and in one female at 1.5 mg/kg bw per day, starting late in the treatment period. These females were all small animals (< 300 g) and are likely to have received a disproportionately high dose of abamectin technical. Mortality incidence was unaffected by treatment at all doses of abamectin technical. At termination, mortality incidences were 52%, 52%, 62%, 42% and 68% in males and 62%, 48%, 68%, 66% and 64% in females, in order of ascending dose level. All incidences of mortality in abamectin-treated groups were not significantly different ($P > 0.05$) from the combined control group incidence. A treatment-related increase in weight gain occurred in both sexes of all treated groups, the effect being inversely related to dose level in the top three doses. Consistently higher weight gains occurred in males throughout the treatment period; at termination, the overall weight gains were significantly ($P < 0.05$) increased by 21%, 9% and 5%, in order of ascending dose level. In females, the effect was confined to the first 60 weeks of treatment, during which time the weight gains of the treated groups were significantly ($P < 0.05$) increased by 26%, 10% and 7%, in order of ascending dose level. Thereafter, the weight gains were lower than the control values; at termination, the overall weight gains of treated females were not significantly different from those of the controls.

The feed consumption of all abamectin-treated groups was unaffected by treatment at all dose levels. Ocular abnormalities observed in both high-dose and control animals during the study were of the type commonly observed in rats, and none was considered to be treatment related in origin. There were no treatment-related changes at any sampling interval in the haematological, serum biochemistry or urine analysis profiles at any dose level. Several individual animals in all study groups showed isolated, abnormal blood or urine parameters; in general, however, these could be related to specific clinical observations or are common geriatric or morbid changes in rats.

There were no treatment-related gross lesions at any dose level either in decedents or in survivors at necropsy after 52 or 104 weeks of treatment. There were no treatment-related changes at any dose level in absolute or relative organ weights after 52 and 104 weeks of treatment. There were no treatment-related histopathological changes, either neoplastic or non-neoplastic, at any dose level after 52 weeks of treatment, including three animals at 2.0 mg/kg bw per day sacrificed because of tremors. No neuromuscular changes were found to account for the clinical signs. There were no treatment-related histopathological changes, either neoplastic or non-neoplastic, at any dose level after 104 weeks of treatment. All tumour incidences in both sexes were not statistically significantly different from the control groups. All histopathological changes observed after 52 and 104 weeks of treatment with abamectin are considered to be common spontaneous findings in rats of the age and strain used.

A NOAEL of 1.5 mg/kg bw per day was identified, based on the occurrence of clinical signs of toxicity at 2.0 mg/kg bw per day in both sexes. There was no increase in tumour incidence in rats in this study.

The study was GLP compliant, and a QA statement was attached (Gordon, 1985b).

2.4 Genotoxicity

Abamectin was tested for genotoxicity in four in vitro assays and one in vivo assay. All the results were negative (Table 11).

Table 11. Overview of genotoxicity tests with abamectin

End-point	Test system	Concentrations/doses tested	Purity (%)	Results	Reference
In vitro					
Mammalian point mutations	Chinese hamster V-79 cells	3, 4, 5, 6 $\times 10^{-3}$ mmol/L (−S9) 3, 4, 4.5, 5 $\times 10^{-2}$ mmol/L (+S9)	94	Negative	Gordon (1983)
Microbial point mutations	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	0, 3, 10, 30, 100, 300, 1 000 µg/plate	94	Negative	Gordon (1986a)
Microbial point mutations	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 and *Escherichia coli* WP2 uvrA	0, 312.5, 625, 1 250, 2 500, 5 000 µg/mL	89.3	Negative	Deparade (2001)
Chromosomal aberrations	Chinese hamster ovary cells	0, 10, 15, 20, 25, 30, 35 $\times 10^{-2}$ mmol/L (−S9) 0, 5, 10, 15, 20, 25 $\times 10^{-3}$ mmol/L (+S9)	94	Negative	Gordon (1986b)
In vivo					
Chromosomal aberrations	CD-1 strain mouse bone marrow	0, 1, 2, 4, 12 mg/kg bw and killed after 6, 24 or 48 h	94	Negative	Blazak (1983)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

Abamectin technical (MK-0936, batch no. L-676,863-00V50, purity not reported) was administered orally to two successive generations (P and F_{1b}) of male and female rats, by gavage, at a dose level of 0 (vehicle only), 0.05, 0.12 or 0.40 mg/kg bw per day. Two litters were produced by both the P generation (F_{1a} and F_{1b}) and the F_{1b} generation (F_{2a} and F_{2b}).

Initially, four groups of 30 male and 30 female rats (Crl:CD(SD) BR strain, approximately 6 weeks old, weight range 113–194 g) were continuously treated daily, by gavage, with abamectin technical for 68 days prior to mating, throughout cohabitation, gestation and lactation for F_{1a} and F_{1b} litters, and subsequently for a minimum 16-day maternal rest period (females) and a further 50 days after weaning of the last F_{1b} litter (males). Selected F_{1b} progeny were similarly treated, but from

weaning until approximately 30 days after the last F_{2b} litter had been weaned. The test substance was administered at 5 mL/kg bw as a solution in sesame oil, at a dose level of 0 (vehicle only), 0.05, 0.12 or 0.40 mg/kg bw per day for 259 days and at least 177 days for P generation males and females, respectively, and for a maximum of 273 days for selected F_{1b} progeny. Individual doses were recalculated weekly for both the P and F_{1b} generations, except for females during gestation and lactation, when treatment volumes were readjusted at 3-day intervals. Samples of abamectin formulations were analysed for test concentration at 5/6-week intervals throughout the study. Achieved concentrations were within acceptable limits (not less than 90% of the nominal concentration), with the exception of the low-dose solution on three occasions, when achieved concentrations were 65–80% of the nominal concentration.

Matings were performed within groups on a 1:1 basis for 14 days, during which time vaginal lavage was performed daily to check for mating (day 0 of gestation = day on which sperm were observed in the vagina). Females failing to mate were rehoused with a proven male for a further 7 days. Litters were examined as soon as possible after birth (day 1 postpartum), and viability and pup weights were recorded on days 1, 4, 7, 14 and 21 postpartum. Litters were culled to 10 pups (five of each sex, where possible) on day 4, by random selection. Maternal behaviour and pup behaviour were recorded on pup weighing days. F_{1a} litters were weaned, killed and discarded without further examination on day 21 postpartum. Following a maternal rest period of at least 16 days, the P generation was remated to produce the F_{1b} generation. P generation females not mating or not producing a litter were killed and discarded after reproductive status had been confirmed. All other P generation females were killed and discarded after weaning of the F_{1b} litters. P generation males were killed, and testes and epididymides were weighed and preserved in Bouin's fluid and then discarded. F_{1b} litters were weaned between days 26 and 30 postpartum, and one or two pups of each sex per litter (32 pups of each sex per group) were randomly selected for extended evaluation. On day 26/27, a further one pup per litter was randomly selected from 10 litters per group for full gross examination, organ weights and microscopic pathological evaluation. All other F_{1b} progeny were killed and discarded. The selected F_{1b} progeny for extended evaluation were mated twice to produce the F_{2a} and F_{2b} generations in a manner similar to that followed for the P generation. Five F_{2b} pups of each sex per group were randomly selected for full gross examination, organ weights and microscopic pathological evaluation, and 10 pups of each sex per group were killed and submitted for skeletal examination. All other F_{2b} pups were killed at weaning, the eyes retained in fixative, and the pups discarded. Thirty days after weaning of the last F_{2b} litter, 10 male and 25 female F_{1b} parental animals per group were randomly selected for necropsy, full gross examination, organ weights and histopathology. The remaining F_{1b} parental animals were killed and discarded, but testes and epididymides were weighed and preserved in Bouin's fluid from the males, and the eyes of both sexes were preserved. All pre-weaned pups found dead were given a gross external and visceral examination, and the carcasses were processed for skeletal examination. Weaned animals that died or were killed in a moribund condition were subjected to necropsy, and gross lesions were processed and examined histologically. Parental clinical signs were recorded daily and body weights were recorded weekly throughout the study and at termination, except for females during gestation and lactation, when body weights were recorded at 3-day intervals. Parental animals dying or killed in a moribund condition were subjected to a full necropsy, gross examination and microscopic evaluation of gross lesions.

Body weights were analysed statistically by Bartlett's test for homogeneity of variance and analysis of variance (ANOVA). Litter data and durations of cohabitation and gestation were analysed by the Kruskal-Wallis test. Where significant, the data were further analysed using Dunn's multiple comparison test. Fertility and mating parameters were analysed by the variance test for homogeneity of the binomial distribution.

P generation. No treatment-related deaths or clinical signs occurred at any dose level, although nine animals died during the study. One, two and two males died in the groups treated at 0, 0.05 and 0.12 mg/kg bw per day, respectively, after receiving 103–216 doses, and two control and two treated females at 0.05 mg/kg bw per day died after receiving 95–171 doses. The causes of death, established by histopathological examination, were diverse; as the distribution of mortality did not show a dose–response relationship, the deaths were considered incidental to treatment. There was a

treatment-related increase in weight gain of male rats treated at 0.40 mg/kg bw per day (Table 12). Thus, at termination, the group mean body weight was increased by 16.2%. A similar, but less marked and transient, effect occurred in males at 0.12 mg/kg bw per day (terminal group mean body weight increased by 5.4%). A similar effect occurred prior to mating in females treated at 0.12 and 0.40 mg/kg bw per day. At the end of the premating period, group mean body weights were increased by 11.2% and 25.8%, respectively. Although increased weight gains at 0.12 and 0.40 mg/kg bw per day are considered treatment related, they are not considered to be adverse effects of treatment. Overall body weight gains of maternal animals during the first and second gestation and lactation periods were unaffected by treatment at all dose levels.

No effects were seen on mating/fertility parameters during breeding for F_{1a} litters. However, during breeding for the F_{1b} generation, the number of high-dose males (0.40 mg/kg bw per day) mating was significantly lower ($P < 0.01$) than in controls. For males, the mean number of days in cohabitation was significantly increased ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) for the low-, middle- and high-dose groups. The statistically significant differences for mating duration for the F_{1b} litters at 0.05 and 0.12 mg/kg bw per day are considered incidental to treatment, as the control group showed an unusually short mating time. Similarly, as observed in males, for 0.40 mg/kg bw per day females, the mean number of days in cohabitation prior to mating was significantly increased ($P < 0.05$). Significantly fewer ($P < 0.01$) 0.40 mg/kg bw per day group females mated compared with controls. Although no measurements of estrous cycling were performed, it has been speculated that this effect on mating may have resulted from estrous cycling irregularities that occurred for P female rats following production of the F_{1a} litters. The effect on fertility and fecundity of P animals of both sexes during breeding for F_{1b} litters was not reproducible in breeding for F_{1a}, F_{2a} and F_{2b} litters. Furthermore, no histopathological correlates were evident in the reproductive organs of either sex of the P generation. Therefore, this is considered to be an isolated finding that is not related to treatment.

There were no treatment-related effects on the incidence of stillborn pups. However, the mortality of neonate rats of F_{1a} and F_{1b} litters increased to significant ($P < 0.01$) levels during lactation, and an associated significant reduction occurred in viability and lactation indices. Pup weight was unremarkable directly after birth and up to day 4. After several days of lactation, it was observed that mean pup weight gain was significantly reduced ($P < 0.01$) at 0.40 mg/kg bw per day in both F_{1a} and F_{1b} litters on days 7, 14 and 21 (Table 13), a finding that was associated with increased numbers of pups that were thin, weak and cold to the touch. No treatment-related skeletal abnormalities occurred in pups dying during lactation.

The other fertility parameters evaluated, fertility index, duration of gestation, gestation index and sex ratio, were unaffected by treatment at all dose levels. Testis and epididymis weights of P generation animals were unaffected by treatment at all dose levels.

F_{1b} generation weanlings. Full postmortem examination, organ weight analysis and histopathological evaluation of five newly weaned F_{1b} pups of each sex per group revealed no treatment-related gross lesions other than the small size of male pups from parental animals treated at 0.40 mg/kg bw per day. There were no treatment-related organ weight changes in any group, although brain weight relative to body weight was elevated at 0.40 mg/kg bw per day as a result of growth retardation. There were no histopathological alterations in the reproductive organs at any dose level. Treatment-related retinal anomalies occurred in 3/4 males and 1/5 females at 0.40 mg/kg bw per day (Table 14). The anomaly was characterized by single or multiple retinal folds of many layers of the retina, which included the pigment epithelium in the centre. Some anomalies appeared as intra-retinal rosettes. The retinal lesion also occurred in one control female and in a male from each of the groups treated at 0.05 and 0.12 mg/kg bw per day.

The retinal anomaly is considered to represent a transient, reversible lesion, possibly related to growth retardation, as the high-dose F_{1b} adults that were of normal size had no retinal anomalies, and only a single incidence occurred in adult animals treated at 0.40 mg/kg bw per day.

F_{1b} generation post-weaning. There were no treatment-related deaths or clinical signs at any dose during the post-weaning administration period. However, two males, one male and one male treated at 0.05, 0.12 and 0.40 mg/kg bw per day, respectively, died or were killed in extremis after

Table 12. Treatment-related effects in the P generation

Parameter	Group mean values			
	0 mg/kg bw per day	**0.05 mg/kg bw per day**	**0.12 mg/kg bw per day**	**0.40 mg/kg bw per day**
Weight gain (g)				
Males, days 0–77	331	333	360*	377**
Males, days 0–259	482	477	508 (5.4%)	560* (16.2%)
Females, days 0–63	116	125	129* (11.2%)	146** (25.9%)
Effects on F_{1a} litters				
No. (%) of dams with liveborn pups	24 (96.0)	26 (100)	28 (100)	25 (100)
Incidence of total litter loss (%)	8.0[a]	3.8	0.0	28.0**
No. of liveborn pups / pup mortality (%)	300 / 6.3	300 / 2.7	330 / 3.0	275 / 42.2**
Lactation index (% survival, days 4–21)	99.5	100	99.2	52.7**
Pup weight (g) ± SD				
Day 1	6.0 ± 0.6	6.0 ± 0.6	6.0 ± 0.5	6.3 ± 0.7
Day 4 (pre-cull)	8.7 ± 1.3	8.9 ± 1.3	9.0 ± 1.5	8.4 ± 1.4
Day 7	12.8 ± 1.2	13.8 ± 1.9	13.8 ± 1.8	10.4 ± 3.0**
Day 14	26.4 ± 2.3	27.5 ± 3.3	28.2± 3.1	17.8 ± 7.8**
Day 21	41.0 ± 3.6	42.6 ± 4.4	42.9 ± 4.6	27.6 ± 11.8**
Effects on P males during F_{1b} mating				
No. of males mating / no. of males cohabiting	25 / 28	26 / 29	27 / 29	19** / 30
Duration of cohabitation (days) ± SD	2.6 ± 2.7	4.1 ± 3.6*	4.4 ± 3.2**	7.2 ± 5.8**
Effects on P females during F_{1b} mating				
No. (%) of dams with prolonged inter-estrus[b]	1 (3.6)	0 (–)	0 (–)	4 (13.3*)
No. (%) of females mating	28 (100)	30 (100)	30 (100)	22 (73.3**)
Duration of cohabitation (days) ± SD	3.8 ± 4.8	4.3 ± 4.2	4.5 ± 3.4	9.5 ± 8.7*
Effects on F_{1b} litters				
No. (%) of dams with liveborn pups	21 (100)	22 (95.6)	26 (100)	16 (100)
Incidence of total litter loss (%)	0.0	0.0	0.0	25.0**
No. of liveborn pups / pup mortality (%)	250 / 2.0	253 / 1.98	303 / 3.96	169 / 33.1**
Lactation index (% survival, days 4–21)	98.0	98.5	99.2	60.0**
Pup weight (g) ± SD				
Day 1	6.2 ± 0.7	6.2 ± 0.6	6.2 ± 0.7	6.6 ± 0.8
Day 4 (pre-cull)	8.8 ± 1.5	8.8 ± 1.5	9.2 ± 1.3	9.0 ± 1.6
Day 7	13.8 ± 2.4	13.8 ± 2.4	14.5 ± 1.8	11.3 ± 3.1**
Day 14	28.9± 4.1	28.9 ± 4.1	29.6 ± 3.0	19.6 ± 9.4**
Day 21	45.1 ± 6.0	45.1 ± 3.4	46.1 ± 4.3	31.4 ± 14.4**

bw: body weight; F_1: first filial generation; P: parental generation; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$

[a] Includes one dam with stillborn litter.

[b] Finding that is not regarded as treatment related, but which explains the observed effects of number and duration of cohabitation during breeding for F_{1b}.

Source: Gordon (1984e)

Table 13. Treatment-related effects in the F_{1b} generation (post-weaning)

Parameter	Group mean values			
	0 mg/kg bw per day	**0.05 mg/kg bw per day**	**0.12 mg/kg bw per day**	**0.40 mg/kg bw per day**
Male body weight (g), day 0	76.4	72.6	73.8	52.6**
Male weight gain (g), days 0–28	187	183	187	159**
Male weight gain (g), days 0–272	600	604	595	651
Female body weight (g), day 0	70.0	68.3	64.5*	50.5**
Effects on F_{2a} litters				
No. (%) of dams with liveborn pups	27 (100)	23 (100)	25 (100)	23 (100)
No. of liveborn pups / pup mortality (%)	284 / 1.8	230 / 1.3	256 / 1.2	209 / 5.7**
Lactation index (% survival, days 4–21)	100	100	99.5	93.9**
Viability index (days 4[a]–14)	100	100	100	94.4**
Pup weight (g) ± SD				
Day 1	6.2 ± 0.7	6.2 ± 0.6	6.1 ± 0.6	6.3 ± 0.7
Day 4 (pre-cull)	8.4 ± 1.0	8.7 ± 1.0	8.8 ± 1.0	8.8 ± 1.0
Day 7	12.9 ± 1.4	13.3 ± 1.4	13.3 ± 1.2	12.0 ± 1.7*
Day 14	26.7 ± 2.5	27.1 ± 2.7	27.1 ± 2.3	20.6 ± 5.3**
Day 21	42.5 ± 3.9	42.9 ± 3.3	42.7 ± 3.4	32.9 ± 7.9**
Effects on F_{2b} litters				
No. (%) of dams with liveborn pups	21 (100)	12 (100)	21 (100)	17 (100)
No. of liveborn pups / pup mortality (%)	213 / 4.2	124 / 1.6	197 / 1.5	162 / 8.6**
Lactation index (% survival, days 4–21)	100	100	99.4	92.8**
Viability index (day 4[a]–14)	100	100	99.4	93.5**
Pup weight (g) ± SD				
Day 1	6.3 ± 0.7	6.5 ± 0.6	6.6 ± 1.1	6.3 ± 0.7
Day 4 (pre-cull)	8.7 ± 1.5	9.1 ± 1.4	9.5 ± 2.0	8.2 ± 1.1
Day 7	13.4 ± 2.3	13.7 ± 2.0	14.2 ± 2.5	11.7 ± 1.5*
Day 14	28.5 ± 4.2	27.3 ± 3.0	27.3 ± 4.4	20.7 ± 4.8**
Day 21	45.9 ± 6.5	42.5 ± 4.5	42.9 ± 7.2	33.2 ± 8.4**

bw: body weight; F_2: second filial generation; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$
[a] Post-cull.
Source: Gordon (1984e)

Table 14. Incidence of treatment-related retinal anomaly in F_{1b} weanlings and adults and F_{2b} weanlings

	Incidence of retinal anomaly							
	0 mg/kg bw per day		**0.05 mg/kg bw per day**		**0.12 mg/kg bw per day**		**0.40 mg/kg bw per day**	
Generation	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
F_{1b} weanlings	0/5	1/5	1/5	0/5	1/5	0/5	3/4	1/5
F_{1b} adults	0/8	0/25	0/10	0/25	0/10	0/25	0/10	1/25
F_{2b} weanlings	3/57	2/51	0/26	1/34	5/88	2/86	10*/63	18*/66

bw: body weight; F: female; F_1: first filial generation; F_2: second filial generation; M: male; *: $P < 0.05$
Source: Gordon (1984e)

29–262 doses. One female treated at 0.05 mg/kg bw per day died during gestation, after 189 doses. No gross or histopathological alterations occurred in the animals, and, as no dose–response relationship was evident, the deaths are considered incidental to treatment. Group mean body weights of males and females at 0.40 mg/kg bw per day and of females at 0.12 mg/kg bw per day were significantly reduced ($P < 0.05$ or $P < 0.01$) at the start of treatment, owing to retarded pre-weaning growth. Treatment-related reduced weight gain ($P < 0.01$) continued in males at 0.40 mg/kg bw per day for 4 weeks, after which weight gain was enhanced, such that terminal body weights were increased by 4.0%. There was no effect on weight gain in the other male groups or in any female groups during the premating, gestation and lactation periods for both the F_{2a} and F_{2b} generations.

At the time of weaning, effects in the F_{2a} and F_{2b} litters were generally less severe than those observed for the F_{1a} and F_{1b} litters. There were no treatment-related effects on the incidence of stillborn pups. However, in both F_{2a} and F_{2b} litters treated at 0.40 mg/kg bw per day, pup mortality was significantly increased ($P < 0.01$) during the course of lactation, and the associated viability and lactation indices were significantly decreased ($P < 0.01$). Pup weights in the high-dose group seemed to be unaffected by treatment directly after birth and for the first few days, but were significantly reduced ($P < 0.05$ or $P < 0.01$) from day 7 to day 21. This was associated with increased numbers of pups that were thin, weak and cold to the touch. These effects were not apparent at lower dose levels. All other fertility parameters for both F_{2a} and F_{2b} litters were unaffected by treatment at all dose levels. No treatment-related skeletal abnormalities occurred in pups dying during lactation. The other fertility parameters evaluated, fertility index, duration of gestation, gestation index, the incidence of stillborn pups and sex ratio, were unaffected by treatment at all dose levels. Testis and epididymis weights of F_{1b} generation animals were unaffected by treatment at all dose levels.

Full postmortem examination, organ weight analysis and histopathological evaluation of 10 males and 25 females per group of F_{1b} adults revealed no treatment-related gross or histomorphological alterations at any dose level other than a single case of retinal anomaly at 0.40 mg/kg bw per day.

F_{2b} generation weanlings. Full postmortem examination, organ weight analysis and histopathological evaluation of five newly weaned F_{2b} pups of each sex per group revealed no treatment-related gross lesions other than the comparably smaller size of pups from parental animals treated at 0.40 mg/kg bw per day. There were no treatment-related organ weight changes in any group, although brain weight relative to body weight was high at 0.40 mg/kg bw per day owing to growth retardation. There were no histopathological alterations in the reproductive organs at any dose level. Examination of the eyes of 98, 50, 164 and 119 animals in the groups treated at 0, 0.05, 0.12 and 0.40 mg/kg bw per day, respectively, revealed a treatment-related increased incidence of retinal anomalies in 10/63 males and 18/66 females at 0.40 mg/kg bw per day (Table 14). The characteristics of this anomaly were identical to those described previously. The retinal lesion occurred at much lower frequencies in the control group and the groups treated at 0.05 and 0.12 mg/kg bw per day, and the occurrence at these dose levels is considered not to be treatment induced. Although the literature describes this effect as a fixation artefact, the observed dose–response relationship and high incidence make this interpretation unlikely.

Directly after birth, all newborn pups showed no adverse treatment-related effects in terms of litter size, survival, viability, body weight and other reproductive parameters. This means that pups in the womb were fully protected against abamectin toxicity by the placental barrier. All the above-mentioned adverse effects observed at 0.40 mg/kg bw per day developed later after the start of lactation and are considered to be a direct consequence of the exposure of the pups to abamectin via the milk. Neonatal rats are particularly susceptible to abamectin toxicity caused by incomplete development of the P-glycoprotein element of the blood–brain barrier at this age. The retarded weight gain in both F_1 and F_2 generation progeny and the transient reduced weight gain in male F_{1b} generation parental animals are a late consequence of reduced weight gain during lactation as well as the increased pup mortality. The increased incidence of transient retinal anomaly in the eyes of F_1 and F_2 generation weanlings can also be explained as a secondary effect due to reduced body weight and reduced growth.

In view of the above, the NOAEL for the parental and reproductive toxicity of abamectin technical in rats was 0.40 mg/kg bw per day, the highest dose tested. For effects on the offspring, the NOAEL was 0.12 mg/kg bw per day, based on the occurrence of transient, reduced weight gain in male F_{1b} generation parental animals, increased pup mortality and retarded weight gain in both F_1 and F_2 generation progeny and an increased incidence of transient retinal anomaly (folds) in the eyes of F_2 generation weanlings at 0.40 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Gordon, 1984e).

(b) Developmental toxicity

Rats

In a dose range–finding study, five groups of 10 naturally mated female rats (CRCD strain, 16 weeks old, 218–288 g) were treated orally, by gavage, with abamectin technical (L-676,863-00V50, purity approximately 94%) in sesame oil at a dose of 0 (vehicle only), 0.25, 0.5, 1.0 or 2.0 mg/kg bw per day from day 6 to day 17 of gestation (day 0 of gestation being the day on which plug or sperm were observed in the vaginal smear). Abamectin technical has been shown to be stable in sesame oil for at least 24 hours at the concentrations used in the study. Samples of abamectin technical in sesame oil, prepared in the first and last weeks of treatment, were analysed for test concentration and found to be acceptable. The animals were observed for clinical signs at least once daily during the treatment period. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16, 18 and 20 of gestation. The animals were killed on day 20, and their pregnancy status was established.

One female treated at 2.0 mg/kg bw per day was killed in a moribund condition on day 18 of gestation. Prior to sacrifice, the animal had lost weight, appeared weak and displayed tremors. All other animals survived to termination, and no other treatment-related clinical signs were apparent. There was a treatment-related increase in maternal weight gain during the treatment period of 15.2–25.8% at dose levels of 0.25, 0.5 and 1.0 mg/kg bw per day, but not at 2.0 mg/kg bw per day. The effect was statistically significant ($P < 0.05$) at 0.25 and 1.0 mg/kg bw per day, but is considered not to be an adverse effect of treatment. Pregnancy incidences were 90% in the control group and 90%, 80%, 100% and 90% in order of ascending dose level of abamectin technical.

The NOAEL for maternal toxicity was identified as 1.0 mg/kg bw per day, based on the occurrence of tremors and morbidity in one animal at 2.0 mg/kg bw per day in the dose range–finding study (Gordon, 1982a).

In the main study, four groups of 25 naturally mated female rats (CRCD strain, 10.5 weeks old, 206–269 g) were treated orally, by gavage, with abamectin technical (MK-0936, batch no. L-676,863-00V50, purity approximately 94%) in sesame oil at a dose level of 0 (vehicle only), 0.4, 0.8 or 1.6 mg/kg bw per day from day 6 to day 19 of gestation (day 0 of gestation being the day on which plug or sperm were observed in the vaginal smear). The animals were observed for clinical signs at least once daily during the treatment period.

Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16, 18 and 20 of gestation. The animals were killed on day 20, and their pregnancy status was established. The ovaries were removed and fixed, and the number of corpora lutea was determined. The fetuses were removed from the uterine horns, individually weighed, sexed and examined for external abnormalities. The uterine horns were examined for the presence of resorptions (classified as early, middle, late or near fetal) and dead fetuses. Visceral examination by dissection was performed on every third fetus of each litter and on all externally abnormal fetuses. The head of every third fetus was fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal abnormalities and variations. Litter data and maternal body weight data were analysed statistically by ANOVA or analysis of covariance (ANCOVA) using a least significant difference procedure after normalization of non-parametric data.

There were no deaths or treatment-related clinical signs at any dose level. Maternal weight gain from day 6 to day 14 of gestation was significantly increased ($P < 0.05$) by 15.8–21.1% at all doses of abamectin technical. The effect is considered to be treatment related, but is not an adverse effect. Thereafter, the weight gain of the treated groups was slightly lower than the control values. At sacrifice on day 20, the overall body weight gain was not significantly increased at any dose level.

There were no treatment-related effects on preimplantation loss, implantation number, incidences of resorptions and dead fetuses, mean fetal weight (sexes combined) or sex ratios at any dose level. The significantly ($P < 0.05$) higher incidence of resorptions in the group treated at 0.8 mg/kg bw per day is considered not to be treatment related, as more than 50% of the total number of resorptions occurred in two litters, and a similar effect was not apparent at 1.6 mg/kg bw per day.

Five externally malformed fetuses occurred: a control fetus with anophthalmia, two fetuses from different litters exposed to 0.8 mg/kg bw per day and a further two fetuses from different litters exposed to 1.6 mg/kg bw per day. At 0.8 mg/kg bw per day, one fetus had gastroschisis, and the other had multiple abnormalities, including anasarca, micrognathia, cleft palate and ectromelia. At 1.6 mg/kg bw per day, one fetus had cleft palate, and the other was a conjoined twin with exencephaly. None of the malformations is considered to be treatment related because of the isolated occurrence of each malformation and because cleft palate, gastroschisis and multiple malformation have occurred spontaneously in historical control groups. There was no evidence of developmental toxicity at any dose level, based on the incidence and nature of visceral and skeletal malformations and variations. Seven, four and three fetuses had one or more malformations in the groups treated at 0.4, 0.8 and 1.6 mg/kg bw per day, respectively, compared with two malformed control fetuses. The higher incidence in the lowest-dose group was due to the occurrence of three cases of hypoplastic rib and three cases of missing vertebrae in two litters. Either all visceral and skeletal malformations in the treated groups occurred in isolation or their distribution between the groups was not dose related. Slightly higher incidences of the variation distended ureter occurred in the treated groups, but higher incidences have occurred in historical control groups.

The incidences of the skeletal variations lumbar ribs and lumbar count variations were higher in the group treated at 1.6 mg/kg bw per day than in the control group. However, the litter distribution of these variations was comparable with the control group distribution (16 and two litters at 1.6 mg/kg bw per day compared with 13 and one control litters, respectively), and higher incidences have occurred in historical control groups. Therefore, none of the increased incidences of variations is considered to be treatment related.

In view of the above, the NOAEL for maternal toxicity and embryo/fetal toxicity for abamectin technical in rats was identified as 1.6 mg/kg bw per day, the highest dose tested. The minimal, transient and non-dose-related increase in maternal weight gain observed at all dose levels was not considered to be an adverse effect.

The study was GLP compliant, and a QA statement was attached (Gordon, 1982a).

Rabbits

In a dose range–finding study, four groups of 10 artificially inseminated female rabbits (New Zealand albino, 6–7 months old, weighing 3.64–4.60 kg) were treated orally, by gavage, with abamectin technical (MK-0936, batch no. L-676,863-00V50, purity approximately 94%) in sesame oil at a dose of 0 (vehicle only), 0.5, 1.0, 2.0 or 3.0 mg/kg bw per day from day 6 to day 18 of gestation (day 0 of gestation is the day of insemination). Abamectin mixtures at the concentrations used in the study have been shown to be stable in sesame oil for at least 24 hours. The animals were observed for clinical signs once daily throughout the study and twice daily during the treatment period. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16, 18, 19 and 28 of gestation. The animals were killed on day 28, and their pregnancy status was established.

One female treated at 3.0 mg/kg bw per day was killed in a moribund condition on day 16 of gestation. Prior to sacrifice, the animal had lost weight and was prostrate, with laboured respiration and discharges from the nose and mouth. All other animals treated at 3.0 mg/kg bw per day survived

to termination but displayed stuporous behaviour 2–5 hours after the fourth and subsequent doses. Some animals in the group also showed a watery yellow-green discharge from the nose and mouth and reduced feed and water consumption (subjective assessment). No deaths or treatment-related clinical signs occurred at the lower dose levels. Four animals aborted prior to caesarean section: one treated at 1.0 mg/kg bw per day on day 20, one treated at 2.0 mg/kg bw per day on day 27, and two treated at 3.0 mg/kg bw per day on days 26 and 27 of gestation. This incidence of abortion is considered not to be treatment related, despite its absence in the control group, as spontaneous abortion has occurred in historical control groups. A statistically significant ($P < 0.05$) body weight loss of 9.5% occurred at 3.0 mg/kg bw per day during the treatment period, with some recovery after day 19; however, at sacrifice on day 28, an overall weight loss of 3.6% had occurred during the gestation period. There was no treatment-related effect on body weight at lower doses of abamectin. The pregnancy incidence was 100% in the control group and 70%, 80%, 90% and 90% in order of ascending dose level of abamectin technical (Gordon, 1982b).

In the main study, four groups of 18 artificially inseminated female rabbits (New Zealand albino, 6–7 months old, weighing 3.47–4.74 kg) were treated orally, by gavage, with abamectin technical (MK-0936, batch no. L-676,863-00V50, purity approximately 94%) in sesame oil at a dose of 0 (vehicle only), 0.5, 1.0 or 2.0 mg/kg bw per day from day 6 to day 27 of gestation (day 0 of gestation is the day of insemination). The animals were observed for clinical signs once daily throughout the study and twice daily during the treatment period. Body weights were recorded on days 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28 of gestation. The animals were killed on day 28, and their pregnancy status was established. The ovaries were removed, and the numbers of corpora lutea were determined. The fetuses were removed from the uterine horns, individually weighed and examined for external abnormalities. All fetuses were killed and the viscera examined by dissection, at which time the sex was determined. The uterine horns were examined for the presence of resorptions (classified as early, middle, late or near fetal) and dead fetuses. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal malformations and variations. Litter data and maternal body weight data were analysed statistically by ANOVA or ANCOVA using a least significant difference procedure after normalization of non-parametric data.

Two deaths and one premature sacrifice occurred in abamectin-treated groups. One animal at 0.5 mg/kg bw per day died on day 26 of gestation, one animal at 1.0 mg/kg bw per day died on day 27 and one animal treated at 2.0 mg/kg bw per day was killed in a moribund condition on day 20. Death was preceded by reduced feed and water consumption in two animals and by blood-stained urine in the cage of the other animal. The relationship of these deaths to treatment with abamectin is equivocal, as a dose-related increase in incidence did not occur, six control animals also showed reduced feed and water consumption, and two controls had blood in the cage during the latter part of gestation. Premature abortion occurred in two females, one female and one female in the groups treated at 0.5, 1.0 and 2.0 mg/kg bw per day, respectively. This incidence of abortions is considered not to be treatment related, as one control animal also aborted. There were no clinical signs of toxicity at any dose level.

The feed consumption and water consumption of all groups were variable; by subjective assessment, the periods of reduced feed and water consumption in the group treated at 2.0 mg/kg bw per day were more prolonged and pronounced than in the other groups. This decreased feed and water consumption in females at 2.0 mg/kg bw per day resulted in a substantial weight loss during the dosing period that was statistically significant ($P < 0.05$) between days 6 and 18 of gestation, compared with controls. Females treated at 2.0 mg/kg bw per day had lost 3.3% of their weight at sacrifice on day 28 compared with the start of treatment. Body weight gain was unaffected by treatment at lower dose levels.

There were no treatment-related effects on preimplantation loss, implantation number, incidences of resorptions and dead fetuses, mean fetal weight (sexes combined) or sex ratios at any dose level. Significantly higher ($P < 0.05$) mean numbers of implantations and dead fetuses in the

group treated at 1.0 mg/kg bw per day are considered not to be treatment related, as there was no dose–response relationship.

One or more malformations occurred in four, four and 13 fetuses from females treated at 0.5, 1.0 and 2.0 mg/kg bw per day, respectively, compared with three abnormal control fetuses (Table 15). The external malformations in the high-dose group comprised two fetuses with cleft palate and two fetuses with omphalocele, all from a single litter, and five fetuses with clubbed forefeet from three other litters. The incidences of these malformations are higher than the concurrent and historical control group incidences and were considered treatment related. In addition, one fetus with clubbed forefeet had a lumbar vertebral malformation, and three of the fetuses in the litter with cleft palate and omphalocele had sternebral malformations, including one of the fetuses with cleft palate. The two other malformations occurring at 2.0 mg/kg bw per day, ventricular septal defect and branched rib, are considered not to be associated with treatment, as higher incidences have occurred in historical control animals. Two fetuses in a litter from a female treated at 1.0 mg/kg bw per day also had clubbed forefeet, but the occurrence is considered not to be treatment related, because higher incidences of the defect have been recorded in historical controls (one fetus from a concurrent control female also had a clubbed forefoot). The incidence and nature of malformations in fetuses from females treated at 0.5 and 1.0 mg/kg bw per day did not indicate an effect of treatment.

At 2.0 mg/kg bw per day, increased incidences of incomplete ossification of sternebrae and metacarpals are considered to reflect a slight treatment-related delay in ossification. A similar effect was not apparent at dose levels of 0.5 and 1.0 mg/kg bw per day.

A NOAEL of 1.0 mg/kg bw per day for maternal toxicity for abamectin technical in rabbits was identified, based on the occurrence of severe maternal toxicity (body weight loss) during gestation at 2.0 mg/kg bw per day. A NOAEL of 1.0 mg/kg bw per day for embryo and fetal toxicity was also identified, based on increased incidences of external malformations (cleft palate, omphalocele and clubbed forefeet) and incomplete ossification of sternebrae and metacarpals at 2.0 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Gordon, 1982b).

2.6 Special studies

(a) Acute neurotoxicity

Groups of 10 male and 10 female fasted Alpk:AP_fSD (Wistar-derived) rats (aged at least 42 days; males 305–362 g and females 197–244 g on the day prior to dosing) were administered abamectin (batch no. VS094KO, purity 96.2%) at a single oral dose of 0 (control), 0.5, 1.5 or 6 mg/kg bw and observed for the following 14 days. The control substance and vehicle for the test substance was sesame seed oil. All animals were observed prior to the start of the study and daily throughout the study for any changes in clinical condition. In addition, a functional observational battery, including quantitative assessments of landing foot splay, sensory perception and muscle weakness, was performed in week −1 and on day 1 (at the time of peak effect), day 8 and day 15. Locomotor activity was also monitored in week −1 and on days 1, 8 and 15. Body weights and feed consumption were measured weekly throughout the study. At the end of the scheduled period, five rats of each sex per group were perfused in situ, brains were weighed and selected nervous system tissues were removed, processed and examined microscopically.

At 6 mg/kg bw, adverse clinical signs were evident at the time of peak effect, 6–7 hours post-dosing on day 1. These signs comprised reduced splay reflex in the majority of animals and splayed and tiptoe gait in one female. These signs were transient, although reduced splay reflex persisted on day 2 or 3 in some females. Motor activity was also reduced on day 1 in females at this dose, but this is consistent with the clinical signs present at this time. Brain weight was lower in females at this dose, but there was no microscopic change in the brain.

At 1.5 mg/kg bw, the only treatment-related effect was reduced splay reflex in two males and three females at the time of peak effect on day 1. There were no treatment-related effects on body

Table 15. Incidence of fetal malformations and variations in the main study on developmental toxicity in rabbits

Parameter	0 mg/kg bw per day	0.5 mg/kg bw per day	1.0 mg/kg bw per day	2.0 mg/kg bw per day	Historical control data for selected fetal alterations			
					No. of fetuses examined	No. of litters examined	No. of fetuses with alteration (%)	No. of litters with alteration (%)
External examination								
No. of fetuses (litters) examined	97 (15)	90 + 1[a] (13)	95 + 5[a] (11)	121 (15)	–	–	–	–
No. of fetuses (litters) with malformations	2 (2)	0 (0)	2 (1)	9 (4)	–	–	–	–
No. of fetuses (litters) with variations	0 (0)	0 (0)	0 (0)	0 (0)	–	–	–	–
No. of fetuses (litters) with cleft palate	0	0	0	2 (1)	–	–	–	–
No. of fetuses (litters) with omphalocele	1	0	0	2 (1)	–	–	–	–
No. of fetuses (litters) with clubbed forefoot	1	0	2 (1)	5 (3)	177	25	7 (4.0)	1 (4.0)
Visceral examination								
No. of fetuses (litters) examined	97 (15)	90 + 1[a] (13)	95 + 5[a] (11)	121 (15)	–	–	–	–
No. of fetuses (litters) with malformations	1 (1)	0 (0)	0 (0)	1 (1)	–	–	–	–
No. of fetuses (litters) with variations	3 (3)	2 (2)	3 (3)	3 (2)	–	–	–	–
No. of fetuses with ventricular septal defect[m]	0	0	0	1	989	135	3 (0.3)	3 (2.2)
No. of fetuses with retrocaval ureter[m]	1	0	0	0	–	–	–	–
No. of fetuses with variation in lung lobe[v]	1	2	2	3	–	–	–	–

Parameter	0 mg/kg bw per day	0.5 mg/kg bw per day	1.0 mg/kg bw per day	2.0 mg/kg bw per day	Historical control data for selected fetal alterations			
					No. of fetuses examined	No. of litters examined	No. of fetuses with alteration (%)	No. of litters with alteration (%)
No. of fetuses with azygous vein variation[v]	1	0	1	0	–	–	–	–
No. of fetuses with gallbladder reduced in size[v]	1	0	0	0	–	–	–	–
Skeletal examination								
No. of fetuses (litters) examined	97 (15)	90 + 1[a] (13)	95 + 5[a] (11)	121 (15)	–	–	–	–
No. of fetuses (litters) with malformations	0 (0)	4 (3)	2 (1)	5 (3)	–	–	–	–
No. of fetuses (litters) with variations	49 (12)	66 (9)	37 (10)	115 (12)	–	–	–	–
No. of fetuses with craniostenosis[m]	0	0	1	0	–	–	–	–
No. of fetuses with vertebral malformation[m]	0	3	1	1	84	10	2 (2.4)	2 (20)
No. of fetuses with branched rib[m]	0	1	0	1	177	25	2 (1.1)	2 (8.0)
No. of fetuses with fused ribs[m]	0	1	0	0				
No. of fetuses (litters) with sternebral malformation[m]	0	0	0	3 (1)	184	26	10 (5.4)	5 (19.2)
No. of fetuses with incompletely ossified vertebra[v]	1	0	0	0	–	–	–	–
No. of fetuses with sternebral variation[v]	1	0	0	0	–	–	–	–
No. of fetuses with incompletely ossified sternebra[v]	17	17	16	42	–	–	–	–

Table 15 (continued)

Parameter	0 mg/kg bw per day	0.5 mg/kg bw per day	1.0 mg/kg bw per day	2.0 mg/kg bw per day	Historical control data for selected fetal alterations			
					No. of fetuses examined	No. of litters examined	No. of fetuses with alteration (%)	No. of litters with alteration (%)
No. of fetuses with incompletely ossified pelvic bone[v]	1	2	0	3	–	–	–	–
No. of fetuses with incompletely ossified metacarpal[v]	8	15	7	33	–	–	–	–
No. of fetuses with incompletely ossified phalanx[v]	19	27	12	31	–	–	–	–
No. of fetuses with incompletely ossified talus calcaneous[v]	2	5	2	6	–	–	–	–
Total no. of malformed fetuses	3	4	4	13[b]	–	–	–	–
% malformed fetuses	3.09	4.40	4.00	10.7	–	–	–	–

bw: body weight; m: malformation; v: variation
[a] Dead fetus(es).
[b] Skeletal malformations partially in the same fetuses with external malformations.
Source: Gordon (1982b)

weight, feed consumption, grip strength, sensory perception, landing foot splay measurements or neuropathology.

In view of the above, it is concluded that single oral administration of abamectin at 6 mg/kg bw caused some clinical signs of neurotoxicity in males and females (principally reduced splay reflex) 6–7 hours after dosing on day 1, a reduction in motor activity in females on day 1 and slightly lower brain weights in females. Oral administration of abamectin at 1.5 mg/kg bw also caused a slightly reduced splay reflex in some males and females 6–7 hours after dosing on day 1. There was no neuropathology at abamectin doses up to 6 mg/kg bw.

The NOAEL in this study was 0.5 mg/kg bw, based on a reduced splay reflex at 1.5 mg/kg bw.

The study was GLP compliant, and a QA statement was attached (Brammer, 2006a).

(b) Subchronic neurotoxicity

Groups of 16 male and 16 female Alpk:APfSD (Wistar-derived) rats (approximately 42 days of age; weight range 252–325 g for males and 186–238 g for females at the start of dose

administration) were dosed orally (by gavage) with abamectin (batch no. VS094KO, purity 96.2%) at 0 (control), 0.4, 1.6 or 4.0 mg/kg bw per day for at least 90 consecutive days. The test substance was formulated in sesame seed oil without correction for purity, on a weekly basis.

All animals were observed prior to the start of the study and daily throughout the study for any changes in clinical condition. In addition, detailed clinical observations, including quantitative assessments of landing foot splay, sensory perception and muscle weakness, were performed on at least 10 rats of each sex per group in weeks −1, 2, 5 and 9 and on all animals in week 14. Locomotor activity was also monitored on at least 10 rats of each sex per group in weeks −1, 2, 5 and 9 and on all animals in week 14. Body weights and feed consumption were measured and feed utilization was calculated weekly throughout the study. Urine samples, collected during week 13, were analysed. At the end of the study, five rats of each sex per group were killed by in situ perfusion fixation, brains were weighed and selected nervous system tissues were removed, processed and examined microscopically. All other surviving rats were killed and examined postmortem. Cardiac blood samples were taken for clinical pathology, selected organs were weighed and specified tissues were taken for histopathological examination.

Administration of abamectin at 4.0 mg/kg bw per day was generally tolerated well for up to 6 weeks, although there was evidence of toxicity characterized by lower body weight in females and adverse clinical signs in both sexes (irregular breathing, upward curvature of the spine, reduced righting reflex, reduced splay reflex and sides pinched in), which increased in incidence and severity with time. In addition, there were effects on hindlimb grip strength in females in weeks 2 and 5. During week 7, animals of both sexes lost weight over several days (from day 42) and showed a significant deterioration in clinical condition. The animals were therefore killed for humane reasons during week 7.

Oral administration of abamectin at 1.6 mg/kg bw per day caused no clinical signs of neurotoxicity, no significant clinical effects in the functional observational battery and no effects on landing foot splay, sensory perception (as shown in the tail flick test), forelimb grip strength or motor activity. There was no effect on perfused brain weight or treatment-related neuropathology.

There was no evidence of systemic toxicity in animals dosed with 0.4 or 1.6 mg/kg bw per day. There were no treatment-related clinical signs, no effects on body weight, feed consumption, ophthalmoscopic findings, haematology or clinical chemistry, or organ weights, and there was no treatment-related histopathology.

In view of the above, it is concluded that oral administration of abamectin at 4.0 mg/kg bw per day was generally well tolerated for up to 6 weeks, although there was evidence of toxicity characterized by a low incidence of adverse clinical signs in both sexes and lower body weight and lower grip strength in females. The animals showed a sudden and progressive weight loss and a significant deterioration in clinical condition during week 7, which necessitated removal of this group from the study.

The NOAEL for systemic toxicity and neurotoxicity was 1.6 mg/kg bw per day, based on reduced body weights in females, clinical signs in both sexes (irregular breathing, upward curvature of the spine, reduced righting reflex, reduced splay reflex) and changes in hindlimb grip strength in females at 4.0 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Brammer, 2006b).

(c) Developmental neurotoxicity

Groups of 30 female Alpk:AP$_f$SD (Wistar-derived) rats (time-mated, 10–12 weeks old, 220–300 g on delivery; actual body weight range 206–310 g) were given abamectin (batch no. VS094KO, purity 96.2%) by gavage at 0, 0.12, 0.20 or 0.40 mg/kg bw per day from day 7 of gestation through parturition and lactation to day 22 postpartum. The control substance and vehicle for the test substance was sesame seed oil. The day of sperm-positive vaginal smear was designated day 1 of gestation. Pups were allocated to the F_1 phase of the study on postpartum day 5, separated from the

dam on day 29 and allowed to grow to adulthood. Evaluations included observations to detect gross neurological and behavioural abnormalities, effects on motor activity, response to auditory startle, assessment of learning and memory, and neuropathological evaluation, including brain weights.

For the parental females, there were no treatment-related clinical observations or effects detected in the functional observational battery. There was a dose-related effect of treatment with abamectin on body weight during gestation. The body weights were significantly higher than those of the controls following the first or second dose. There was also a treatment-related effect on body weight postpartum, although the effect was not dose related for the 0.20 and 0.40 mg/kg bw per day groups. Following the last dose on day 22 postpartum, the difference in body weights between the control and abamectin-treated animals was reduced by day 29, indicating a recovery from the transient effect of abamectin.

For the abamectin-treated groups, maternal feed consumption was higher than in the control group during gestation through to day 5 postpartum.

There was no effect of treatment with abamectin on the proportion of pups born live, litter size on day 1 or the proportion of male pups. To day 5 postpartum, there was no effect of maternal treatment with abamectin on pup clinical condition, body weight or survival.

There was no effect of maternal treatment with abamectin on the clinical condition of the F_1 animals, and no treatment-related effects in the functional observational battery were detected at any time point.

For days 5–22 postpartum, the mean body weights of the F_1 males and F_1 females in the abamectin-treated groups were higher than those in the control group, although not in a dose-related manner. For days 29–63 postpartum, the body weights of the F_1 animals in the abamectin-treated groups were lower than those in the control group, although the differences from control values were statistically significant for the 0.20 and 0.40 mg/kg bw per day groups only (Table 16).

The mean day of age when vaginal opening occurred was statistically significantly later for the females in the 0.20 and 0.40 mg/kg bw per day groups, in comparison with the control group (Table 17).

Table 16. Intergroup comparison of F_1 body weights at selected time points

	Adjusted mean body weight (g)							
	Males				**Females**			
Day	**0 mg/kg bw per day**	**0.12 mg/kg bw per day**	**0.20 mg/kg bw per day**	**0.40 mg/kg bw per day**	**0 mg/kg bw per day**	**0.12 mg/kg bw per day**	**0.20 mg/kg bw per day**	**0.40 mg/kg bw per day**
5	9.4	10.2**	10.4**	10.1*	9.2	9.9*	9.7*	9.7
29	93.4	92.4	92.0	87.1**	88.3	86.5	84.1**	81.0**
63	350.1	342.7	340.0*	323.2**	219.3	212.7	208.6**	197.5**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)
Source: Moxon (2005)

Table 17. Intergroup comparison of selected developmental landmarks

Observation	**0 mg/kg bw per day**	**0.12 mg/kg bw per day**	**0.20 mg/kg bw per day**	**0.40 mg/kg bw per day**
Day of vaginal opening	35.5	36.5	37.4**	38.3**
Body weight at landmark (g)	125.4	127.6	128.6	126.8

bw: body weight; **: $P < 0.01$
Source: Moxon (2005)

There was no effect of maternal treatment with abamectin on locomotor activity, the auditory startle response or learning or memory of the F_1 animals at any time point.

There was no effect of maternal treatment with abamectin on brain weight or histology of the F_1 animals at 12 or 63 days of age.

In view of the above, it can be concluded that abamectin at dose levels of 0.12, 0.20 and 0.40 mg/kg bw per day was associated with higher maternal body weight and feed consumption during the dosing period. Treatment-related effects on the offspring were confined to the 0.20 and 0.40 mg/kg bw per day groups and included lower body weights post-weaning and hence a slight delay in the time of vaginal opening. There was no effect of abamectin on the function or morphology of the nervous system.

On the basis of the results of this study, there is no evidence of developmental neurotoxicity at abamectin dose levels of up to 0.40 mg/kg bw per day. The NOAEL for offspring toxicity was 0.12 mg/kg bw per day, based on reduction in body weight and delay in vaginal opening at 0.20 mg/kg bw per day and above.

The study was GLP compliant, and a QA statement was attached (Moxon, 2005).

In a supplementary developmental neurotoxicity study performed to provide brain morphometry data, groups of 30 time-mated female Alpk:AP_fSD (Wistar-derived) rats were given abamectin by gavage at 0, 0.12, 0.20 or 0.40 mg/kg bw per day from day 7 of gestation through parturition and lactation to day 22 postpartum. The control substance and vehicle for the test substance was sesame seed oil. The day of sperm-positive vaginal smear was designated day 1 of gestation. Pups were allocated to the F_1 phase of the study on postpartum day 5, separated from the dam on day 29 and allowed to grow to adulthood. Evaluations included observations to detect gross neurological and behavioural abnormalities, effects on motor activity, response to auditory startle, assessment of learning and memory, and neuropathological evaluation, including morphometry and brain weights.

Maternal treatment with 0.40 mg/kg bw per day was associated with high pup mortality, resulting in an insufficient number of litters/pups available to meet the objectives of the study. All dams and pups in this group were therefore removed from the study (between days 15 and 38 postpartum) prior to scheduled termination.

For the parental females, there were no treatment-related deaths, clinical observations or effects detected in the functional observational battery.

There was a treatment-related increase in body weight and feed consumption during gestation; the effect was dose related. There was also a treatment-related effect on body weight on day 1 postpartum, with higher mean values for the 0.20 and 0.40 mg/kg bw per day groups, although there was no effect on subsequent days. For days 5–23 postpartum, feed consumption was lower for the 0.40 mg/kg bw per day group. There was no effect of 0.12 or 0.20 mg/kg bw per day on feed consumption postpartum.

There was no effect of treatment with abamectin on the proportion of pups born live, litter size on day 1 or the proportion of male pups. To day 5 postpartum, there was no effect of maternal treatment with abamectin on clinical condition or survival of pups, although the body weight of the males in the 0.40 mg/kg bw per day group was lower than that of the controls. A similar difference was not seen in females.

After day 5, maternal treatment with 0.40 mg/kg bw per day was clearly associated with toxicity, and the pups showed signs of dehydration, tremors and lower body weights, resulting in the premature death of 31% (59/190) of the animals. The group was therefore terminated prematurely.

There was no effect of maternal treatment with abamectin at 0.12 or 0.20 mg/kg bw per day on the clinical condition of the F_1 animals, and no treatment-related effects were detected in the functional observational battery at any time point.

Table 18. Intergroup comparison of F_1 body weights at selected time points

	Adjusted mean body weight (g)							
	Males				Females			
Day	0 mg/kg bw per day	0.12 mg/kg bw per day	0.20 mg/kg bw per day	0.40 mg/kg bw per day	0 mg/kg bw per day	0.12 mg/kg bw per day	0.20 mg/kg bw per day	0.40 mg/kg bw per day
5	9.3	9.5	10.1**	9.6	9.0	9.2	9.6*	9.4
8	15.4	15.3	15.2	13.1**	14.8	15.0	14.8	12.8**
29	91.5	87.3*	86.5*	61.6**	87.5	81.9**	81.1**	55.6**
63	354.6	335.2**	326.6**	354.6	218.7	206.1**	202.9**	218.7

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)
Source: Moxon (2007)

Table 19. Intergroup comparison of vaginal opening

Observation	0 mg/kg bw per day	0.12 mg/kg bw per day	0.20 mg/kg bw per day
Day of vaginal opening	32.0	33.3*	33.3*
Body weight at landmark (g)	102.0	102.7	101.7

bw: body weight; *: $P < 0.05$
Source: Moxon (2007)

For the pups in the 0.12 and 0.20 mg/kg bw per day groups, mean body weights were slightly lower than those of the control group from day 29 through to day 63, and the decrease was dose related (Table 18). For this reason, a slight delay in the time of vaginal opening was also observed (Table 19). There was no effect of treatment on the age when preputial separation occurred.

There was no effect of maternal treatment with abamectin at 0.12 or 0.20 mg/kg bw per day on locomotor activity, auditory startle response, learning or memory of the F_1 animals at any time point.

There was no effect of maternal treatment with abamectin at 0.12 or 0.20 mg/kg bw per day on brain weight, histopathology or morphometry in the F_1 animals on day 12 or 63.

In conclusion, abamectin was evaluated at dose levels of 0.12, 0.20 and 0.40 mg/kg bw per day in this study, although the highest dose level of 0.40 mg/kg bw per day was toxic to the offspring following maternal exposure and resulted in the premature termination of the selected offspring in this group. All dose levels were associated with higher maternal body weight and feed consumption during gestation. For the 0.12 and 0.20 mg/kg bw per day groups, slightly lower body weights were observed post-weaning, with an associated slight delay in the time of vaginal opening. There was no effect of abamectin on the function or morphology of the nervous system. Although there was a reduction in body weights of offspring at all doses, the days of vaginal opening were within the normal range.

Under the conditions of this study, at 0.12 and 0.20 mg/kg bw per day, body weights of pups post-weaning were statistically significantly lower and the time of vaginal opening was statistically significantly later compared with the control group. However, the effects observed at 0.12 mg/kg bw per day were marginal and within the range of normal biological variation and were therefore not considered to be toxicologically significant. Hence, the NOAEL for offspring toxicity was 0.12 mg/kg bw per day. There was no effect on function or morphology of the nervous system at dose levels up to 0.40 mg/kg bw per day, the highest dose tested.

The study was GLP compliant, and a QA statement was attached (Moxon, 2007).

(d) Acute oral and reproductive toxicity in CF-1 mice

Acute oral toxicity studies in CF-1 mice

Acute oral toxicity and exploratory acute oral toxicity studies with abamectin in CF-1 mice are summarized in Table 20.

Table 20. Summary of acute oral toxicity and exploratory acute oral toxicity studies with abamectin

Species	Strain	Sex	Study	Dose (mg/kg bw)	Purity (%)	LD_{50} (mg/kg bw)	Reference
Mouse[a]	CF-1	Female pregnant	Acute oral	0, 5, 10, 20, 40, 80	94	11.8	Gordon (1986b)
Mouse[b]	CF-1	Female non-pregnant	Acute oral	0, 5, 10, 20, 40, 80	94	15.0	Gordon (1986b)
Mouse[c]	CF-1	Female genotyped as homozygous positive (+/+)	Exploratory acute oral for expression of P-glycoprotein	0, 10, 20, 40, 80	Not specified	28	Hall (1997)
Mouse[c]	CF-1	Female genotyped as heterozygous (+/−)	Exploratory acute oral for expression of P-glycoprotein	0, 10, 20, 40, 80	Not specified	14	Hall (1997)

bw: body weight; LD_{50}: median lethal dose

[a] Symptoms included tremors, bradypnoea and clonic convulsions.

[b] Symptoms included loss of righting reflex and bradypnoea.

[c] Symptoms included tremors, bradypnoea and decreased activity. Death occurred at 40 mg/kg bw.

Ten-day reproductive toxicity study in CF-1 mice

Four groups of 20 naturally mated, singly housed, female mice (albino CF-1 strain, 12 weeks old, weight range 26.8–33.4 g) were treated orally with a mixture of tritiated (< 0.5% total) and unlabelled abamectin technical (MK-0936, batch nos L-676,895-01S25 and L-676,895-00V54, purity > 98% and > 88%, respectively), by admixture in the diet, at a constant concentration of 0 (vehicle only), 0.33, 1.0 or 2.0 ppm from day 6 to day 15 of gestation (day 0 being day on which vaginal plug observed). Target dose levels were 0, 0.1, 0.3 and 0.6 mg/kg bw per day, which were achieved (0, 0.10, 0.33 and 0.61 mg/kg bw per day, respectively) on days 6–11. However, on days 12–16 of gestation, as a result of lower feed consumption, achieved dose levels were reduced to 0, 0.06, 0.16 and 0.33 mg/kg bw per day, respectively. The individual dose levels achieved from day 12 to 16 in the group treated at 0.1 mg/kg bw per day ranged from 0.04 to 0.13 mg/kg bw per day, with three animals receiving 0.1 mg/kg bw per day during this period.

Clinical signs were recorded daily from day 6 to day 17 of gestation. Body weights were recorded on days 0, 6, 11, 16 and 17 of gestation, and feed consumption was measured. The animals were killed on day 17 of gestation, and the unopened uteri were examined to estimate the numbers of implantation sites, resorptions and live fetuses. Dead fetuses were classified as resorptions. Maternal feed consumption and body weight data were analysed statistically by ANOVA or ANCOVA using a least significant difference procedure after normalization of non-parametric data by the Rankit method.

Two females treated at 0.6 mg/kg bw per day were killed in poor condition after 3 days of treatment, having developed marked tremors. Three females treated at 0.3 mg/kg bw per day showed hunched posture and tremors after 6 or 7 days of treatment and were also killed in poor condition. There were no clinical signs or animals killed prematurely at 0.1 mg/kg bw per day. There were no treatment-related effects on maternal weight gain during gestation at any dose level. However, the mean weight gains of all treated groups were significantly higher ($P < 0.05$) than the control values from day 16 to day 17, resulting in a treatment-related increase in terminal body weights of 3.1–5.5%.

There were no consistent treatment-related effects on feed consumption and no treatment-related effects on reproductive status, as assessed by pregnancy incidence, number of implantations and numbers of live fetuses and resorptions.

In view of the above, a NOAEL for maternal toxicity of 0.1 mg/kg bw per day (time-weighted average 0.08 mg/kg bw per day) for abamectin technical was identified, based on the occurrence of poor condition and hunched posture and/or tremors at dose levels of 0.3 and 0.6 mg/kg bw per day. A NOAEL for reproductive parameters (pregnancy incidence, number of implantations and fetal viability) of 0.6 mg/kg bw per day (time-weighted average 0.48 mg/kg bw per day), the highest dose tested, was identified.

The study was GLP compliant, and a QA statement was attached (Gordon, 1984f, 1986a).

(e) Comparison of abamectin sensitivity and P-glycoprotein levels in CF-1 and CD-1 mice

To identify "abamectin-sensitive" and "abamectin-insensitive" CF-1 mice, two groups of mice (49 male and 50 female CF-1 strain mice and five male and five female CD-1 strain mice) were treated orally, by gavage, with abamectin (batch no. L-676,863-000V093, purity 96.0%) at 0.8 mg/kg bw per day as a solution in sesame oil at a treatment volume of 5 mL/kg bw for 4 days. Two further groups (five male and five female CF-1 strain mice and five male and five female CD-1 strain mice) received sesame oil at 5 mL/kg bw per day for 4 days, as vehicle control groups. All animals were weighed pre-dosing and observed for signs of neurotoxicity several times each day. Animals that showed severe tremors and/or ataxia at this dose level were killed and exsanguinated. A total of three vehicle control mice (one male and two females) of the same strain were killed and exsanguinated at the same time. All remaining vehicle control animals and 20 randomly selected CF-1 strain insensitive mice were killed on day 4. Brain and small intestine were removed at necropsy. Sections of cerebral cortex, cerebellum and jejunum were frozen in isopentane/liquid nitrogen for immunohistochemical analysis of P-glycoprotein by an adaptation of a published method (Schinkel et al., 1994). Following staining with diaminobenzidine and haematoxylin, the slides were microscopically examined blind for relative staining intensity of P-glycoprotein against control sections of each tissue. Further samples of brain (cerebrum and cerebellum) and small intestine were cooled by ice for subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) western immunoblot analysis of P-glycoprotein content. Crude membrane protein fractions, as the source of P-glycoprotein, were prepared by a modification of the method of Gerlach et al. (1987). Following completion of the immunohistochemical studies, five insensitive CF-1 strain mice of each sex per group and a random sample of naive CD-1 strain mice (five of each sex or 10 females per group) were treated with single oral doses of abamectin ranging from 1.0 to 10 mg/kg bw to determine their sensitivity to abamectin for comparison with the sensitive CF-1 subgroup.

Treatment with abamectin at 0.8 mg/kg bw per day induced clinical signs of neurotoxicity, whole-body tremors and slight ataxia only in CF-1 strain mice. The signs first occurred within 1.5 hours of treatment on day 1. Within 4 hours of treatment, 12 female and five male CF-1 mice, 17% of those tested, showed severe signs of toxicity, comprising dyspnoea, lateral recumbency, tremors and coma following handling. These animals, identified as sensitive to abamectin toxicity, were killed and subjected to necropsy, together with two females and one male from each of the other groups. No clinical signs of toxicity occurred in any of the other CF-1 strain mice during the 4-day treatment period.

Immunohistochemical analyses of brain and small intestine showed that 11 of the 12 CF-1 strain females and all the CF-1 strain males identified as sensitive had no detectable P-glycoprotein in any of the tissues examined. The other sensitive CF-1 female had a minimal amount of P-glycoprotein in the brain sections. In contrast, the 20 CF-1 strain insensitive individuals evaluated for the presence of P-glycoprotein all had detectable P-glycoprotein, from minimal to intense staining, in all tissues examined of both sexes. Similarly, all CD-1 strain mice of both sexes treated with abamectin showed moderate to intense staining in all tissues examined, with the exception of a single female with

minimal staining in the jejunum. CD-1 control animals showed comparable staining to the treated animals, indicating that abamectin does not influence P-glycoprotein levels in this strain.

The results of the western immunoblot analyses confirmed the results of the immunohistochemical visualization of P-glycoprotein. CD-1 strain animals subjected to SDS-PAGE showed intense banding of P-glycoprotein in all three tissues, whereas the two CF-1 strain sensitive animals evaluated showed no detectable P-glycoprotein. Insensitive CF-1 strain mice showed levels of P-glycoprotein similar to those of CD-1 strain mice.

The CF-1 strain insensitive mice and CD-1 strain mice rechallenged with abamectin at either 1.0 or 2.5 mg/kg bw showed no clinical signs of toxicity. Rechallenge with 5.0 or 10 mg/kg bw produced transient slight tremors and ataxia in 6/10 and 8/10 CF-1 strain insensitive mice. The effect occurred 5–6 hours after treatment and persisted for approximately 19 hours in all animals, with the exception of one animal treated at 10 mg/kg bw, which continued to show clinical signs for a further 24 hours.

In view of the above, it is concluded that approximately 17% of a random population of CF-1 strain mice are sensitive to abamectin toxicity, but CD-1 strain mice are uniformly insensitive. Sensitivity in CF-1 strain mice is directly related to the absence of P-glycoprotein in brain and small intestine. CF-1 strain mice expressing higher levels of P-glycoprotein are more resistant than P-glycoprotein-deficient individuals to abamectin toxicity.

The study was not GLP compliant (Lankas, 1994).

(f) P-glycoprotein developmental expression in rat pups

Exploratory study of P-glycoprotein developmental expression in rat pups

The purpose of this study was to determine levels of P-glycoprotein, immunohistochemically and by western blot analysis, in the brain and intestine of adult rats, gestation day 20 rat fetuses, and rat pups on postnatal days 2, 5, 8, 11, 14, 17 and 20. There were 40 mated F_0 females and four unmated females assigned to this study (approximately 10 weeks of age; weight range 200–300 g). None was treated with a test or control substance. During cohabitation, females were housed with untreated males of the same strain in a ratio of 1:1. Mating was confirmed by the presence of seminal plug(s) in the cage pan and/or vagina. Mortality of F_0 females was checked daily. Body weights and feed consumption were not recorded. Scheduled live fetuses from F_0 females were weighed, sexed and given external examinations. Selected F_1 offspring were sexed and examined externally on postnatal day 0 and weighed at sacrifice.

Four F_0 females were sacrificed by carbon dioxide asphyxiation on gestation day 20. Within each litter, one male and one female fetus were terminated by rapid induction of hypothermia, and the brain and jejunum were snap frozen in isopentane/liquid nitrogen and cryosectioned for immunohistochemical analysis for P-glycoprotein. In addition, frozen samples of brain and jejunum were stored and later analysed for P-glycoprotein by western blot analysis. The brain, jejunum and uterus of the four dams were sampled, sectioned and analysed as above, immunohistochemically and by western blot technique.

F_1 offspring. On postnatal day 0, litters were culled to 10 pups each (five of each sex, fostering as needed). On postnatal days 2, 5, 8, 11, 14, 17 and 20, one male and one female pup from each of four litters (total of four pups of each sex per day) were sacrificed by rapid induction of hypothermia (days 2 and 5) or carbon dioxide asphyxiation (days 8, 11, 14, 17 and 20) and sampled and sectioned as described above for immunohistochemical analysis of P-glycoprotein in the brain and jejunum. Selected samples were analysed for P-glycoprotein by western blot analysis. To ensure random, unbiased sample collection, pups were selected such that they were sourced from as many litters as possible.

Four adult non-pregnant females were terminated by carbon dioxide asphyxiation, and the uteri were sampled, sectioned and analysed as described above for pregnant females.

The frozen sections were processed immunohistochemically using methods adapted from those of Schinkel et al. (1994) for P-glycoprotein. Slides were examined microscopically using a blind procedure and scored for relative staining intensity for P-glycoprotein.

At a later date, western blots produced in the study were analysed using densitometry of the P-glycoprotein bands to quantify the levels of protein. A molecular dynamics scanning densitometer using a 5-minute film exposure with chemiluminescent detection was used.

There were no deaths or abortions during the study. External examination of fetuses and pups did not reveal any anomalies.

P-glycoprotein staining was observed in a diffuse pattern on the endothelial cell surface of capillaries in the cerebrum and cerebellum and the brush border of jejunal epithelial cells. In contrast to the non-pregnant rats, which had no uterine P-glycoprotein expression, the pregnant rats had a moderate amount of P-glycoprotein present on the luminal surface of the uterine epithelium. The P-glycoprotein observed microscopically in the brain and uterus was confirmed by western blot analysis. F_1 pups of all ages, including the gestation day 20 fetuses, showed P-glycoprotein staining in the brain. Expression appeared weaker in the gestation day 20 fetuses and younger pups compared with the older postnatal days 17 and 20 pups. However, P-glycoprotein staining of the jejunal epithelial cells was not apparent until approximately postnatal day 8. Minimal to very slight staining was seen in the postnatal day 8 pups' intestinal epithelial cells. P-glycoprotein staining appeared more intense in the older pups by approximately postnatal day 17, but did not achieve the intensity found in the F_0 adult females, even by postnatal day 20. The intensity of the P-glycoprotein seen microscopically in the F_1 pups was further supported by western blot analysis.

Many of the pups approximately 8 days old or younger did not have P-glycoprotein staining along the jejunal epithelial brush border. The absence of intestinal P-glycoprotein in these young pups may allow greater absorption of drugs, which suggests that higher plasma drug levels may be possible. Moreover, it can be hypothesized that higher plasma drug levels, coupled with less P-glycoprotein in the blood–brain barrier, may produce higher drug levels in the brain, resulting in clinical evidence of neurotoxicity, including death. Less abamectin may be absorbed from the intestine, and there is less accumulation in the brain, as the P-glycoprotein barrier develops in the intestinal tract and brain. Thus, it appears that young rat pups (8 days old) are potentially more susceptible to avermectin-related neurotoxicity because of differences in the development of the P-glycoprotein transport system. These results may explain the enhanced neonatal toxicity of avermectins to rats and the postnatal development of a blood–brain barrier to these substances in this species.

Quantification by scanning densitometry of western blots of brain samples shows that levels of P-glycoprotein in the gestation day 20 fetus are very low (approximately 11% of the adult value), as are levels in neonatal rats through to postnatal day 11. The apparent increase in the level for the rat fetus (11% of the adult value) compared with the early postnatal samples (5% of the adult value) is probably a reflection of qualitative differences of the protein as a function of age. The western blots show that the rat fetal protein migrates in a more diffuse pattern compared with the neonatal samples. As this protein undergoes significant post-translational processing (i.e. glycosylation), it is possible that the fetal protein is not a fully mature, functional protein, equivalent to that in older animals. However, it is clear that the neonatal animals through to postnatal day 14 have significantly lower levels of P-glycoprotein in the brain compared with older neonates and adults. These differences in P-glycoprotein correlate with the increased neonatal toxicity to the avermectins observed from approximately day 4 through to day 14 in multigeneration studies.

In view of the above, it was concluded that the blood–brain barrier, formed by the expression of P-glycoprotein on the luminal surface of endothelial cells of cerebral and cerebellar capillaries (and tight junctions of these endothelial cells), is not fully developed in neonatal rats. This barrier develops to full (adult) extent during the first 20 days. Furthermore, the expression of P-glycoprotein in the jejunal epithelial brush border does not start before postnatal day 8. Neonatal rats, with limited or no blood–brain barrier, have increased susceptibility to avermectin toxicity (Cukierski, 1995).

Examination of developmental expression of P-glycoprotein levels in rat pups

To examine the expression and localization of P-glycoprotein in the brain of adult rats and neonatal rat pups during their early development, female rats (Crj:Wistar) pregnant for 19 days were anaesthetized under ether inhalation, and rat fetuses were removed from the uteri by caesarean sections. Rats on postnatal days 1, 3, 7, 14, 21, 28, 56 and 84 were anaesthetized under ether inhalation, and the brains were dissected. Five rats were used at each time period. Brains were homogenized and separated by centrifugation into a cytosolic fraction (supernatant) and a membrane fraction (pellet). Aliquots were separated by SDS-PAGE, and P-glycoprotein was marked by a specific antibody (anti-human P-glycoprotein antibody C-19, Santa Cruz, CA, USA). For quantitative analysis, the X-ray film was scanned with a charge-coupled device colour scanner and then analysed by image analysis. The protein levels of P-glycoprotein determined were presented as the percentage of the protein level in the adult rat brain (postnatal day 84 = 12 weeks).

Immunohistochemistry was performed on brain slices to determine the localization of P-glycoprotein in relation to brain capillaries and astrocytes.

In the adult rat brain (postnatal day 84), P-glycoprotein was detected in the membrane fraction of the cerebral cortex, cerebellum and hippocampus. Comparison between the membrane and cytosolic fractions shows that P-glycoprotein was predominantly detected in the membrane fraction.

Although P-glycoprotein was detected in the adult rat brain, it was not detectable in the early stages of postnatal development in the cerebral cortex, cerebellum or hippocampus. P-glycoprotein was first detected from postnatal day 7 in the membrane fraction of all three areas of the brain. From postnatal day 7 onwards, levels gradually increased, reaching a plateau on postnatal day 28. The observed expression profile of P-glycoprotein in the membrane fraction was similar in the cerebral cortex, hippocampus and cerebellum.

Immunolocalization of P-glycoprotein in brain slices confirmed that P-glycoprotein was not detectable until postnatal day 7. Intense staining was seen on postnatal day 21. Double immunostaining of P-glycoprotein with von Willebrand factor (a marker for capillaries) and glial fibrillary acidic protein (a marker for astrocytes) showed that P-glycoprotein was co-localized with the capillaries, but not the astrocytes.

These findings suggest that P-glycoprotein may act as part of the blood–brain barrier, and its expression may increase synchronously with other blood–brain barrier components: namely, formation of the tight junction–based endothelium and inclusion of other energy-dependent transport systems.

It can be concluded that adult rats show intense P-glycoprotein immunoreactivity. The expression and localization of P-glycoprotein in the brain during the early development of rats were examined. P-glycoprotein was undetectable in the embryo and early stages of postnatal development. It was first detected on postnatal day 7 and then gradually increased to reach a plateau by day 28 at levels approximating those seen in adult rats. There is evidence that P-glycoprotein expression increases with postnatal development and is localized in the brain capillaries, suggesting a role for P-glycoprotein in the blood–brain barrier.

The study was not GLP compliant (Matsuoka et al., 1999).

(g) Oral toxicity and plasma level study in monkeys

In an in-house investigative study, two groups of two male and two female rhesus monkeys (*Macaca mulatta*, 2–3 years of age, weight range 2.4–3.2 kg, supplied by Charles River Primates, USA) were treated orally, by gavage, with repeated single doses of abamectin (MK-0936, batch no. L-676,863-00V50, purity 94% by HPLC) or ivermectin (MK-0933, batch no. L-640,471-000W076, purity 97.3%) in sesame oil at 2- to 3-week intervals for a total of 13 doses. The dosing schedule was as follows: weeks 1, 4 and 6: 0.2 mg/kg bw; week 8: 0.5 mg/kg bw; week 10: 1.0 mg/kg bw; week 13: 2.0 mg/kg bw; week 15: 4.0 mg/kg bw; week 17: 2.0 mg/kg bw; week 19: 6.0 mg/kg bw; week 21: 8.0 mg/kg bw; week 24: 8.0 mg/kg bw; week 27: 12.0 mg/kg bw; and week 29: 24.0 mg/kg bw.

After the first two doses of either abamectin or ivermectin, treatment of the groups was crossed over in order to elucidate slight pupil dilation in two animals treated with ivermectin. Thereafter, the groups continued to receive the same test substance. Formulations prepared for each week, with the exception of week 4, were analysed for test substance concentration. All formulations, with the exception of the week 24 ivermectin sample, which had a mean concentration of 84% of the nominal concentration, were within 10% of the nominal concentration. Clinical signs were recorded daily. Feed consumption was estimated 5 times/week. Body weights were recorded twice each week up to week 9 and weekly thereafter. Ocular examinations for mydriasis were performed using a penlight light source pre-dosing and at 2, 4 and 24 hours post-dosing for all doses except 24.0 mg/kg bw (for which 8- and 48-hour examinations were also included). Plasma samples for analysis of test substance concentration were prepared from blood withdrawn 15 and 30 minutes and 1, 2, 4, 8, 24, 48, 72 and 96 hours following treatment at 2.0 mg/kg bw in week 17. Plasma samples were also taken 8, 24 and 48 hours after treatment at 8.0 mg/kg bw in week 24 and 8, 24, 48, 72 and 96 hours following treatment at 24.0 mg/kg bw in week 29. Analysis of plasma for abamectin or ivermectin concentration was performed by the modified liquid chromatography–fluorescence method.

Both abamectin and ivermectin produced emesis at 2.0 mg/kg bw, with a dose-related increase in incidence and decrease in time of onset. No treatment-related clinical signs occurred at doses less than 2.0 mg/kg bw. Therefore, emesis is considered to be the most sensitive indicator of toxicity in rhesus monkeys and was used to determine the minimum toxic dose. The minimum toxic dose (LOAEL) for both test substances is considered to be 2.0 mg/kg bw. Mydriasis is considered to be a less sensitive indicator of toxicity because a marked effect occurred only in response to abamectin at 24.0 mg/kg bw and because of interanimal variability. Pupil dilation and/or decreased pupil constriction occurred at dose levels of 6.0 mg/kg bw for abamectin and 12.0 mg/kg bw for ivermectin, but there was no relationship between dose level and time of onset and duration of effect. The incidence of mydriasis was higher in the group treated with abamectin than in the ivermectin-treated group. Three animals in each group displayed transient reduced activity or sedation after treatment at 24.0 mg/kg bw. The effect had a variable time of onset and persisted for up to 25 hours. The occurrence and severity of sedation or reduced activity did not correlate with mydriatic effects. There were no other treatment-related clinical signs and no effect on feed consumption or body weight at any dose level in either group.

Plasma concentrations of abamectin and ivermectin were similar for 4 hours after a dose of 2.0 mg/kg bw, but thereafter the plasma concentrations of ivermectin were generally higher than those of abamectin.

The peak plasma concentrations of abamectin and ivermectin occurred 4–24 hours and 8–48 hours post-treatment, respectively. The shapes of the plasma concentration–time plots suggested that the peak concentrations occurred between 8 and 24 hours for both compounds. The highest plasma concentrations were recorded 8 hours after treatment with doses of 8.0 and 24.0 mg/kg bw for both compounds. At 8, 24 and 48 hours after treatment with abamectin, the ratios of plasma concentrations between 2.0 and 8.0 mg/kg bw were 2.0, 0.8 and 2.5, respectively. The equivalent values for ivermectin were 2.8, 1.4 and 1.5, respectively. The ratios of plasma concentrations between 8.0 and 24.0 mg/kg bw at 8, 24 and 48 hours after treatment were 2.6, 4.2 and 3.4, respectively, for abamectin and 2.5, 2.6 and 3.6, respectively, for ivermectin. Therefore, there was an approximate proportionality between dose and plasma concentration for both compounds. There was a poor correlation between plasma levels in individuals and the occurrence of emesis and the incidence and severity of mydriasis.

Clinical signs of toxicity elicited by abamectin and ivermectin are confined to emesis and mydriasis at dose levels up to 12.0 mg/kg bw and, additionally, sedation at 24.0 mg/kg bw. The most sensitive indicator of abamectin and ivermectin toxicity in rhesus monkeys is emesis, as clinical signs of toxicity seen in mice and rats (tremors and convulsions) did not occur. The minimum toxic dose (LOAEL) of both abamectin and ivermectin was 2.0 mg/kg bw, and the NOAEL was 1.0 mg/kg bw. The maximum plasma concentrations of both abamectin and ivermectin occurred between 8 and 24 hours after oral administration. Plasma concentrations increased with increasing dose, but generally less than proportionally with dose. Approximate 5-fold and 7-fold increases in the plasma

concentrations of abamectin and ivermectin, respectively, over those produced by the minimum toxic dose did not elicit more severe clinical signs of toxicity, other than slight to moderate sedation.

The study was GLP compliant, and a QA statement was attached (Gordon, 1985c).

(h) Studies on metabolites

The 2000 JMPR defined the residue for compliance with maximum residue limits (MRLs) for animal commodities as the sum of avermectin B_{1a} and 8,9-*Z*-avermectin B_{1a}. The residue for compliance with MRLs for plant commodities and for estimating dietary exposure was defined as the sum of avermectin B_{1a}, avermectin B_{1b}, 8,9-*Z*-avermectin B_{1a} and 8,9-*Z*-avermectin B_{1b}. 8,9-*Z*-avermectin B_{1a} (also referred to as 8,9-*Z*-B_{1a}, Δ-8,9-isomer, NOA 427011 and L-652,280) and 8,9-*Z*-avermectin B_{1b} are the photodegraded products of abamectin. 8,9-*Z*-avermectin B_{1b} is present in smaller proportions and is assumed to have toxicological properties similar to those of 8,9-*Z*-B_{1a}.

Acute toxicity of 8,9-Z-B_{1a}

Studies on the acute toxicity of 8,9-*Z*-B_{1a} are summarized in Table 21.

Genotoxicity of 8,9-Z-B_{1a}

8,9-*Z*-B_{1a} (batch no. L-652,280-000N005, purity 91.6%) in dimethyl sulfoxide solvent was tested in four histidine-auxotrophic strains (TA97a, TA98, TA100 and TA1535) of *Salmonella typhimurium* and three tryptophan-auxotrophic strains of *Escherichia coli* (WP2, WP2 uvrA and WP2 uvrA pKM101) by preincubation for 48 hours at concentrations of 0 (solvent control), 10, 30, 100, 300, 1000 and 3000 µg/plate.

8,9-*Z*-B_{1a} did not produce any 2-fold or greater dose-related increase in the number of revertant colonies at any dose level in any of the strains tested. There was no inhibition of the background lawn growth of any strain at any dose level. Precipitation occurred on all plates at 3000 µg/plate. The positive control materials produced the expected increase in the numbers of revertant colonies in all strains.

In view of the above, 8,9-*Z*-B_{1a} and/or its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used in the study at doses up to 3000 µg/plate, a concentration at the limit of solubility.

The study was GLP compliant, and a QA statement was attached (Gordon, 1988c).

One-generation reproductive toxicity of 8,9-Z-B_{1a}

Four groups of 20 female Sprague-Dawley rats (Crl:CD(SD) BR strain, approximately 10 weeks old, weight range 192–242 g) were treated orally, by gavage, for 15 days prior to mating, throughout cohabitation and gestation, and through lactation until day 20 with 8,9-*Z*-B_{1a} (batch no. L-652,280-000N005, purity 91.6%) as a solution in sesame oil at a dose level of 0 (vehicle only), 0.06, 0.12 or 0.40 mg/kg bw per day. Individual doses were based on the most recent body weight. All maternal animals were observed for clinical signs twice daily, one observation being 1–5 hours after treatment. Maternal body weights were recorded weekly during the premating and cohabitation periods, on days 1, 6, 12, 16, 18 and 20 of gestation and daily thereafter until parturition, and on days 0, 7, 14 and 20 of lactation. Maternal feed consumption was measured at 5-day intervals twice during premating, 3 times during gestation and twice during lactation. After 15 days of treatment, the females were paired 1:1 with untreated males of the same strain for a maximum of 14 days. Vaginal lavage was performed daily to detect sperm (day 0 of gestation = the day on which sperm were observed in the vagina). The females were allowed to litter naturally, with observation of parturition behaviour 4 times daily from day 21 to day 24. Neonatal pups were examined externally on the day of birth. Dead pups were given a visceral examination limited to trachea and oesophagus only. The litters were culled to eight pups per litter on day 3 of lactation by random selection, but with a balanced sex ratio

Table 21. Summary of acute toxicity studies with 8,9-Z-B_{1a}, a metabolite of abamectin

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw)	Reference
Mouse	CF-1	M + F	Oral[a]	> 99	> 5 000	Gordon (1984g)
Mouse	CF-1	M + F	Oral[b]	> 99	> 48	Gordon (1984g)
Mouse	Crl:CF1 (BR)	M + F	Oral[c]	> 99	> 80	Gordon (1986c)
Mouse	CD-1	F	Oral[d]	Not specified	217	Lynch (1996)
Mouse	CF-1	M	Oral[e]	Not specified	20	Lynch (1996)

bw: body weight; F: female; LD_{50}: median lethal dose; M: male

[a] Tested at dose levels of 1250, 2500 and 5000 mg/kg bw (polar photodegradate of abamectin). No deaths occurred in the animals treated at any dose level, and clinical signs were confined to the group treated at 5000 mg/kg bw. All mice in the group showed decreased activity and bradypnoea within 10 minutes of treatment, and this persisted for approximately 4 hours. All animals, except one female, gained weight during the subsequent observation period. There were no treatment-related gross changes at necropsy.

[b] Tested at dose levels of 6, 12, 24 and 48 mg/kg bw (non-polar metabolite). One male treated at 12 mg/kg bw died approximately 4.5 hours after treatment. Prior to death, the animal had decreased activity and bradypnoea; at necropsy, the cause of death was established as dose maladministration. Treatment-related clinical signs were absent in all dose groups, but the sesame oil controls and the groups treated at 24 and 48 mg/kg bw showed slight perianal oil staining. One female treated at 6 mg/kg bw had pale extremities and had lost weight on day 14. At necropsy, the animal had a blood clot on the ovary. All other animals, except one female, gained weight during the observation period. No treatment-related gross changes were evident at necropsy in any dose group.

[c] Tested at dose levels of 0, 5, 10, 20, 40 and 80 mg/kg bw. Clinical signs of toxicity, including decreased activity, bradypnoea, ataxia and ptosis, occurred within 60–90 minutes of treatment in all treated groups except for females receiving 5 mg/kg bw. The symptoms persisted until day 2 in both sexes treated at 80 mg/kg bw and in the males treated at 40 mg/kg bw. Mortality incidences were 10%, 10%, 20%, 0% and 30% in the male groups and 0%, 10%, 10%, 0% and 10% in the female groups, in order of ascending dose level. All deaths occurred within 24 hours of treatment, starting 137 minutes post-treatment. There were no deaths or clinical signs in the vehicle control animals. All surviving animals gained weight during the observation period. There were no treatment-related gross lesions at necropsy, and all gross and histological lesions were considered to be incidental to treatment with the 8,9-*Z* isomer of avermectin B_{1a}. The LD_{50} value was estimated to be > 80 mg/kg bw for animals of both sexes, without a significant difference between the sexes.

[d] This was an exploratory acute oral toxicity study using dose levels of 50, 90, 162, 292 and 525 mg/kg bw. All female CD-1 mice treated at 292 and 525 mg/kg bw died within 2 days of treatment, but there were no deaths in the groups treated at 50, 90 or 162 mg/kg bw. Prior to death, the animals treated at 292 and 525 mg/kg bw were moribund within 5 hours of treatment and showed decreased activity, bradypnoea and, in some animals, tremors on handling. Urine staining was also evident in animals treated at 525 mg/kg bw. Animals treated at 162 mg/kg bw showed decreased activity and bradypnoea from day 2 to day 3 and hunched posture on day 3. Animals treated at 90 mg/kg bw showed decreased activity and unkemptness on day 2 only. No clinical signs occurred at 50 mg/kg bw.

[e] This was an exploratory acute oral toxicity study using dose levels of 10, 20 and 30 mg/kg bw. There were one, three and two deaths in male CF-1 mice, in order of ascending dose level. Clinical signs of decreased activity and bradypnoea at all dose levels and ptosis at 30 mg/kg bw occurred within 30 minutes of treatment. Decedents were moribund within 3 hours of treatment, and some animals treated at 20 or 30 mg/kg bw showed tremors on handling. Urine staining was evident at 30 mg/kg bw. Survivors were of normal appearance by day 2.

where possible. Excess pups were killed and discarded without further examination. Pup weight and sex were recorded on days 0, 7, 14 and 21 of lactation, and clinical signs were recorded daily until sacrifice on day 21 postpartum.

Postmortem examination of F_1 progeny was limited to the eyes, which were preserved and examined histologically. Complete retinal cross-sections of both eyes were examined. Females of the P generation not giving birth were killed and subjected to necropsy on day 24 of presumed gestation. All other maternal animals were killed and subjected to gross necropsy and metrial gland count on days 21–23 postpartum. Litter data, maternal body weights, time to mating and duration of gestation were analysed statistically.

No deaths or treatment-related clinical signs occurred at any dose level during the course of the study. There were no treatment-related effects on maternal weight gain or feed consumption and no treatment-related gross findings at necropsy at any dose level. There were no treatment-related effects at any dose level on mating performance as assessed by mean day of mating, numbers of matings and pregnancies, and duration of gestation. There were no treatment-related effects on the

external appearance of pups. One pup from the 0.12 mg/kg bw per day group had anencephaly and ethmocephaly at birth, and one pup from the 0.06 mg/kg bw per day group, with a domed head, was found to be severely hydrocephalic at necropsy on day 21. There were no other external malformations. Pup growth, survival and sex ratios were unaffected by treatment at all dose levels. Although postimplantation survival was significantly reduced ($P < 0.05$) in the 0.12 mg/kg bw per day group (84.9%) compared with the control value of 94.6%, the difference was due largely to one female bearing one dead pup at birth. As there was no dose–response relationship, the difference is considered to be incidental to treatment. Gross and histomorphological examination of the eyes of F_1 generation progeny revealed no treatment-related effects.

In view of the absence of effects on female fertility and reproductive performance at all doses, a NOAEL for maternal toxicity and reproductive effects in rats for the 8,9-*Z* isomer of avermectin B_{1a} was identified as 0.40 mg/kg bw per day, the highest dose tested. The NOAEL for offspring toxicity was also 0.40 mg/kg bw per day, the highest dose tested, based on the absence of effects on external appearance, viability and growth of F_1 generation progeny at all doses.

The study was GLP compliant, and a QA statement was attached (Gordon, 1988b).

Developmental toxicity of 8,9-Z-B_{1a}

Seven groups of 7–13 naturally mated female mice (Crl:CF1 (BR) strain, 82 days old, weight range 23.2–30.9 g) were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with the 8,9-*Z* isomer of avermectin B_{1a} (batch no. L-652,280-00N03, purity 99%) in sesame oil at a dose level of 0 (vehicle only), 1.5, 3.0, 6.25, 12.5, 25.0 or 50.0 mg/kg bw per day from day 6 to day 15 of gestation (day 0 = day on which vaginal plug was observed). Treatment at dose levels of 3.0 mg/kg bw per day and higher was discontinued after a single dose due to mortality. Clinical signs were recorded daily immediately after dosing and also 1–5 hours after dosing on weekdays. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 17 of gestation. The animals were killed on day 17 of gestation and subjected to gross necropsy examination. The uterus was examined to determine pregnancy status, and implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Animals dying during the study were subjected to gross necropsy examination and determination of reproductive status only. Maternal body weight data and litter parameters were analysed statistically.

There were two treatment-related deaths in each of the groups treated at 3.0, 12.5, 25.0 and 50.0 mg/kg bw per day and three deaths in the group treated at 6.25 mg/kg bw per day after one dose. The animals in these groups were killed and discarded on days 6–8 of gestation. A single treatment-related death also occurred at 1.5 mg/kg bw per day. Death occurred on day 8 of gestation after two doses. There were no other deaths during the study. There were no clinical signs of toxicity other than in some animals that died after one dose that were comatose or moribund prior to death. The overall weight gain during the treatment period at 1.5 mg/kg bw per day was 9.2% lower than in the controls, but the difference was not statistically significant ($P > 0.05$). No gross lesions were evident at necropsy in maternal animals treated at 1.5 mg/kg bw per day. A treatment-induced increase in the incidence of external malformations was apparent at 1.5 mg/kg bw per day. A total of 24 fetuses (28.9%) from four litters had cleft palate, and a further fetus from a litter affected by cleft palate had exencephaly and omphalocele. The overall external malformation incidence of the group treated at 1.5 mg/kg bw per day was 30.1% compared with a control incidence of 0.0%.

In view of the above, the 8,9-*Z* isomer of avermectin B_{1a} elicits both maternal toxicity and a teratogenic response at 1.5 mg/kg bw per day. NOAELs for maternal and fetal toxicity could not be identified, as a treatment-related maternal death and an excess incidence of cleft palates occurred at the lowest dose level tested.

The study was GLP compliant, and a QA statement was attached (Gordon, 1986d).

In a subsequent study, five groups of 12 naturally mated female mice (Crl:CF1 (BR) strain, 10 weeks old, weight range 21.8–27.1 g) were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with the 8,9-*Z* isomer of avermectin B_{1a} (batch no. L-652,280-00N03, purity 99%) in sesame oil at a dose level of 0 (vehicle only), 0.05, 0.1, 0.5 or 1.0 mg/kg bw per day from day 6 to day 15 of gestation (day 0 = day on which vaginal plug was observed). Treatment at dose levels of 3.0 mg/kg bw per day and higher was discontinued after a single dose due to mortality. Clinical signs were recorded daily immediately after dosing and also 1–5 hours after dosing on weekdays. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 17 of gestation. The animals were killed on day 17 of gestation and subjected to gross necropsy examination. The uterus was examined to determine pregnancy status, and implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Animals dying during the study were subjected to gross necropsy examination and determination of reproductive status only. Maternal body weight data and litter parameters were analysed statistically.

Two deaths (one at 1.0 mg/kg bw per day and the other at 0.5 mg/kg bw per day) occurred during the study, both of which are considered to be treatment related. A female treated at 1.0 mg/kg bw per day was found dead on day 10 after receiving four doses. Clinical signs prior to death were restricted to lethargy and weight loss. One female treated at 0.5 mg/kg bw per day was killed in poor condition on day 11 of gestation. Marked weight loss occurred from the start of treatment, and tremors were evident for 2 days before sacrifice. No other deaths or clinical signs of toxicity occurred at any dose level. Other than in the two animals that died or were sacrificed prematurely, maternal body weight gain was unaffected by treatment at all dose levels. However, the mean weight gains from day 6 to day 17 of gestation in the groups treated at 0.05 and 1.0 mg/kg bw per day were significantly ($P < 0.05$) lower than in the control group (by 18.4%), owing to significantly lower litter sizes. There were no treatment-related gross changes at necropsy in maternal animals of all treatment groups. The mean number of implantations per female in the group treated at 1.0 mg/kg bw per day (9.8) was significantly lower ($P < 0.05$) than the control value of 12.5. Fewer implantations per female also occurred in the group treated at 0.05 mg/kg bw per day. These differences from the controls are considered not to be treatment induced, as implantation was essentially complete at the start of treatment on day 6 of gestation. The incidence of resorptions was higher than the control incidence in all treated groups, but the differences were not statistically significant ($P > 0.05$), and their magnitude did not increase with dose. As a consequence of lower implantation numbers and higher incidences of resorption, the mean number of live fetuses per female was significantly reduced ($P < 0.05$) in the groups treated at 0.05 and 1.0 mg/kg bw per day (8.7 and 8.3, respectively, compared with 11.3 in the controls). As both contributing factors are considered not to be treatment induced, the reduced live litter size is considered not to be related to treatment. Mean fetal weights were unaffected by treatment at all dose levels.

Increased incidences of exencephaly and cleft palate occurred at dose levels of 0.1 mg/kg bw per day and higher, but there was no correlation between incidence and dose level. Cleft palate occurred at incidences between 1.10% (one fetus) and 11.3% (13 fetuses from two litters), compared with a control incidence of 0%. Exencephaly occurred at incidences between 1.74% (two fetuses from one litter) and 4.40% (four fetuses from three litters), compared with a single occurrence (0.73%) in the control group. The similarity of malformations occurring in the treated groups indicated treatment-related teratogenicity at dose levels of 0.1 mg/kg bw per day and higher. Cleft palate and exencephaly did not occur at 0.05 mg/kg bw per day. Two other fetuses from treated litters showed external malformations, one fetus with a malformed tail (1.0 mg/kg bw per day) and one fetus with polydactyly (0.05 mg/kg bw per day). These malformations are not considered to be treatment related.

In view of the above, a NOAEL for maternal toxicity for the 8,9-*Z* isomer of avermectin B_{1a} in the mouse was identified as 0.1 mg/kg bw per day, based on the occurrence of maternal death at levels of 0.5 mg/kg bw per day and higher. The 8,9-*Z* isomer of avermectin B_{1a} is teratogenic in the CF-1 mouse at dose levels of 0.1 mg/kg bw per day and higher, based on excess incidences of cleft palate and exencephaly. A NOAEL for embryo and fetal toxicity was identified as 0.05 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Gordon, 1986e).

In another study, four groups of 25 naturally mated female mice (Crl:CF1 (BR) strain, 10 weeks old, 19.2–28.7 g) were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with 8,9-*Z*-B_{1a} (batch no. L-652,280-00N03, purity 99%) in sesame oil at a dose level of 0 (vehicle only), 0.015, 0.03 or 0.06 mg/kg bw per day from day 6 to day 15 of gestation (day 0 of gestation = day on which vaginal plug was observed). Clinical signs were recorded daily immediately after dosing and also 1–5 hours after dosing on weekdays. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 17 of gestation. The animals were killed on day 17 of gestation and subjected to gross necropsy examination. The uterus was examined to determine pregnancy status, and implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Visceral examination by dissection was performed on every third fetus of each litter and on all externally abnormal fetuses. The head of every third fetus was fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal malformations and variations. Maternal body weight data and litter parameters were analysed statistically.

There were no deaths or treatment-related clinical signs at any dose level. However, one female treated at 0.06 mg/kg bw per day had only metrial glands at the implantation sites at necropsy, and the animal is considered to have aborted between days 10 and 12 of gestation, when its body weight decreased by 1.7 g. Body weight gain was unaffected by treatment at all dose levels. Litter parameters were unaffected by treatment at all dose levels, and embryonic survival, the proportion of resorbed or dead fetuses and fetal weights were not significantly different ($P > 0.05$) from the control values. There were no treatment-related effects on the incidences of fetal abnormalities or variations at any dose level. However, the overall incidences of abnormal fetuses in all groups treated with 8,9-*Z*-B_{1a} (4.27%, 3.46% and 2.36%, in order of ascending dose) were higher than the control incidence of 1.50%. The difference is considered not to be an effect of treatment, as no dose–response relationship was apparent, and the incidences of individual malformations are considered to reflect spontaneous variation. Three fetuses in each of the groups treated at 0.03 and 0.06 mg/kg bw per day had exencephaly, in three and two litters, respectively. Although the incidences of 1.30% and 1.42%, respectively, are higher than the concurrent control group (0%) and the historical control group (0.27%) incidences, exencephaly has occurred in individual historical control groups at up to 1.64% of fetuses from 14.3% of litters. Furthermore, there is no dose–response relationship for the occurrence of exencephaly, as the incidence of the malformation in the study by Gordon (1986a) was 1.20% (one fetus out of seven litters) at a dose level of 1.5 mg/kg bw per day. A single fetus in the group treated at 0.015 mg/kg bw per day showed cleft palate, which is considered to be a spontaneous occurrence in the absence of cleft palate at higher dose levels. One litter each at 0.015 and 0.03 mg/kg bw per day contained fetuses with hindlimb extension (four and one fetuses, respectively), but the malformation also occurred in a control fetus.

Sternebral, vertebral and rib malformations occurred at low incidence in the treated groups, but none was considered treatment induced, as they also occurred spontaneously in historical control groups. The incidences of visceral and skeletal variations, including the incidences of fetuses with incomplete ossification, were comparable with those of the controls, although sternebral and vertebral sites occurred at slightly higher incidences than in controls.

The NOAEL for both maternal and embryo/fetal toxicity, including teratogenicity, was 0.06 mg/kg bw per day, the highest dose tested, for the 8,9-*Z* isomer of avermectin B_{1a} in the CF-1 mouse.

The study was GLP compliant, and a QA statement was attached (Gordon, 1986f).

In another study, five groups of 25 naturally mated female mice (Crl:CF1 (BR) strain, 9.5 weeks old, 21.5–28.5 g) were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with 8,9-*Z*-B_{1a} (batch no. L-652,280-00N03, purity 99%) in sesame oil at a dose level of 0 (vehicle only), 0.015, 0.03, 0.1 or 0.5 mg/kg bw per day from day 6 to day 15 of gestation (day 0 of gestation = day on which vaginal plug was observed). Clinical signs were recorded daily immediately after dosing and also 1–5 hours after dosing on weekdays. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 17 of gestation, and feed consumption was measured periodically. The animals were

killed on day 17 of gestation and subjected to gross necropsy examination. The uterus was examined to determine pregnancy status, and implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Visceral examination by dissection was performed on every third fetus of each litter and on all externally abnormal fetuses. The head of every third fetus was fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal malformations and variations. Maternal body weight changes and litter parameters (implantations per female, resorptions plus dead fetuses per implantation site, live fetuses per female and live fetal weight) were analysed statistically.

One animal treated at 0.5 mg/kg bw per day was killed in a moribund condition after receiving six doses. The animal had marked weight loss, anorexia, lethargy and chromodacryorrhoea prior to death. No other deaths or treatment-related clinical signs occurred during the study. Maternal body weight and feed consumption were unaffected by treatment at all dose levels, and there were no treatment-related gross changes at necropsy. Litter parameters were unaffected by treatment at all dose levels, and embryonic survival and fetal weights were not significantly different ($P > 0.05$) from the control values. The proportion of resorbed or dead fetuses per implantation in the group treated at 0.03 mg/kg bw per day (16.3%) was significantly ($P < 0.05$) higher than the control value (9.0%). The difference is considered not to be treatment induced, as there was no effect on the parameter in the group treated at 0.1 mg/kg bw per day (9.3% resorbed or dead fetuses per implantation). Thus, no dose–response relationship was evident.

The higher proportion of resorbed or dead fetuses per implantation in the group treated at 0.5 mg/kg bw per day (14.3%) was not statistically significant ($P > 0.05$). The overall incidences of malformed fetuses were 3.86%, 3.78%, 3.23% and 12.4%, in order of ascending dose, compared with a control incidence of 2.30%. An increased incidence of cleft palate occurred in the group treated at 0.5 mg/kg bw per day (24 fetuses in six litters – 10.3%) compared with the control group incidence of 0%, indicating treatment-related teratogenicity. Six fetuses from a single litter treated at 0.1 mg/kg bw per day also had cleft palate. As the group fetal incidence of 2.15% is in excess of the historical control mean incidence (0.28%) and the concurrent control incidence, it is considered to be an effect of treatment. The occurrence of single fetuses with cleft palate in the groups treated at 0.015 and 0.03 mg/kg bw per day is considered not to be treatment induced, as the incidences (0.35% and 0.42%, respectively) are similar to the historical control incidence. Exencephaly occurred at slightly higher incidences in the groups treated at 0.015, 0.03 and 0.5 mg/kg bw per day (0.70%, 2.09% and 0.85%, respectively) compared with the control incidence (0.38%), but the malformation did not occur at 0.1 mg/kg bw per day. As there was no statistically significant ($P > 0.05$) dose–response relationship, the higher incidence at 0.5 mg/kg bw per day is considered not to be treatment related. All other external malformations, open eyelid, micrognathia, cleft lip and tail malformation, occurred in isolation and are considered unrelated to treatment. The incidences of visceral and skeletal malformations did not indicate an effect of treatment at any dose level. At the two highest dose levels, 0.1 and 0.5 mg/kg bw per day, all but one malformation (two fetuses with sternebral malformation at 0.1 mg/kg bw per day) occurred in single fetuses. Higher numbers of fetuses with sites of incomplete ossification at 0.03 and 0.1 mg/kg bw per day are considered incidental to treatment because the incidence at the highest dose level, 0.5 mg/kg bw per day, was similar to the control incidence.

In view of the above, a NOAEL for maternal toxicity for the 8,9-*Z* isomer of avermectin B_{1a} in CF-1 mice was identified as 0.1 mg/kg bw per day, based on the occurrence of one treatment-related death at 0.5 mg/kg bw per day. A teratogenic effect, characterized by an increased incidence of cleft palate, occurs in response to treatment with the 8,9-*Z* isomer of avermectin B_{1a} at 0.5 mg/kg bw per day. The minimum teratogenic dose level (LOAEL) is 0.1 mg/kg bw per day, based on a slight increase in the incidence of cleft palate. Therefore, a clear NOAEL for embryo/fetal toxicity in CF-1 mice was identified as 0.03 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Gordon, 1986g).

Table 22. Combined data for observation of excencephaly in oral maternal toxicity/teratogenicity studies with the 8,9-Z isomer of avermectin B_{1a} in CF-1 mice

Dose (mg/kg bw per day)	Reference	Litters examined	Litters with exencephaly		Fetuses examined	Fetuses with exencephaly	
			N	%		*N*	%
Control	Gordon (1986d)	13	0	0	164	0	0
	Gordon (1986e)	12	1	8.3	137	1	0.73
	Gordon (1986f)	22	0	0	200	0	0
	Gordon (1986g)	23	1	4.3	261	1	0.38
0.015	Gordon (1986f)	22	0	0	211	0	0
	Gordon (1986g)	24	1	4.2	285	1	0.35
0.03	Gordon (1986f)	23	3	13.0	231	3	1.3
	Gordon (1986g)	23	2	8.6	239	5	2.1
0.05	Gordon (1986e)	12	0	0	104	0	0
0.06	Gordon (1986f)	22	2	9.1	212	3	1.4
0.1	Gordon (1986e)	11	1	9.1	115	2	1.7
	Gordon (1986g)	24	0	0	279	0	0
0.5	Gordon (1986e)	9	3	33.3	91	4	4.3
	Gordon (1986g)	23	1	4.3	234	1	0.43
1.0	Gordon (1986e)	11	1	9.1	91	2	2.3
1.5	Gordon (1986f)	7	1	14.3	83	1	1.2
Historical control	From eight studies between 1984 and 1987		Mean	4.3		Mean	0.7
			Minimum	0		Minimum	0
			Maximum	16.7		Maximum	4.1

The common developmental defects seen in the above developmental toxicity studies were combined and compared with the historical controls (Table 22 and Table 23).

In an oral developmental toxicity study in CD-1 mice, four groups of 22 naturally mated female mice (Crl:CD-1 (BR) strain, 12 weeks old, weight range 23.8–32.4 g) were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with the 8,9-*Z* isomer of avermectin B_{1a} (batch no. L-652,280-000N010, purity 98.1%) in sesame oil at a dose level of 0 (vehicle only), 0.75, 1.5 or 3.0 mg/kg bw per day from day 6 to day 15 of gestation (day 0 of gestation = day on which vaginal plug was observed). Periodic analysis of the formulations demonstrated acceptable achieved concentrations (within 10% of the nominal concentration). Clinical signs were recorded on day 0 of gestation and twice daily from day 6 to day 18 of gestation. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 18 of gestation, and feed consumption was measured at 3-day intervals from day 3 of gestation. Surviving animals were killed on day 18 of gestation and subjected to a gross necropsy examination of the thoracic and abdominal cavities. The uterus was examined to determine pregnancy status, and corpora lutea were enumerated. Implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Placentas were examined for gross changes. Visceral examination by dissection was performed on approximately one half of the fetuses in each litter and on all externally abnormal fetuses. The heads of these fetuses were fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal abnormalities and variations.

Table 23. Combined data for observation of cleft palate in oral maternal toxicity/teratogenicity studies with the 8,9-Z isomer of avermectin B_{1a} in CF-1 mice

Dose (mg/kg bw per day)	Reference	Litters examined	Litters with cleft palate		Fetuses examined	Fetuses with cleft palate	
			N	%		*N*	%
Control	Gordon (1986d)	13	0	0	164	0	0
	Gordon (1986e)	12	0	0	137	0	0
	Gordon (1986f)	22	0	0	200	0	0
	Gordon (1986g)	23	0	0	261	0	0
0.015	Gordon (1986f)	22	1	4.5	211	1	0.47
	Gordon (1986g)	24	1	4.2	285	1	0.35
0.03	Gordon (1986f)	23	0	0	231	0	0
	Gordon (1986g)	23	1	4.3	239	1	0.42
0.05	Gordon (1986e)	12	0	0	104	0	0
0.06	Gordon (1986f)	22	0	0	212	0	0
0.1	Gordon (1986e)	11	2	18.2	115	13	11.3
	Gordon (1986g)	24	1	4.2	279	6	2.2
0.5	Gordon (1986e)	9	1	11.1	91	1	1.1
	Gordon (1986g)	23	6	26.1	234	24	10.3
1.0	Gordon (1986e)	11	4	36.4	91	7	7.7
1.5	Gordon (1986g)	7	4	57.1	83	24	28.9
Historical control	From 11 studies between 1983 and 1987		Mean	2.9		Mean	0.3
			Minimum	0		Minimum	0
			Maximum	14.3		Maximum	1.3

bw: body weight

There were no deaths and no treatment-related clinical signs during the study. There were no treatment-related effects on maternal body weight gain or feed consumption. There were no treatment-related gross findings at necropsy in maternal animals. Pregnancy indices, embryo survival parameters and fetal weights were unaffected by treatment. There were no treatment-related external, visceral or skeletal alterations in fetuses. Minor group differences in the low incidence of cleft palate showed no dose–response relationship and were well within the historical control range for CD-1 mice. There was a slight increase in the incidence of postaxial pseudopolydactyly in the 3.0 mg/kg bw per day group: 11 fetuses (3.5%), compared with two fetuses (0.7%) in the concurrent control group and none in the historical control group. However, the increased incidence of this minor alteration was not considered to be treatment related, as the increase was restricted to mainly one litter, which had nine of the 11 fetuses with the alteration.

In view of the above, it is concluded that there was no evidence of maternal toxicity or embryo/fetal toxicity up to 3.0 mg/kg bw per day when the 8,9-*Z* isomer of avermectin B_{1a} was administered to CD-1 mice. Hence, the NOAELs for both maternal and embryo/fetal toxicity were 3.0 mg/kg bw per day, the highest dose tested.

The study was GLP compliant, and a QA statement was attached (Wise, 1996a).

Developmental toxicity of 8,9-Z-B_{1a} in a pregnant subpopulation of mice sensitive and insensitive to the tremor-inducing properties of abamectin

This study was undertaken to ascertain the developmental toxicity of the 8,9-*Z* isomer of avermectin B_{1a} to a pregnant subpopulation of CF-1 mice sensitive or insensitive to the tremor-inducing properties of abamectin technical. Subpopulations of mice sensitive and insensitive to the tremor-inducing properties of abamectin were identified. Initially, 276 female mice (Crl:CF1 (BR) strain, 9–10 weeks old, 21.7–32.3 g) were treated with a single gavage dose of abamectin technical (batch no. L-676,863-000V098, purity 97.1%) at 0.4 mg/kg bw in sesame oil. The animals were observed for clinical signs approximately hourly on the day of treatment, daily for 3 days thereafter and then twice weekly until mating. Following preliminary identification of insensitive individuals, the subpopulation was treated with a further oral abamectin dose of 0.8 mg/kg bw in sesame oil to confirm its insensitive status. The insensitive animals and the surviving sensitive animals were naturally mated 2–3 weeks later for a developmental toxicity study. All animals that died or were killed were discarded without necropsy.

Animals selected for the developmental toxicity study were maintained for 2–3 weeks prior to mating. Four groups of 25 naturally mated insensitive female mice were treated orally, by gavage, at a constant treatment volume of 10 mL/kg bw with the 8,9-*Z* isomer of avermectin B_{1a} (batch no. L-652,280-000N007, purity 79.8%) in sesame oil at a dose level of 0 (vehicle only), 0.5, 1.0 or 1.5 mg/kg bw per day from day 6 to day 15 of gestation (day 0 of gestation = day on which vaginal plug was observed). Two further groups of sensitive mice were similarly treated with 0 (four animals) or 0.2–1.0 mg/kg bw per day (18 animals). (The dose level was increased to 0.3 mg/kg bw per day after 1–3 doses. Two days later, the dose level was increased to 0.5 mg/kg bw per day. One day later, the dose level was increased to 1.0 mg/kg bw per day for 1 day. Dosing was then suspended for 2 days and recommenced at 0.75 mg/kg bw per day in 6/18 females of normal appearance. Twelve animals with adverse clinical signs were killed, examined for pregnancy status and discarded.) Periodic analysis of the formulations demonstrated acceptable achieved concentrations (within 10% of the nominal concentration). Clinical signs were recorded on day 0 of gestation and twice daily from day 6 to day 18 of gestation. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16 and 18 of gestation, and feed consumption was measured at 3-day intervals from day 3 of gestation. Surviving animals were killed on day 18 of gestation and subjected to a gross necropsy examination of the thoracic and abdominal cavities. Gross lesions were preserved for possible histological examination. The brain (cerebrum and cerebellum) was removed and frozen in isopentane/liquid nitrogen. The brains of all surviving sensitive animals and of seven insensitive vehicle control animals and 11 insensitive animals treated at 1.5 mg/kg bw per day were processed for P-glycoprotein immunohistochemistry. In addition, one half of each brain from the sensitive animals was also submitted to western immunoblot analysis of P-glycoprotein. Slides were examined microscopically using a blind procedure and scored for relative staining intensity for P-glycoprotein after examining appropriate control sections to evaluate background staining. The uterus was examined to determine pregnancy status, and corpora lutea were enumerated. Implantations were counted and classified as resorptions, dead fetuses or live fetuses. All fetuses were examined externally, weighed and sexed. Placentas were examined for gross changes. Visceral examination by dissection was performed on approximately one half of the fetuses in each litter and on all externally abnormal fetuses. The heads of these fetuses were fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal abnormalities and variations.

In the preliminary sensitivity testing phase, 27% of animals tested displayed clinical signs of neurotoxicity, comprising tremors or recumbency. Subsequently, 69% of the sensitive animals died or were killed in extremis, leaving 23 sensitive survivors. The survivors displayed clinical signs for up to 4 days, but were of normal appearance thereafter. Twenty-two animals mated, of which four were allocated to the vehicle control group and 18 allocated to be treated at 0.2–1.0 mg/kg bw per day. One hundred insensitive females were allocated to the study and mated. Treatment-related deaths and clinical signs were confined to the sensitive group treated at 0.2–1.0 mg/kg bw per day. Twelve animals were killed and discarded on days 9–15 of gestation after establishing pregnancy status. A further animal in this group died, and another was killed in extremis on day 17. A vehicle control

insensitive animal died of apparent dose maladministration. Clinical signs were apparent when the dose level administered was increased to 1.0 mg/kg bw per day. Recumbency or decreased activity occurred in all animals after one or two doses at 1.0 mg/kg bw per day.

Four animals survived to day 18, but three were recumbent for at least 2 days before necropsy. Treatment-related effects on maternal body weight gain and feed consumption were confined to the sensitive group treated at 0.2–1.0 mg/kg bw per day. A 74.2% decrease in weight gain occurred from day 6 to day 16, resulting in a group mean body weight of 39.0 g compared with the vehicle control sensitive group value of 53.3 g. Feed consumption was markedly reduced from day 11 of gestation. Decreases of 42.4% and 83.0% occurred on days 11 and 17 of gestation, respectively, compared with the vehicle control sensitive group. The overall group mean feed consumption values of the treated insensitive groups were 6.40, 6.36 and 6.50 g/day compared with the vehicle control insensitive group value of 6.54 g/day. There were no treatment-related gross changes in maternal animals at any dose level. Three of the four sensitive animals treated at 0.2–1.0 mg/kg bw per day produced dead fetuses only, which is considered to be a consequence of prolonged maternal inactivity. In the insensitive groups, there were no treatment-related effects on litter parameters at any dose level, as assessed by preimplantation and postimplantation losses, mean numbers of corpora lutea, implantations and live fetuses, fetal weights and sex ratios. There was a treatment-related increase in the incidence of cleft palate in the insensitive groups at all dose levels. The effect was dose related and affected 4.4%, 7.1% and 19.9% of fetuses, in order of ascending dose level, compared with a control incidence of 2.6%. A high incidence of cleft palate (45.5%) occurred in the single litter from the sensitive animal treated at 0.2–1.0 mg/kg bw per day. The incidence of the variation postaxial pseudopolydactyly was increased in all treated insensitive groups (8.8–14.7%) compared with a control incidence of 5.1%. This is considered not to be treatment related, as the incidences lie within, or very close to, the historical control range of 5.8–13%. The nature, incidence and distribution of other external malformations and variations and all visceral and skeletal malformations and variations in the treated insensitive groups did not suggest an effect of treatment. Minimal to slight diffuse P-glycoprotein staining on the endothelial cell surface of cerebral and cerebellar capillaries occurred in all insensitive female mice. In contrast, no P-glycoprotein expression occurred on the luminal surface of endothelial cells of sensitive female mice.

In view of the above, the developmental toxicity of the 8,9-*Z* isomer of avermectin B_{1a} to a subpopulation of CF-1 mice sensitive to the tremor-inducing properties of abamectin could not be evaluated, as only a single viable litter was produced. However, developmental toxicity, characterized by excess incidences of cleft palate, was produced by the 8,9-*Z* isomer of avermectin B_{1a} in apparently insensitive CF-1 mice at dose levels of 0.5, 1.0 and 1.5 mg/kg bw per day. Therefore, a NOAEL for embryo and fetal toxicity could not be established. The isomer did not produce maternal toxicity at dose levels up to 1.5 mg/kg bw per day.

The study was GLP compliant, and a QA statement was attached (Wise, 1996b).

Exploratory study of oral developmental toxicity of 8,9-Z-B_{1a}

To undertake an exploratory study of the oral developmental toxicity of the 8,9-*Z* photoisomer in CF-1 mice of known P-glycoprotein genotype, female mice of known genotype for the *mdr1* gene encoding the P-glycoproteins and mated to male mice of known genotype were used. Prior to initiation of the study, male and female mice (strain Crl:CF-1 (BR)) were submitted for restriction fragment length polymorphism Southern blot analysis to determine the genotype for the *mdr1* gene. The females (11 weeks old, body weight range 22.8–30.7 g) were mated with sexually mature males to provide groups of 12 mated females producing fetuses of genotypes predictable by the principles of Mendelian inheritance.

The five groups of 12 mated females were treated orally, by gavage, with the 8,9-*Z* photoisomer of avermectin B_{1a} (batch no. L-652,280-000N009, purity 94.3%) in sesame oil at a dose level of 0 (vehicle only) or 1.5 mg/kg bw per day from day 6 to day 15 of gestation (day 0 of gestation = day on which vaginal plug was observed). Clinical signs were recorded daily on day 0 of gestation and twice daily from day 6 to day 18 of gestation. Body weights were recorded on days 0, 6,

8, 10, 12, 14, 16 and 18 of gestation. Surviving animals were killed on day 18 of gestation, and the uterus was examined to determine pregnancy status. A distal tail segment was retained from each maternal animal for possible reanalysis of P-glycoprotein genotype. Implantations were counted and classified as resorptions, live fetuses or dead fetuses. All fetuses were weighed and examined for external malformations, including the palate. One hindlimb of each fetus was removed for possible analysis of P-glycoprotein genotype. At least four litters per group were genotyped using DNA isolated from the hindlimb. Placentas from one vehicle control (−/−), one 1.5 mg/kg bw per day female (+/+) and two vehicle controls (+/−) were retained for western blotting biochemical analysis of P-glycoprotein. The heads and placentas of fetuses from four vehicle control (+/−) females and four females treated at 1.5 mg/kg bw per day (+/− × −/−) were frozen in isopentane/liquid nitrogen for P-glycoprotein immunohistochemistry of brain endothelium, palate epithelium, placental trophoblasts and placental yolk sac endothelium. The frozen sections were analysed immunohistochemically for P-glycoprotein using an adaptation of an external reference (Schinkel et al., 1994). Slides were examined microscopically using a blind procedure and scored for relative staining intensity for P-glycoprotein compared with control sections. The brain of one female treated at 1.5 mg/kg bw per day (+/+) was retained for possible immunohistochemical analysis.

No deaths or treatment-related clinical signs or body weight effects occurred during the study. The weight gain from day 6 to day 18 of the vehicle control group (+/− × +/−) was 12.4–20.8% higher than in all other study groups, but all 8,9-*Z* isomer–treated groups showed weight gains comparable with those of the second vehicle control (−/− × −/−) group. There were no treatment-related effects on embryo survival or litter parameters in any treated group, as assessed by mean number of implantation sites, postimplantation loss, mean number of live fetuses per litter, fetal weight and sex ratio. There were increased incidences of cleft palate in two of the three groups treated at 1.5 mg/kg bw per day. The +/− female × +/+ male group produced 18 fetuses with cleft palate (litter mean incidence 12.0%), and the +/− female × −/− male group produced 80 fetuses with cleft palate (litter mean incidence 58.0%), compared with litter mean incidences of 0.83% and 0% in the vehicle control groups (+/− × +/− and −/− × −/−, respectively). The incidence of cleft palate was 0% in the homozygous positive group (+/+ × +/+) treated at 1.5 mg/kg bw per day. The incidence of postaxial pseudopolydactyly was higher in the +/− × −/− group (litter mean incidence 19.0%) compared with incidences in the vehicle control groups of 2.8% and 8.3%. The difference is considered incidental to treatment, as the high litter mean incidence was due primarily to a single affected fetus. All other observed abnormalities occurred in isolation and are considered unrelated to treatment with 8,9-*Z*-B_{1a}.

Genotyping of fetuses from at least four litters per group confirmed the hypothesis that the markers correlating with sensitivity to abamectin follow normal Mendelian inheritance. When homozygous positive males were mated with homozygous positive females, all progeny were homozygous positive (+/+). Similarly, when homozygous negative males were mated with homozygous negative females, all progeny were homozygous negative (−/−). When heterozygous males and females were mated, the litters contained progeny with all predicted genotypes, +/+, +/− and −/−, in the expected ratio of approximately 1:2:1, respectively.

None of the +/+ genotype fetuses derived from treated females in either of the groups in which the genotype occurred had cleft palate, whereas 30/31 (97%) −/− genotype fetuses exposed to 8,9-*Z*-B_{1a} had cleft palate, and 29/70 (41%) of the +/− genotype fetuses were affected.

Histopathological evaluation of fetal brain revealed diffuse P-glycoprotein staining on the endothelial cell surface of cerebral and cerebellar capillaries in most of the heterozygous (+/−) and homozygous positive (+/+) fetuses. In addition, minimal to slight P-glycoprotein staining occurred on the surface of trophoblasts in the placental labyrinth and on the apical surface of epithelial cells of the yolk sac in many of the fetuses with these genotypes. In contrast, no P-glycoprotein expression occurred on the luminal surface of endothelial cells, trophoblasts in the placental labyrinth or yolk sac epithelial cells in most of the homozygous (−/−) fetuses. No P-glycoprotein staining occurred in the epithelial lining of the oral cavity in the palate region of any fetuses in the study. Western blotting analysis of placentas for *mdr1a* P-glycoprotein confirmed the results of the immunohistochemical analysis; the amount of P-glycoprotein in the placenta varies with fetal genotype. Thus, the highest

amounts were detected in the +/+ placentas, with lesser amounts in the +/− placentas, and P-glycoprotein was absent in −/− placentas.

In view of the above, the dose level of 1.5 mg/kg bw per day of the 8,9-*Z* isomer of avermectin B_{1a} was identified as the NOAEL for maternal toxicity and reproductive indices, but elicited embryotoxicity, manifested as increased incidences of cleft palate in sensitive fetuses. Fetal sensitivity to the induction of cleft palate is influenced by genotype for the *mdr1* gene encoding for the P-glycoprotein, a gene governed by Mendelian inheritance. Fetal genotype for the *mdr1* gene influences the extent of expression of P-glycoprotein in the brain, placental trophoblasts and yolk sac epithelium. The homozygous positive, heterozygous and homozygous negative genotypes express decreasing amounts of P-glycoprotein, and there is an inverse relationship with the incidence of cleft palate.

The study was GLP compliant, and a QA statement was attached (Lankas, 1996a,b).

Developmental toxicity of 8,9-Z-B_{1a} in rats

Four groups of 25 naturally mated female rats (Crl:CD(SD) BR strain, approximately 2.5 months old, 201–269 g) were treated orally, by gavage, with the 8,9-*Z* isomer of avermectin B_{1a} (batch no. L-652,280-000N005, purity 91.6%) in sesame oil at a dose level of 0 (vehicle only), 0.25, 0.5 or 1.0 mg/kg bw per day from day 6 to day 17 of gestation (day 0 of gestation = day on which plug or sperm in vaginal smear were observed). The animals were observed for clinical signs daily from day 6 to day 20 of gestation and also 1–5 hours after dosing during the treatment period. Body weights were recorded on days 0, 6, 8, 10, 12, 14, 16, 18 and 20 of gestation. The feed consumption of all animals was measured at 3-day intervals from day 3 to day 20 of gestation. The animals were killed on day 20 and subjected to a gross postmortem examination. Maternal gross lesions were preserved and subsequently examined microscopically. The pregnancy status was established, and the number of corpora lutea was determined. The uterine horns were examined, the number of implantation sites was enumerated and the implantations were classified as live fetuses, dead fetuses or resorptions. The fetuses were removed from the uterine horns and individually weighed, sexed and examined for external malformations. Visceral examination by dissection was performed on every third fetus of each litter and on all externally abnormal fetuses. The head of every third fetus was fixed in Bouin's fluid for subsequent examination by free-hand sectioning. All fetuses were fixed, cleared, stained with alizarin red and examined for skeletal malformations and variations. Litter data (preimplantation loss, implantation number, postimplantation loss, litter size and fetal weight) and maternal body weight data were analysed statistically.

There were no deaths and no treatment-related clinical signs during the study. Females treated at 0.5 and 1.0 mg/kg bw per day showed a treatment-related enhanced body weight gain during the treatment period. The mean weight gains of these groups were 8.9% higher than in the control group and statistically significant ($P < 0.05$). There was no effect on weight gain in the group treated at 0.25 mg/kg bw per day. Feed consumption was unaffected by treatment at all dose levels. There were no treatment-related gross changes at necropsy in maternal animals, and pregnancy incidences were comparable in all groups. There were no treatment-related effects at any dose level on preimplantation and postimplantation losses, live litter size, sex ratio or fetal weights. Preimplantation loss in the group treated at 0.25 mg/kg bw per day (13.4%) was significantly higher ($P < 0.05$) than the control value (7.9%), but because there was no dose–response relationship and because implantation is essentially complete at the commencement of dosing, it is considered to be incidental to treatment. There was no evidence of developmental toxicity, either embryonic/fetal growth retardation or teratogenicity, at any dose level, based on the incidences of external, visceral and skeletal malformations, variations and unossified centres. The overall incidences of malformed fetuses in the treated groups were 1.81%, 3.21% and 2.56%, in order of ascending dose level, compared with a control incidence of 1.69%. The fetal malformations observed in treated groups, clubbed forefoot, hindlimb rotation, situs inversus, missing vertebrae and hypoplastic rib, either were single occurrences or occurred at an incidence comparable with that of the control group. The incidences of fetal variations and sites of incomplete ossification were comparable with those of the control group.

In view of the above, the NOAEL for maternal effects and embryo/fetal toxicity, including teratogenicity, was identified as 1.0 mg/kg bw per day, the highest dose tested, for the 8,9-*Z* isomer of avermectin B_{1a}, based on an absence of adverse effects at all doses.

The study was GLP compliant, and a QA statement was attached (Gordon, 1988a).

3. Observations in humans

Manufacturing employees are medically examined by company physicians at the beginning of their employment and then routinely once a year. Abamectin was manufactured at the Syngenta (formerly Novartis) formulation site at Monthey, Switzerland, from 1998 to 2000 and at the Syngenta (formerly Novartis) formulation site at Rezende, Brazil, from 1999 to 2000, with annual production volumes (formulation) in the range of 90–900 tonnes. Questionnaires have also been completed by the responsible heads of formulation sites and company physicians. No adverse health effects have been reported that could be related to abamectin (Syngenta, 2015).

Human intoxication with abamectin is uncommon. However, there are a number of published reports regarding acute poisoning due to abamectin, mainly ingested for suicidal purposes. Data on humans related to poisoning are consistent with studies on experimental animals and do not demonstrate high acute toxicity. Humans show a low susceptibility towards the toxicity of abamectin. However, depending on the dose consumed, there are variable signs and symptoms, basically due to effects on the nervous system. These include altered mental status, drowsiness, hypotension, tachycardia and dermal erythema (Aminiahidashti, Jamali & Gorji, 2014); mild poisonings led mainly to mild gastrointestinal symptoms (nausea, vomiting and diarrhoea) or short-lasting central nervous system depression (dizziness, drowsiness and weakness) (Chung, 1999); tremor and vomiting (Soyuncu et al., 2007); nystagmus, which may be affected due to the vestibular cerebellar system (Wang et al., 2013); neurological toxicity and respiratory failure (Bansod et al., 2013); and acute onset of confusion, blurred vision and unsteady gait, restlessness and agitation, and partial ptosis of both eyes (Karunatilake et al., 2012). The therapy for abamectin poisoning is mainly symptomatic and supportive. Despite the lack of specific therapy, the follow-up of severely poisoned patients showed an uneventful recovery. Therefore, the prognosis is good, unless complications occur from severe uncontrolled aspiration.

No epidemiological studies on abamectin were identified in the literature.

Comments

Susceptibility of CF-1 mice and neonatal rats to abamectin

In mammals, abamectin is a substrate for P-glycoprotein, which is a member of the adenosine triphosphate (ATP)–binding cassette subfamily B (ABCB1), also known as multidrug resistance protein 1 (MDR1). P-glycoprotein is extensively expressed in the intestinal epithelium, liver cells, cells of the proximal tubule of the kidney and capillary endothelial cells of the brain (blood–brain barrier), placenta, ovaries and testes. As an efflux transporter, P-glycoprotein acts as a protective barrier to keep xenobiotics out of the body by excreting them into bile, urine and intestinal lumen and prevents the accumulation of these compounds in the brain and gonads, as well as the fetus. Therefore, some test animals (e.g. CF-1 mice) in which genetic polymorphisms compromise P-glycoprotein expression are particularly susceptible to abamectin-induced toxicity. As a result, studies on CF-1 mice were not considered relevant for the human risk assessment.

In rats, P-glycoprotein was undetectable in the embryo and early stages of postnatal development. In the brush border of intestinal epithelial cells, expression of P-glycoprotein was absent to minimal on postnatal day 8 and was more intense but did not reach adult levels by postnatal day 20 (Cukierski, 1995; Lankas, 1996a). In the brain capillaries, expression of P-glycoprotein was minimal in fetuses on gestation day 20 and in younger pups until postnatal day 11, with subsequent increases to a plateau at adult levels by postnatal days 20–28 (Cukierski, 1995; Lankas, 1996a; Matsuoka et al.,

1999). In humans, P-glycoprotein expression in fetal intestinal epithelium and brain capillaries is at the adult level at week 28 of gestation (van Kalken et al., 1992), and therefore results of reproductive toxicity studies in neonatal rats should be interpreted accordingly.

Biochemical aspects

In rats, orally administered radiolabelled avermectin B_{1a} was rapidly and almost completely absorbed, based on a comparison with urinary excretion after intravenous administration. Maximum concentrations in blood were achieved within 4–8 hours after administration. Radiolabel was widely distributed. Elimination of radiolabel occurred predominantly by non-biliary excretion into the gastrointestinal tract and excretion in the faeces, whereas urinary excretion accounted for only 0.5–1.4% of the dose. Elimination accounted for 80–101% of the administered dose within 96 hours. Rate of oral absorption, tissue distribution and excretion were independent of the dose level, treatment regimen and/or sex; however, the depletion of tissue residues was approximately 2-fold more rapid in males than in females. There was no evidence for tissue accumulation on repeated administration (Alvaro et al., 1984; Hassler, 2001, 2003; Briswalter, 2003a).

In toxicokinetic studies in genotyped subpopulations of CF-1 mice (i.e. *mdr1a* P-glycoprotein–deficient mice and mice with normal expression of *mdr1a* P-glycoprotein), plasma levels of avermectin B_{1a} were higher in *mdr1a* P-glycoprotein–deficient mice than in mice with normal expression of *mdr1a* P-glycoprotein. Distribution and elimination appeared to be slower in *mdr1a* P-glycoprotein–deficient mice, with concentrations in the brain tissue of *mdr1a* P-glycoprotein–deficient mice up to 160 times higher than in mice with normal expression of *mdr1a* P-glycoprotein. The primary route of excretion was via faeces (Gledhill, 2008).

Metabolism of avermectin B_{1a} in the rat was moderate to extensive and proceeded predominantly via demethylation, hydroxylation, cleavage of the oleandrosyl ring and oxidation reactions. The metabolite pattern in urine, faeces and bile was complex but qualitatively independent of the sex and dose, with some quantitative variations. Eleven metabolites were isolated. Unchanged avermectin B_{1a} and the metabolites 3″-*O*-desmethyl-avermectin B_{1a} (3″DM), 24a-hydroxy-avermectin B_{1a} (24aOH), 27-hydroxy-avermectin B_{1a} (27OH), 3″-*O*-desmethyl, 24a-hydroxy-avermectin B_{1a} (3″DM,24aOH) and 3″-*O*-desmethyl, 27-hydroxy-avermectin B_{1a} (3″DM,27OH) represented the majority of the faecal radioactivity (Alvaro et al., 1984; Hassler, 2003).

Toxicological data

Abamectin was of high acute oral toxicity, with an LD_{50} of 8.7 mg/kg bw in rats with sesame oil as the vehicle (Robertson, 1981a) and an LD_{50} of 214 mg/kg bw in rats with water as the vehicle (Glaza, 2001). The acute inhalation toxicity of abamectin was also high, with an LC_{50} of > 0.034 and < 0.21 mg/L in rats (Ruddock, 2001a; Noakes, 2003). The dermal LD_{50} in both rats and rabbits was greater than 2000 mg/kg bw (Gordon, 1984a; Sieber, 2011). Abamectin was not irritating to the eye (Glaza, 2000) or the skin of rabbits (Robertson, 1981b). It was not a dermal sensitizer in guinea-pigs (Ruddock, 2001b).

In short- and long-term toxicity studies performed in rats and dogs, clinical signs observed as a response to treatment were tremors in rats and dogs and mydriasis or absent pupil reflex in dogs. There were no histopathological changes in the tissues of the central and peripheral nervous systems. The clinical signs are considered to be an exaggerated pharmacological response to the interaction of abamectin with the gamma-aminobutyric acid (GABA)–benzodiazepine receptor chloride channel complex. Treatment-related histopathological alterations in dogs were confined to the hepatobiliary system. Based on the severity of clinical signs of neurotoxicity and mydriasis and the doses at which death occurs, the dog was more sensitive than the rat to abamectin.

In an 8-week dietary toxicity study in rats with mean achieved dose levels of 0, 0.5, 1.2, 1.9, 2.2 and 5.0 mg/kg bw per day, deaths occurred at 2.2 and 5.0 mg/kg bw per day. The NOAEL was 1.2 mg/kg bw per day, based on the occurrence of clinical signs and reduced body weight gain at 1.9 mg/kg bw per day and above (Gordon, 1984b). No 90-day toxicity study in rats was provided.

In 12-week, 18-week and 53-week toxicity studies in dogs, a very steep dose–response curve for abamectin was noted. In the 12-week feeding study in dogs with dietary concentrations of abamectin adjusted to provide dose levels of 0, 0.25, 0.5, 1.0 and 4.0/2.0 mg/kg bw per day, the NOAEL was 0.5 mg/kg bw per day, based on the occurrence of mydriasis starting in week 1 of treatment at 1.0 mg/kg bw per day. Markedly reduced feed consumption, body weight loss and clinical signs of intoxication were observed at 2.0 mg/kg bw per day and above (Gordon, 1984c).

In the 18-week gavage study in dogs administered abamectin at a dose of 0, 0.25, 0.5, 2.0 or 8.0 mg/kg bw per day, the NOAEL was 0.25 mg/kg bw per day, based on mortality, clinical signs of toxicity, reduced body weight gain or body weight loss, and histopathological changes in the liver at 0.5 mg/kg bw per day. Signs of toxicity were observed starting on day 7 at 0.5 mg/kg bw per day, on day 3 at 2.0 mg/kg bw per day and on day 1 (within 3 hours after dosing) at 8.0 mg/kg bw per day (Allen & Robertson, 1976; Allen, 1982).

In the 53-week feeding study in dogs with dietary concentrations of abamectin adjusted to provide dose levels of 0, 0.25, 0.5 and 1.0 mg/kg bw per day, the NOAEL was 0.25 mg/kg bw per day, based on mydriasis at 0.5 mg/kg bw per day. Signs of toxicity were observed starting in the first week at 0.5 and 1.0 mg/kg bw per day (Gordon, 1984d).

The overall NOAEL for the three dog studies was 0.25 mg/kg bw per day, based on mortality (one animal), clinical signs of toxicity (including mydriasis) and reduced body weight gain or body weight loss at 0.5 mg/kg bw per day.

In a 93-week toxicity and carcinogenicity study in mice, with dietary concentrations of abamectin adjusted to provide dose levels of 0, 2.0, 4.0 and 8.0 mg/kg bw per day, the NOAEL was 4.0 mg/kg bw per day, based on increased mortality in males and reduced body weight gain in females at 8.0 mg/kg bw per day. There was no increase in tumour incidence (Gordon, 1985a).

In a 104-week chronic toxicity and carcinogenicity study in rats, with dietary concentrations of abamectin adjusted to provide dose levels of 0, 0.75, 1.5 and 2.0/2.5 mg/kg bw per day, the NOAEL was 1.5 mg/kg bw per day, based on the occurrence of clinical signs of toxicity at 2.0/2.5 mg/kg bw per day. There was no increase in tumour incidence (Gordon, 1985b).

The Meeting concluded that abamectin is not carcinogenic in mice or rats.

Abamectin has been tested for genotoxicity in an adequate range of assays, both in vitro (Gordon, 1983, 1986a,b; Deparade, 2001) and in vivo (Blazak, 1983). There was no evidence of genotoxicity.

The Meeting concluded that abamectin 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 abamectin is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats, abamectin (in sesame oil) was administered orally by gavage at a dose level of 0, 0.05, 0.12 or 0.40 mg/kg bw per day. The NOAEL for parental toxicity and reproductive toxicity was 0.40 mg/kg bw per day, the highest dose tested. For offspring toxicity, the NOAEL was 0.12 mg/kg bw per day, based on increased pup mortality and retarded weight gain in both F_1 and F_2 generation progeny and an increased incidence of transient retinal folds in the eyes of F_2 generation weanlings at 0.40 mg/kg bw per day (Gordon, 1984e).

In a developmental toxicity study in rats, abamectin (in sesame oil) was administered orally by gavage at a dose level of 0, 0.4, 0.8 or 1.6 mg/kg bw per day. The NOAEL for maternal toxicity and embryo/fetal toxicity was 1.6 mg/kg bw per day, the highest dose tested (Gordon, 1982a).

In a developmental toxicity study in rabbits, abamectin (in sesame oil) was administered orally by gavage at a dose level of 0, 0.5, 1.0 or 2.0 mg/kg bw per day. The NOAEL for maternal toxicity was 1.0 mg/kg bw per day, based on the occurrence of severe maternal toxicity (body weight loss) during gestation at 2.0 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 1.0 mg/kg bw per day, based on increased incidences of external malformations (cleft palate,

omphalocele, clubbed forefeet) and incomplete ossification of sternebrae and metacarpals at 2.0 mg/kg bw per day (Gordon, 1982b).

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

In an acute neurotoxicity study in rats, abamectin (in sesame oil) was administered orally by gavage at a dose level of 0, 0.5, 1.5 or 6 mg/kg bw. The NOAEL for acute neurotoxicity was 0.5 mg/kg bw, based on a reduced splay reflex at 1.5 mg/kg bw. This change was seen at 6–7 hours after dosing on day 1 and was consistent with the neurotoxic effects observed at higher doses. No neuropathological changes were observed at dose levels up to 6 mg/kg bw (Brammer, 2006a).

In a 90-day neurotoxicity study in rats, abamectin (in sesame oil) was administered orally by gavage at a dose level of 0, 0.4, 1.6 or 4.0 mg/kg bw per day. The NOAEL for systemic toxicity and neurotoxicity was 1.6 mg/kg bw per day, based on reduced body weights in females, clinical signs in both sexes (irregular breathing, upward curvature of the spine, reduced righting reflex, reduced splay reflex) and changes in hindlimb grip strength in females at 4.0 mg/kg bw per day (Brammer, 2006b).

In a developmental neurotoxicity study in rats, abamectin (in sesame oil) was administered orally by gavage to parental females once daily from day 7 of gestation until day 22 postpartum at a dose level of 0, 0.12, 0.20 or 0.40 mg/kg bw per day. In parental females, body weights and feed consumption were slightly increased at all dose levels. In offspring, lower body weights post-weaning and hence a delay in time of vaginal opening were observed at 0.20 mg/kg bw per day and above. The NOAEL was 0.12 mg/kg bw per day. There was no effect on function or morphology of the nervous system at dose levels up to 0.40 mg/kg bw per day, the highest dose tested (Moxon, 2005).

In a supplementary developmental neurotoxicity study in rats performed to provide brain morphometry data, abamectin (in sesame oil) was administered orally by gavage to parental females once daily from day 7 of gestation until day 22 postpartum at a dose level of 0, 0.12, 0.20 or 0.40 mg/kg bw per day. In parental females, body weights and feed consumption were slightly increased at all dose levels during gestation. In offspring at 0.40 mg/kg bw per day, clinical signs and increased mortality were observed in the pre-weaning period, and therefore all dams and pups in this group were removed from the study on days 15–38 postpartum. At 0.20 mg/kg bw per day, body weights of pups post-weaning were statistically significantly lower and the time of vaginal opening was statistically significantly later compared with the control group. The NOAEL for offspring toxicity was 0.12 mg/kg bw per day. There was no effect on function or morphology of the nervous system at dose levels up to 0.40 mg/kg bw per day, the highest dose tested (Moxon, 2007).

In a gavage study in rhesus monkeys, the most sensitive indicator of abamectin toxicity was emesis, as clinical signs of toxicity seen in mice and rats (tremors and convulsions) did not occur. The minimum toxic dose (LOAEL) of abamectin was 2.0 mg/kg bw, and the NOAEL was 1.0 mg/kg bw (Gordon, 1985c).

The Meeting concluded that abamectin is neurotoxic.

Toxicological data on metabolites and/or degradates

The 8,9-*Z* isomer of avermectin (also referred to as the Δ-8,9-isomer, NOA 427011 and L-652,280) is a photodegraded product of abamectin.

An exploratory acute oral toxicity study with the 8,9-*Z* isomer of avermectin B_{1a} in CD-1 mice and CF-1 mice gave LD_{50} values of 217 and 20 mg/kg bw, respectively (Lynch, 1996).

The 8,9-*Z* isomer of avermectin B_{1a} did not induce gene mutations in bacteria, with or without metabolic activation (Gordon, 1988c).

In a one-generation reproductive toxicity study with the 8,9-*Z* isomer of avermectin B_{1a} in rats using dose levels of 0, 0.06, 0.12 and 0.40 mg/kg bw per day, the NOAEL for maternal toxicity, reproductive effects and effects on offspring was 0.40 mg/kg bw per day, the highest dose tested (Gordon, 1988b).

In a developmental toxicity study with the 8,9-*Z* isomer of avermectin B_{1a} in CD-1 mice using dose levels of 0, 0.75, 1.5 and 3.0 mg/kg bw per day, the NOAEL for maternal effects and embryo/fetal toxicity, including teratogenicity, was 3.0 mg/kg bw per day, the highest dose tested (Wise, 1996a).

In a developmental toxicity study with the 8,9-*Z* isomer of avermectin B_{1a} in rats using dose levels of 0, 0.25, 0.5 and 1.0 mg/kg bw per day, the NOAEL for maternal effects and embryo/fetal toxicity, including teratogenicity, was 1.0 mg/kg bw per day, the highest dose tested (Gordon, 1988a).

The Meeting concluded that the 8,9-*Z* isomer of avermectin B_{1a} is of no greater toxicity than the parent abamectin.

A metabolite, 24-hydroxymethyl abamectin, was found in liver and milk in smaller proportions than the parent. The Meeting concluded that 24-hydroxymethyl abamectin is of no greater toxicity than the parent, because it has been found in amounts of more than 10% in excreta and therefore has been tested in studies with the parent.

Human data

From reports on health records of manufacturing plant personnel, no adverse health effects were noted. A number of reports on intentional poisoning in humans available in the literature showed low susceptibility of humans to abamectin, with variable dose-related neurological signs and symptoms.

Although administration of abamectin to mice, rats and dogs at relatively low dose levels was associated with clinical signs of central nervous system toxicity (including mydriasis, tremors, convulsions, ataxia and bradycardia), it was shown that rhesus monkeys and humans are less sensitive to abamectin, at least following acute exposure (Gordon, 1985c).

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

Toxicological evaluation

The Meeting established a new ADI of 0–0.001 mg/kg bw, based on the NOAEL of 0.12 mg/kg bw per day for lower body weights and delayed time of vaginal opening observed at 0.20 mg/kg bw per day in post-weaning pups in the two developmental neurotoxicity studies in rats, using a safety factor of 100. The Meeting withdrew the existing ADI of 0–0.002 and also concluded that the additional information available to this Meeting regarding the critical period in development and species differences does not justify the application of a reduced safety factor to the NOAEL.

The ADI also applies to the 8,9-*Z* isomer of avermectin B_{1a} and the 24-hydroxymethyl metabolite of abamectin.

The Meeting established an ARfD of 0.003 mg/kg bw, based on the overall NOAEL of 0.25 mg/kg bw per day for clinical signs in dogs (mydriasis) observed in the first week of treatment at 0.5 mg/kg bw per day. This ARfD also applies to the 8,9-*Z* isomer of avermectin B_{1a} and the 24-hydroxymethyl metabolite of abamectin.

Levels relevant to risk assessment of abamectin and the 8,9-Z isomer of avermectin B_{1a}

Species	Study	Effect	NOAEL	LOAEL
Abamectin				
Mouse	Ninety-three-week study of toxicity and carcinogenicity[a]	Toxicity	4.0 mg/kg bw per day	8.0 mg/kg bw per day
		Carcinogenicity	8.0 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	1.5 mg/kg bw per day	2.0 mg/kg bw per day
		Carcinogenicity	2.0 mg/kg bw per day[b]	–
	Two-generation reproductive toxicity study[c]	Reproductive toxicity	0.40 mg/kg bw per day[b]	–
		Parental toxicity	0.40 mg/kg bw per day[b]	–
		Offspring toxicity	0.12 mg/kg bw per day	0.40 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	1.6 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1.6 mg/kg bw per day[b]	–
	Acute neurotoxicity study[a]	Neurotoxicity	0.5 mg/kg bw	1.5 mg/kg bw
	Developmental neurotoxicity studies[a,d]	Developmental neurotoxicity	0.40 mg/kg bw[b]	–
		Toxicity	0.12 mg/kg bw	0.20 mg/kg bw
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1.0 mg/kg bw per day	2.0 mg/kg bw per day
		Embryo and fetal toxicity	1.0 mg/kg bw per day	2.0 mg/kg bw per day
Dog	Twelve-week,[a] 18-week[c] and 53-week[a] studies of toxicity[d]	Toxicity	0.25 mg/kg bw per day	0.5 mg/kg bw per day
8,9-*Z* isomer of avermectin B_{1a}				
CD-1 mice	Developmental toxicity study[c]	Maternal toxicity	3.0 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	3.0 mg/kg bw per day[b]	–
Rat	One-generation study of reproductive toxicity[a]	Reproductive toxicity	0.40 mg/kg bw per day[b]	–
		Parental toxicity	0.40 mg/kg bw per day[b]	–
		Offspring toxicity	0.40 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
Rat	Developmental toxicity study[c]	Maternal toxicity	1.0 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1.0 mg/kg bw per day[b]	–

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

Estimate of acceptable daily intake (ADI)

0–0.001 mg/kg bw

Estimate of acute reference dose (ARfD)

0.003 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 abamectin

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, absorption almost complete, T_{max} 4–8 h
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	> 80% within 96 h, almost exclusively in faeces
Metabolism in animals	Moderate to extensive; by demethylation, hydroxylation, cleavage of the oleandrosyl ring and oxidation reactions
Toxicologically significant compounds in animals and plants	Abamectin, 8,9-*Z* isomer of avermectin B_{1a}, 24-hydroxymethyl abamectin
Acute toxicity	
Rat, LD_{50}, oral	8.7 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 0.034 and < 0.21 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson and Kligman test)
Short-term studies of toxicity	
Target/critical effect	Nervous system/clinical signs, mortality
Lowest relevant oral NOAEL	0.25 mg/kg bw (dog)
Lowest relevant dermal NOAEL	No data

Lowest relevant inhalation NOAEC	0.5 µg/L (30-day rat study)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Increased mortality in males and reduced weight gain (mouse), clinical signs (rat)
Lowest relevant NOAEL	1.5 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	No reproductive effects
Lowest relevant parental NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)
Lowest relevant offspring NOAEL	0.12 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)
Developmental toxicity	
Target/critical effect	External malformations at maternally toxic dose (rabbit)
Lowest relevant maternal NOAEL	1.0 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	1.0 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	0.5 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	1.6 mg/kg bw per day (rat)
Developmental neurotoxicity NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)
Studies on 8,9-*Z* isomer of avermectin B_{1a} (photodegraded product)	
Developmental toxicity	
Target/critical effect	No developmental effects
Lowest relevant maternal NOAEL	3.0 mg/kg bw per day (highest dose tested; CD-1 mouse)
Lowest relevant embryo/fetal NOAEL	3.0 mg/kg bw per day (highest dose tested; CD-1 mouse)
Lowest relevant maternal NOAEL	1.0 mg/kg bw per day (highest dose tested; rat)
Lowest relevant embryo/fetal NOAEL	1.0 mg/kg bw per day (highest dose tested; rat)
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)
Lowest relevant offspring NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)
Lowest relevant reproductive NOAEL	0.40 mg/kg bw per day (highest dose tested; rat)

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

Summary

	Value	Study	Safety factor
ADI	0–0.001 mg/kg bw	Developmental neurotoxicity studies (rat)	100
ARfD	0.003 mg/kg bw	Twelve-, 18- and 53-week toxicity studies (dog)	100

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ACETOCHLOR

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Explanation

Acetochlor is the International Organization for Standardization (ISO)–approved common name for 2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 34256-82-1. It belongs to the group of chloroacetanilide compounds, which are used as herbicides. Acetochlor is a pre-emergence

herbicide used against grasses and broadleaf weeds in corn, soya beans, sorghum and peanuts grown in high organic content. It inhibits protein synthesis in shoot meristems and root tips.

Acetochlor 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.

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, unless otherwise indicated.

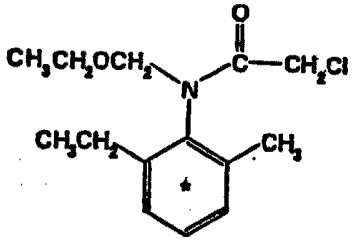
Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion of acetochlor were studied in rats following a single oral low dose, a single oral high dose and a single oral low daily dose repeated for 14 days followed by a radioactive dose.

Fig. 1 shows the structure of [phenyl-U-^{14}C]acetochlor.

Fig. 1. Structure of [phenyl-U-^{14}C]acetochlor



* Denotes position of ^{14}C label.

1.1 Absorption, distribution and excretion

(a) Oral administration

In an absorption, distribution and excretion study, CD Sprague-Dawley rats (five of each sex) were administered [phenyl-U-^{14}C]acetochlor (purity at least 97.6%) in a single oral gavage dose of 10 mg/kg body weight (bw) in polyethylene glycol 400. A further five male and five female rats were dosed at 10 mg/kg bw for the determination of plasma concentrations at various intervals up to 240 hours.

Less than 0.15% of the administered dose was excreted in carbon dioxide in the preliminary study, so carbon dioxide was not collected in the main study. Over 5 days, males excreted 70.6% of the dose in urine and 22.8% of the dose in faeces. Females eliminated 77.0% in urine and 13.2% in faeces (Table 1). Over half of the administered dose was excreted within 24 hours after dosing (59.5% in males and 66.5% in females). The majority of the excretion in faeces occurred in the first 48 hours. The radioactivity retained in tissues and organs accounted for 3.9% of the administered dose on day 5 in males and 3.7% in females. The whole blood sample taken at termination contained 1.8% and 2.0% of the dose for males and females, respectively. The remaining carcass contained about 0.7% of the administered dose. Tissue concentrations after 5 days were highest in whole blood (4.5–6.2 µg acetochlor equivalents/g), but the very low levels in plasma showed that the radioactivity was associated with the cellular fraction of blood. Much lower concentrations – approximately 0.5 µg equivalents/g or lower – were present in spleen, heart, lungs and liver.

Table 1. Recoveries of administered radioactivity over 5 days after administration of ^{14}C-labelled acetochlor in a single oral dose of 10 or 200 mg/kg bw or a single dose of 10 mg/kg bw following 14 daily oral doses of non-radiolabelled acetochlor at 10 mg/kg bw

	% of administered radioactivity excreted over 5 days (mean of five rats)					
	10 mg/kg bw[a]		200 mg/kg bw[b]		10 mg/kg bw repeated for 14 days followed by a radioactive dose[c]	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**
Urine	69.8	75.8	51.1	63.8	64.3	73.7
Faeces	22.8	13.2	37.2	26.9	26.1	16.5
Cage wash	0.8	1.2	0.5	0.9	0.7	0.9
Tissues/organs	3.9	3.7	4.1	3.5	3.2	3.5
Total recovered	97.3	94.0	97.3	95.1	94.3	94.6

bw: body weight
[a] Data extracted from Hawkins, Kirkpatrick & Dean (1988)
[b] Data extracted from Hawkins, Kirkpatrick & Dean (1989a).
[c] Data extracted from Hawkins, Kirkpatrick & Dean (1989b).

The mean plasma concentrations reached peaks at 7 hours after dosing in both male (1.27 ± 0.32 µg/g) and female (1.62 ± 0.09 µg/g) rats. The plasma concentrations were consistently higher in females than in males. Mean concentrations appeared to decline biphasically (Hawkins, Kirkpatrick & Dean, 1988).

In an absorption, distribution and excretion study, CD Sprague-Dawley rats (five of each sex) were administered [phenyl-U-^{14}C]acetochlor (purity > 98.9%) in a single oral gavage dose of 200 mg/kg bw in polyethylene glycol 400. A further five male and five female rats were dosed at 200 mg/kg bw for the determination of plasma concentrations at various intervals up to 360 hours.

Over 5 days, males excreted 51.6% of the dose in urine and 37.2% of the dose in faeces. Females eliminated 64.7% in urine and 26.9% in faeces. Over half of the administered dose was excreted within 24 hours after dosing (43.1% in males and 51.7% in females) (Table 1). The majority of the excretion in faeces occurred in the first 48 hours. The radioactivity retained in tissues and organs accounted for 4.1% of the administered dose at day 5 in males and 3.5% in females. The whole blood sample taken at sacrifice contained 2.1% and 1.9% of the dose for males and females, respectively. The remaining carcass contained a mean of 0.9% of the administered dose for males and 0.7% for females. Tissue concentrations after 5 days were highest in whole blood (112 and 105 µg acetochlor equivalents/g for males and females, respectively). The mean ratios of whole blood to plasma concentrations were 140 and 110 for males and females, respectively. The next higher concentrations were found in heart, lungs, kidneys and liver (about 7.6–23 µg/g).

The mean plasma concentrations reached peaks at 12 hours after dosing in both male (25.0 ± 4.6 µg/g) and female (41.9 ± 9.6 µg/g) rats. The plasma concentrations were consistently higher in females than in males. Mean concentrations appeared to decline biphasically (Hawkins, Kirkpatrick & Dean, 1989a).

In a repeated-dose absorption, distribution and excretion study, [phenyl-U-^{14}C]acetochlor (purity > 98%) was administered in a single oral gavage dose of 10 mg/kg bw in polyethylene glycol 400 to CD Sprague-Dawley rats (five of each sex) that had previously been administered non-radiolabelled acetochlor (purity not reported) in 14 daily oral doses of 10 mg/kg bw.

Over 5 days, males excreted 65.0% of the dose in urine and 26.1% of the dose in faeces. Females eliminated 74.6% in urine and 16.5% in faeces (Table 1). Over half of the administered dose was excreted within 24 hours after dosing (57.2% in males and 67.1% in females). The majority of the excretion in faeces occurred in the first 48 hours. The radioactivity retained in tissues and organs accounted for 3.2% of the administered dose at day 5 in males and 3.5% in females. The whole blood sample taken at sacrifice contained 1.5% and 1.9% of the dose for males and females, respectively. The remaining carcass contained about 0.6% of the dose for males and females. Tissue concentrations after 5 days were highest in whole blood (4.41 and 4.72 µg acetochlor equivalents/g for males and females, respectively). The mean ratios of whole blood to plasma concentrations were 100 and 98 for males and females, respectively. The next highest concentrations were found in heart, spleen, lungs, kidneys and liver (about 0.38–1.1 µg/g).

In this repeated-dose study, acetochlor was well absorbed and rapidly excreted, with no pronounced sex differences. The toxicokinetics of acetochlor were not modified by a repeated dose and were almost the same as after either a high or a low single dose (Hawkins, Kirkpatrick & Dean, 1989b).

A study was designed to determine the overall tissue distribution and localization, especially in the nasal turbinate tissue, of ^{14}C-labelled acetochlor residues following oral administration to rats and mice. The nasal turbinate is a target tissue in rats. The details of this study are provided in section 2.6 on the mode of action of acetochlor.

Acetochlor and/or its metabolites were found to accumulate specifically in the olfactory region of the nasal turbinates in the rat and remained detectable for at least 7 days. No specific accumulation of acetochlor and/or its metabolites was observed in the olfactory region of the nose in mice (Kraus & Wilson, 1996).

A study was conducted to characterize the absorption and routes and rates of excretion and to determine the bioaccumulation of ^{12}C, ^{13}C and ^{14}C isotopically labelled (homogeneous mixture) acetochlor administered by oral intubation to young adult male and female Sprague-Dawley rats. The study design is shown in Table 2.

Only 0.04% of the administered dose was recovered in exhaled air in group A animals over 7 days; therefore, the radioactivity in exhaled air in groups B, C and D was not measured. Excretion of radioactivity in urine and faeces in treated groups is shown in Table 3. The cumulative rate of excretion (urine and faeces combined) in male and female rats in treated groups is shown in Table 4.

Table 2. Study design and sampling

Group	No. of rats	Dose (mg/kg bw)	Dosing regimen	Excretion period (days)	Samples for radioactivity determination
A	3 M, 3 F	400	Single dose	7	Expired air only (preliminary test)
B	5 M, 5 F	10	Single dose	7	Urine, faeces, blood, tissues and quantification of metabolites[a]
C	5 M, 5 F	400	Single dose	7	Urine, faeces, blood, tissues and quantification of metabolites[a]
D	5 M, 5 F	10	14 daily doses of cold material, then single dose of radioactive material	7	Urine, faeces, blood, tissues and quantification of metabolites[a]

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

[a] Results of metabolite studies described in section 1.2 below.

Source: Carr et al. (1983)

Table 3. Excretion of radioactivity in various groups of acetochlor-treated rats[a]

	% of administered radioactivity excreted over 7 days					
	Group B		Group C		Group D	
	Males	Females	Males	Females	Males	Females
Urine	31.5	43.4	46.7	49.7	59.4	67.8
Faeces	50.1	40.4	28.9	26.6	25.6	19.3
Tissues/organs	3.2	3.0	3.5	3.7	2.6	2.8
Residual carcass	1.25	1.05	1.66	1.25	0.725	0.69
Cage wash	0.5	0.5	0.8	0.7	0.3	0.3
Minimum absorbed	36.5	48.0	52.7	55.4	63.0	71.6
Total excreted	81.6	83.7	75.6	76.3	85.1	87.1
Total recovered	86.6	88.2	81.6	82.0	88.7	90.9

[a] Average five rats of each sex.
Source: Carr et al. (1983)

Table 4. Cumulative rate of excretion of radioactivity (urine and faeces combined) in various groups of acetochlor-treated rats in 7 days[a]

	% of administered radioactivity excreted over 7 days					
	Group B		Group C		Group D	
Day	Males	Females	Males	Females	Males	Females
0.5	16.7	22.8	7.2	6.0	30.3	43.4
1	46.5	45.3	24.1	17.8	39.6	34.7
2	13.4	12.7	37.2	47.5	10.9	6.9
3	3.4	1.4	4.2	2.4	2.4	1.0
4	0.6	0.7	1.4	1.2	0.9	0.4
5	0.4	0.4	0.7	0.6	0.5	0.3
6	0.3	0.2	0.5	0.4	0.3	0.2
7	0.3	0.2	0.3	0.4	0.2	0.2
Total	81.6	83.7	75.6	76.3	85.1	87.1

[a] Average five rats of each sex.
Source: Carr et al. (1983).

As shown in Table 4, the combined excretion in faeces and urine of group B was 81.6% and 83.7% of the dose for males and females, respectively. The mean percentages of the administered dose retained in tissues were 3.24% and 3.04% for males and females, respectively (Table 3). The combined excretion in faeces and urine of group C was 75.6% and 76.3% of the dose for males and females, respectively (Table 4). The mean percentages of the administered dose retained in tissues were 3.50% and 3.69% for males and females, respectively (Table 3). The combined excretion in faeces and urine of group D was 85.1% and 87.1% of the dose for males and females, respectively (Table 4). The mean percentages of the administered dose retained in tissues was 2.58% and 2.79% for males and females, respectively (Table 3). Almost all excretion of the radiolabel occurred within the first 48 hours (Table 3).

Table 5. Elimination half-lives for males and females of groups B, C and D[a]

	Elimination half-life (h)			
	α phase		β phase	
Group	**Males**	**Females**	**Males**	**Females**
B	7.13	5.77	162	182
C	10.4	9.3	249	286
D	7.1	5.4	129	186

[a] Average five rats of each sex.
Source: Carr et al. (1983).

The urinary and faecal excretion combined showed biphasic elimination, where α is the rapid whole-body elimination phase and β is the slow elimination phase (Table 5).

Analysis of blood revealed higher levels of radioactivity in whole blood than in plasma. Analysis of the red cell fraction of the blood showed that the retained radioactivity was bound to haemoglobin molecules. The mean percentage of the dose retained in whole blood in all test groups ranged from 1.95% to 2.95% (Carr et al., 1983).

Twelve male and six female Sprague-Dawley rats were administered a single oral dose of ^{14}C-phenyl-labelled acetochlor at 10 or 200 mg/kg bw in polyethylene glycol 600. The bile ducts of four of the male rats (two each at 10 and 200 mg/kg bw) were cannulated 24 hours prior to dosing. The dosing regimen and samples collected are shown in Table 6.

Excretion of the 200 mg/kg bw dose of ^{14}C-labelled acetochlor in this study is shown in Table 7. These results show that the majority of the dose was excreted within 24 hours, with no marked sex differences apparent. However, the data indicate that a greater proportion of the dose was excreted via faeces compared with urine, which contrasts with excretion data for similarly dosed, but individually housed, rats in previous studies (Hawkins, Kirkpatrick & Dean, 1989a,b,c). This may be due to contamination of faeces with urine.

Male bile duct–cannulated rats given 10 mg/kg bw excreted on average 85.1% of the dose in bile, 8.0% in urine and 4.2% in faeces over 48 hours. At 200 mg/kg bw, an average 80.2% of the dose was eliminated via bile, 8.5% in urine and 3.9% in faeces (Table 8). Approximately 84% of the administered dose was excreted in bile within 24 hours, indicating that the biliary route of elimination was very important (Jones, 1990).

Table 6. The dosing regimen and sampling scheme

Dose (mg/kg bw)	**No. of rats**	**Sex**	**Sampling period (h)**	**Samples**
200	8	M	72	Urine, faeces, cage wash
200	6	F	72	Urine, faeces, cage wash
10	2	M	48	Bile, urine, faeces, cage wash
200	2	M	48	Bile, urine, faeces, cage wash

bw: body weight; F: female; M: male
Source: Jones (1990)

Table 7. Excretion profiles for rats given a single oral dose of labelled acetochlor at 200 mg/kg bw

Sample	Sampling interval (h)	Males	Females
Urine	0–24	20.1	27.25
	24–48	5.28	6.46
	48–72	2.56	1.92
	0–72	27.85	35.63
Faeces	0–24	35.3	24.15
	24–48	13.15	15.87
	48–72	2.18	2.64
	0–72	50.63	42.66
Cage wash	0–72	0.5	6.62
Total recovery		78.98	84.91

bw: body weight
Source: Jones (1990)

Table 8. Mean recoveries (n = 2) of administered radioactivity in male bile duct–cannulated rats over 48 hours after a single oral dose of ^{14}C-labelled acetochlor at 10 or 200 mg/kg bw

		% of the dose	
Fraction	**Sampling interval (h)**	**10 mg/kg bw**	**200 mg/kg bw**
Urine	0–48	8.0	8.5
Bile	0–48	85.1	80.2
Faeces	0–48	4.2	3.9
Total	0–48	97.4	92.5
Bile	0–4	24.4	27.8
	4–8	29.3	18.0
	8–12	16.0	14.2
	12–24	14.3	18.1
	24–36	1.06	1.94
	36–48	0.08	0.12

bw: body weight
Source: Jones (1990)

(b) Other routes of administration

The dermal penetration of acetochlor from a topically applied formulated product containing acetochlor (Harness herbicide formulation) was investigated in rhesus monkeys. Studies were first conducted to determine the extent of urinary and faecal elimination of radioactivity following administration of ^{14}C-labelled acetochlor via three parenteral routes: intravenous, intramuscular and subcutaneous. The results of the parenteral studies were then used to correct for incomplete urinary and faecal excretion in the topical study to determine an estimate of the extent of dermal penetration of acetochlor from Harness herbicide formulation.

In this study, four male rhesus monkeys received 1 mg of radioactive acetochlor formulation in propylene glycol as the vehicle via the intravenous, intramuscular and subcutaneous routes. Urine

and faeces were collected at selected time points and analysed for radioactivity. In a percutaneous absorption study, six male rhesus monkeys each received radioactive acetochlor formulation (Harness; emulsifiable concentrate formulation) on their abdomen. Acetochlor (1119 mg) was applied to 12 cm^2 of abdominal area from which hair had been lightly clipped. After 24 hours, the application site was washed twice with soap and water. Urinary and faecal samples were collected at selected time points for 7 days.

The percentage of radioactivity excreted in faeces in 7 days was negligible. The percentages of radioactivity excreted in the urine in 7 days were 87.7%, 96.7% and 83.2% following administration by the intravenous, intramuscular and subcutaneous routes, respectively. The radioactivity in the urine in 7 days following dermal application was 9.2%, corrected for incomplete excretion via the intramuscular route. Peak time periods for the excretion of radioactivity were 0–4 hours, 0–4 hours, 4–24 hours and 36–96 hours for the intravenous, intramuscular, subcutaneous and dermal applications, respectively. For the dermal route, 7 days was not sufficient to reach background radioactivity levels. Pharmacokinetic analysis of the semi-log plot of the percentage of dose excreted in urine versus time revealed biphasic elimination (α phase and β phase) of radioactivity in urine after parenteral administration and a one-phase elimination of radioactivity in urine after dermal application. The absorption half-lives were 4.4 and 25.4 hours following subcutaneous and dermal administration, respectively. The elimination half-life for the dermal route was 92.5 hours.

In summary, the dermal absorption of acetochlor was low (9.2%) based on urinary excretion, and 7 days was not sufficient time to reach background levels of radioactivity (Maibach, 1983).

A study was conducted to determine the route and rate of elimination of acetochlor following intravenous administration to rhesus monkeys at two different doses and to characterize acetochlor metabolites in monkey urine (see section 1.2 below). A specific purpose of the study was to provide analytical information that could be used in the calculation of a systemic body burden from acetochlor residues in urine from European applicator exposure monitoring studies.

Two groups of male rhesus monkeys were administered ^{14}C-phenyl-labelled acetochlor intravenously, using propylene glycol/ethanol/saline (60:10:30) as the dose vehicle, at a dose level of 0.005 or 0.05 mg/kg bw. Three monkeys were treated at each dose level, and urine and faeces were collected at various times up to 5 days. The metabolism cages were rinsed with a minimal amount of water after each collection of urine and faeces. The first cage wash was added to the urine specimen. The second cage wash was retained separately as the cage rinse. The final cage rinse was performed with 50:50 (volume per volume) methanol/water.

Total recoveries of the administered dose ranged from 77.3% to 88.6%. Elimination of the dose was rapid for both groups, with 75.9–87.8% of the dose excreted within 48 hours. Excretion in the urine, which includes cage washes, was the major route of elimination and accounted for 76.9–84.2% of the dose for the group treated at 0.005 mg/kg bw, whereas elimination in the faeces accounted for 4.4–7.6% of the dose. For the group treated with 0.05 mg/kg bw, the amount excreted in the urine ranged from 72.8% to 76.1%, whereas the amount eliminated in the faeces ranged from 3.7% to 5.2%. The data also show that the majority of the radioactivity excreted in urine was excreted within the first 6 hours and that the total amount excreted in urine (> 98%) was excreted within the first 48 hours for all animals in each group. Based on the similarity between the two dose groups, an average of 77.2% of the administered dose was excreted in the urine, and the study author suggested that a correction factor of 77.2% should be used for European applicator exposure monitoring studies for acetochlor (Kurtzweil, 2014).

1.2 Biotransformation

Metabolites are identified in this monograph by European Union (EU) reference numbers included in parentheses after the metabolite names.

(a) Oral administration

In a study in which acetochlor was administered by oral intubation to young adult male and female Sprague-Dawley rats, described in section 1.1 above (see also Table 2 in that section), the high-performance liquid chromatographic (HPLC) profiles of radioactivity in pooled urine samples were comparable between sexes and test groups. The urinary metabolites that could be isolated and identified are presented in Table 9, together with their average percentage of dose for each sex.

The isolation and identification of metabolites in faeces were difficult because of the complexity of the faecal metabolite mixture. No individual metabolite was found to be predominant, and the individual metabolites identified were each less than 1% of the dose. The distribution of radioactivity over the different faecal fractions isolated is given in Table 10.

No metabolites could be characterized that were bound to the solid faecal residues. HPLC analysis of the *n*-hexane-soluble fraction revealed the metabolites shown in Table 11.

Table 9. Metabolites identified in urine of rats treated with acetochlor (10 and 400 mg/kg bw)[a]

Metabolite (trivial name[b])	% of dose		HPLC fraction (metabolite no.)	EU reference no.
	Males	Females		
sec-Amide mercapturic acid	5–14	8–23	6 (3)	40
p-Amino phenyl sulfate	2–3.6	2.2–5	3A (4)	37
1-Hydroxyethyl *sec*-amide mercapturic acid	2–3.6	0.7–1.7	3B (7)	38
sec-Amide mercapturic acid sulfoxide	1.2–3.2	1.4–2.4	4 (8)	39
1-Hydroxyethyl *sec*-amide mercapturic acid sulfoxide	0.5–1.8	1.0–1.7	7 (9)	57
Hydroxymethyl *sec*-amide methyl sulfoxide	0.9–2.1	2.7–4.1	8 (11)	41
1-Hydroxyethyl *sec*-amide methyl sulfone	1.1–2.5	1.1–2.7	9B (12)	42
2-Hydroxyethyl *sec*-amide methyl sulfone	0.8–0.9 (only C, D)	0.7–1.4	9C (14)	43
tert-Amide mercapturic acid	–	0.5–1.0	10A (15)	44
Hydroxymethyl *sec*-amide methyl sulfone	0.3–1.0	0.5–1.0	10B (16)	45
sec-Amide methyl sulfoxide	0.5–1.3	2.3–2.7	12 (17)	46
sec-Amide methyl sulfone	0.1 (only C, D)	0.5	14 (18)	10
sec-Amide chloride	0.2–0.4	0.4–0.9	15 (19)	8
sec-Amide methyl sulfide	–	–	15 (20)	36

bw: body weight; EU: European Union; HPLC: high-performance liquid chromatography

[a] Average five rats of each sex. See Table 2 for group descriptions.

[b] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Carr et al. (1983).

Table 10. Percentage of dose represented by the faecal fractions isolated[a]

	% of dose	
Faecal fraction	**Males**	**Females**
Faecal-bound residues	4.9–7.4	4.9–6.4
Hexane-soluble fraction	2.3–6.7	2.6–4.8
Water-soluble fraction	15.9–35.8	10.6–27.4

[a] Average five rats of each sex.
Source: Carr et al. (1983).

Table 11. Faecal metabolites identified in the n-hexane-soluble fraction of pooled faecal samples of rats collected up to 72 hours[a]

Metabolite (trivial name[b])	**% of dose**		**HPLC fraction (metabolite no.)**	**EU reference no.**
	Males	**Females**		
Acetochlor	< 1 (from traces to 6%)	< 1 (from traces to 6%)	3 (1)	1
sec-Amide chloride	< 1	< 1	1 (19)	8
sec-Amide-S-S-*sec*-amide	< 1	< 1	6 (24)	58
sec-Amide-S-S-*tert*-amide	< 1	< 1	5 (25)	47
sec-Amide-S-*tert*-amide	< 1	< 1	4 (27)	48
sec-Amide-S-O-*tert*-amide	< 1	< 1	2 (28)	49
sec-Amide-S-*sec*-amide	< 1	< 1	2 (29)	50

EU: European Union; HPLC: high-performance liquid chromatography
[a] Average five rats of each sex.
[b] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.
Source: Carr et al. (1983)

The faecal metabolite that could be isolated and identified in the *n*-hexane fraction at 12 hours after treatment is *sec*-amide mercapturic acid (EU reference no. 40). However, this metabolite could not be detected in the combined 72-hour faecal sample. The water-soluble fraction was subjected to Raney nickel desulfuration after extraction with ethyl acetate at neutral and acidic pH. The HPLC analysis of the reaction products indicated the presence of thioethers and dithioethers of *sec*-amide, *tert*-amide and 1-hydroxy *sec*-amide metabolites.

Glutathione (GSH) conjugation of acetochlor occurs through displacement of the chlorine atom from the chloromethyl group. The GSH residue is further metabolized to form the corresponding mercapturic acid conjugate. The most abundant metabolite that was isolated and identified in urine is the *sec*-amide mercapturic acid conjugate (5–23% of the dose). This metabolite was detected in faeces at 12 hours after administration. The identification of hydroxylated side-chain and sulfoxide analogues confirmed this route of metabolism. The mercapturic acid conjugate *tert*-amide mercapturic acid (EU reference no. 44) resulting from conjugation of acetochlor with GSH has also been found in urine, but in much lower concentrations (0.5–1.0% of the dose in females only). In the rat, GSH and mercapturic acid conjugates are excreted in the bile and are further metabolized in the gastrointestinal tract by the C–S lyases of the microbial flora. The thiol products that result from this reaction may either be reabsorbed or react with each other to form thioethers and dithioethers, which remain in the gastrointestinal tract and are excreted with the faeces. The identification of thioethers and dithioethers

of *sec*-amides and *tert*-amides confirmed this route of metabolism. The thiols that are formed in the gastrointestinal tract as a result of C–S lyase activity have not been found, but the *S*-methylated derivative (EU reference no. 36) originating from its mercapturic acid analogue (EU reference no. 40) has been identified in urine. This points to enterohepatic circulation of the thiols and further metabolism by *S*-methylation and *S*-oxidation. The identification of the *S*-oxidation products and their hydroxylated side-chain products confirms enterohepatic circulation.

A much less important metabolic pathway is the formation of *N*-dealkylated acetochlor or *sec*-amide chloride (EU reference no. 8). As this metabolite has been identified in urine as well as in faeces, there are two possible routes by which it is formed. The first route involves oxidative *N*-dealkylation of acetochlor. The other route is more complicated and consists of an *O*-dealkylation step of the *N*-ethoxymethyl side-chain followed by glucuronic acid conjugation. The glucuronic acid conjugate is excreted in the bile and deconjugated in the gastrointestinal tract by bacterial glucuronidase activity. This reaction yields the *N*-hydroxymethyl compound, which is unstable and degrades to the *sec*-amide chloride (EU reference no. 8). This metabolite can be reabsorbed from the gastrointestinal tract and further metabolized through GSH conjugation. The last metabolite that was identified is the sulfate conjugate of *p*-amino methylethylphenol (EU reference no. 37). This conjugate is indicative of the formation of a *p*-aminophenol through a combination of aniline *para*-hydroxylation and amide bond cleavage of metabolite precursors.

Only a trace amount of unchanged acetochlor (average < 1% of the administered dose) was found in the faeces. This indicates that most of the radioactivity that has been measured in the faeces originates from biliary excretion.

In summary, acetochlor was rapidly absorbed from the gastrointestinal tract, and the ^{14}C radioactivity was rapidly excreted in the urine and faeces, which contained 76–85% of the dose for males and 76–87% of the dose for females, depending on size and number of doses. The elimination pathways did not appear to be affected by the sex of the animal, and the percentages were essentially the same, despite a 40-fold difference in dose levels. The accumulation of ^{14}C radioactivity in body tissues or organs was very small, which accounted for about 1% of the administered dose. The whole-body elimination takes place in a rapid (α) phase, with a half-life of 5.4–10.4 hours, and a slow late (β) phase, with a half-life of 129–286 hours. The slow elimination phase can be explained by the significant amount of radioactivity that is still bound to the red cell fraction of the blood at 7 days after dosing (2–3% of the administered radioactivity).

The predominant route of metabolism of acetochlor in the rat is the GSH conjugation pathway. Through this pathway, *S*-conjugates are formed, which are excreted in the bile and undergo cleavage of the C–S bond in the gastrointestinal tract. The emerging thiol products are then reabsorbed or condensed to form thioethers and dithioethers, which are excreted in the faeces. The reabsorbed thiols are methylated and further metabolized by *S*-oxidation and side-chain hydroxylation. In addition to the metabolites that are representative of the GSH conjugation pathway, *N*-dealkylation of acetochlor and formation of *p*-amino methylethylphenol (EU reference no. 37) were also identified. From the very low percentage (< 1% of the dose on average) of unchanged acetochlor that could be recovered from the faeces, it can be concluded that acetochlor is very well absorbed from the gastrointestinal tract (Carr et al., 1983).

A study was conducted to investigate the nature of excretory biotransformation products of acetochlor in rats following oral administration of the ^{14}C-labelled compound at nominal doses of 10 and 200 mg/kg bw. The dosing of animals and distribution and excretion of radioactivity have been described in detail above (see section 1.1; Hawkins, Kirkpatrick & Dean, 1988, 1989a,b).

The urine samples collected for 24 hours were pooled for each subgroup of five animals of the same sex. The metabolites were isolated from the urine by means of extraction using C18 Bond Elut sorbent columns. The methanol column eluates were analysed by thin-layer chromatography (TLC) in solvent systems H (chloroform/methanol/acetic acid, 90:10:3) and J (chloroform/methanol/formic acid/water, 70:25:3:3) without further concentration. A fraction of the urine samples was

subjected to incubation with β-glucuronidase/sulfatase before column extraction. The methanol eluates from the urine samples treated with the enzyme mix were analysed only by TLC with solvent system H. The faecal samples collected for 24 hours were pooled for each subgroup of five animals of the same sex. The pooled homogenates were extracted sequentially with ethyl acetate, acetonitrile and acetonitrile/water (7:3). The ethyl acetate extracts were concentrated and analysed by TLC with solvent system H. The acetonitrile extracts (with and without water) were purified by sorbent extraction as described for urine.

The TLC analysis showed that urine contained at least 15 radiolabelled components with a wide range of polarities. No unchanged acetochlor was detected in urine (Table 12). The pretreatment with non-radiolabelled acetochlor did not significantly affect the qualitative or quantitative profile of urinary metabolites, but a quantitative difference was observed when rats were administered higher doses (200 mg/kg bw). All urine samples contained a single major radioactive component (EU reference no. 40) of low mobility in solvent system H (H1; Table 12) and high mobility in solvent system J (J9; Table 13). This component co-chromatographed with the reference compound mercapturic acid conjugate of acetochlor after removal of the ethoxymethyl side-chain from the nitrogen. This component accounted for 22–27% (male) or 28–37% (females) in H-J TLC systems at the dose of 10 mg/kg bw. This metabolite accounted for a lower (9–16%) proportion of the 200 mg/kg bw dose, and this was the main quantitative difference between the urinary profiles at the high and low dose levels. Other metabolites that were identified were *sec*-amide methyl sulfoxide (EU reference no. 46) and *tert*-amide thiolactic acid (EU reference no. 70). The cysteine conjugate of acetochlor, *sec*-amide cysteine (EU reference no. 67), appeared to be a minor metabolite. Comparative chromatography of metabolites before and after incubation with enzymes indicated that glucuronide and sulfate conjugates were relatively minor. The incubation of urine with β-glucuronidase/sulfatase enzymes resulted in a small reduction (19.1–12.8% of the dose) in polar components of the solvent system H, indicating that sulfate and/or glucuronide conjugates were minor components in urine. When urine samples were incubated with enzymes and analysed using solvent system H, component H9 co-chromatographed with the reference compound (EU reference no. 5), which resulted from *N*-dealkylation of acetochlor and retained the chlorine atom. Approximately 80% of the radioactivity in 0- to 24-hour faecal samples was extracted into ethyl acetate and acetonitrile/water. These extracts contained 22% (males) and 16% (females) of the administered dose at 200 mg/kg bw and showed complex patterns of metabolites, none of which was formally characterized. Unchanged acetochlor appeared to be a minor faecal metabolite.

In summary, following oral administration to male and female rats, acetochlor was extensively metabolized, and the metabolites were excreted predominantly in urine. No parent compound was detected in the urine. The major routes of metabolism involved *N*-dealkylation and displacement of the chlorine atom from the chloromethyl group by GSH. The GSH conjugate was found to be further degraded to mercapturic acid and thiolactic acid conjugates. The presence of *sec*-amide methyl sulfoxide (EU reference no. 46) in the urine is indicative of biliary excretion of GSH conjugates and their degradation products (cysteine and mercapturic acid conjugates), cleavage of the C–S bond by β-lyase of the gut microflora and *S*-methylation after reabsorption of the thiols from the gastrointestinal tract. The metabolite profiles were similar after administration of ^{14}C-labelled acetochlor at 10 or 200 mg/kg bw and were not affected by pretreatment of the rats with non-radiolabelled acetochlor prior to administration of the radioactive dose (Hawkins, Kirkpatrick & Dean, 1989c).

The biotransformation of acetochlor was investigated in a study in which 12 male and six female Sprague-Dawley rats were administered a single oral dose of ^{14}C-phenyl-labelled acetochlor at 10 or 200 mg/kg bw in polyethylene glycol 600 and the bile ducts of four of the male rats (two each at 10 and 200 mg/kg bw) were cannulated 24 hours prior to dosing. This study (Jones, 1990) was described in section 1.1 above (see also Table 6).

Table 12. Quantification of acetochlor metabolites expressed as percentages of a single oral dose (10 or 200 mg/kg bw) or a single oral ^{14}C dose (10 mg/kg bw) after 14 unlabelled doses (10 mg/kg bw) to rats, analysed with solvent system H

Metabolite (trivial name[a])	% of administered dose						TLC fraction	EU reference no.
	10 mg/kg bw		200 mg/kg bw		10 mg/kg bw (14 + 1 days)			
	M	F	M	F	M	F		
	21.1	19.3	16.2	15.8	20.2	21.9	Polar fraction	
sec-Amide mercapturic acid	22.1	29.8	8.9	10.5	22.1	28.3	H1	40
tert-Amide thiolactic acid	4.4	3.0	4.2	2.2	4.0	2.7	H2	70
	2.1	2.6	1.3	2.0	1.8	2.3	H3/H4	
	4.9	4.7	6.2	6.0	4.4	4.4	H5	
sec-Amide methyl sulfoxide	3.5	5.0	4.3	12.0	3.4	5.4	H6	46
			1.1	2.6			H7	
	0.4	1.0	0.4		0.7	1.0	H8	
	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	H9/H10	

bw: body weight; EU: European Union; F: female; M: male; TLC: thin-layer chromatography

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Hawkins, Kirkpatrick & Dean (1989c)

Table 13. Quantification of acetochlor metabolites expressed as percentages of a single oral dose (10 or 200 mg/kg bw) or a single oral ^{14}C dose (10 mg/kg bw) after 14 unlabelled doses (10 mg/kg bw) to rats, analysed with solvent system J

Metabolite (trivial name[a])	% of administered dose						TLC fraction	EU reference no.
	10 mg/kg bw		200 mg/kg bw		10 mg/kg bw (14 + 1 days)			
	M	F	M	F	M	F		
	1.5	0.8	1.3	1.0	0.9	0.6	Polar fraction	
	6.9	6.1	5.2	7.3	5.0	4.6	J1	
sec-Amide cysteine	4.5	3.0	4.0	2.8	3.8	6.3	J2–J4	67
	3.7	4.1	2.8	3.2	2.7		J5/6	
	6.1	5.9	3.3	1.9	6.9	4.8	J7	
	3.7	3.5	3.4	2.0	3.5	4.8	J8	
sec-Amide mercapturic acid	24.8	32.3	12.1	15.9	27.3	37.2	J9	40
	3.9	3.5	5.6	4.1	3.4	2.5	J10	
sec-Amide methyl sulfoxide, *tert*-amide thiolactic acid	2.8	4.5	3.2	10.8	2.1	4.1	J11	46, 70
	0.6	1.2	1.2	1.8	0.8		J12	
	0.2	0.3	0.5	0.5	0.8	1.0	J13	

bw: body weight; EU: European Union; F: female; M: male; TLC: thin-layer chromatography

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Hawkins, Kirkpatrick & Dean (1989c)

Metabolites were isolated from urine and bile samples by solid sorbent extraction with Bond Elut C-8 columns using dichloromethane, ethyl acetate and ethanol as eluents. Only dichloromethane was used for the elution of bile metabolites following incubation with β-glucuronidase to hydrolyse glucuronic acid conjugates.

Metabolites were isolated from faeces by extraction of the homogenates with ethyl acetate, acetonitrile and acetonitrile/water (1:1). The combined acetonitrile fractions were evaporated and the metabolites extracted with Bond Elut C-8 columns using methanol as the eluent.

The quantification and characterization of the metabolite fractions are shown in Table 14 for urine and Table 15 for bile.

Table 14. Quantification of characterized acetochlor metabolites in urine of non-bile duct–cannulated rats expressed as percentages of the administered radioactivity after a single oral dose

	% of administered dose					
	10 mg/kg bw		200 mg/kg bw			EU reference
Metabolite (trivial name[a])	M	F	M	F	Fraction	no.
sec-Amide mercapturic acid	24.8	32.3	12.1	15.9	U9	40
sec-Amide methyl sulfoxide	< 2.8	< 4.3	< 3.2	< 10.8	U11	46

bw: body weight; EU: European Union; F: female; M; male

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Jones (1990)

Table 15. Quantification of characterized acetochlor metabolites in bile of male bile duct–cannulated rats expressed as percentages of the administered radioactivity after a single oral dose

	% of administered dose					
	10 mg/kg bw		200 mg/kg bw			EU reference
Metabolite (trivial name[a])	M	F	M	F	Fraction	no.
sec-Amide glutathione	3.8	–	3.8	–	B5	51
tert-Amide glutathione	8.9	–	13.8	–	B7	54
O-dealkylated acetochlor *O*-glucuronide	30.2	–	41.1	–	B9	52
tert-Amide mercapturic acid	5.7	–	3.6	–	B15	44

bw: body weight; EU: European Union; F: female; M; male

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Jones (1990)

No qualitative sex differences in metabolite profiles were apparent, although differences were apparent in the relative proportions of the metabolites. In non-bile duct–cannulated rats, the major urinary metabolite (U9) accounted for approximately 25% and 32% of a 10 mg/kg bw dose and 12% and 16% of a 200 mg/kg bw dose for males and females, respectively. The remaining urinary radioactivity was resolved into at least 11 minor metabolites. Urine collected from bile duct–cannulated rats contained at least five metabolites, none of which corresponded to the major metabolite found in non-bile duct–cannulated rats. The cannulated rats were examined for bile metabolite profile. There were no marked differences when the rats were dosed with 10 or 200 mg/kg bw. Two major metabolites were identified: B9, which accounted for 30.2% and 41.1% of the dose at 10 and 200 mg/kg bw, respectively; and B7, representing about 8.9% and 13.8% of the dose at 10 and 200 mg/kg bw, respectively. The remaining 10–13 components represented less than 5% of the dose.

Acetochlor was both rapidly and extensively absorbed following a single oral dose of either 10 or 200 mg/kg bw. This was clearly shown in the excretion data for bile duct–cannulated rats, as 30% of the administered dose was eliminated within 4 hours, and more than 84% of a 10 mg/kg bw dose was eliminated via bile within 24 hours. No marked dose level effect was apparent, as more than 80% of a 200 mg/kg bw dose was eliminated via bile. The small amounts of dose excreted in faeces by bile duct–cannulated rats showed that an oral dose of acetochlor was almost quantitatively absorbed. Hence, the majority of faecal radioactivity in non-cannulated rats was of biliary origin.

As non-bile duct–cannulated rats excreted more than 70% of a 10 mg/kg bw oral dose in urine, the elimination of approximately 85% of a similar dose via bile shows that enterohepatic recirculation of acetochlor metabolites was extensive. The absence of the major urinary metabolite (U9) in the urine of bile duct–cannulated rats also indicates that this metabolite was derived via enterohepatic recirculation. The identification of intermediary metabolites in bile showed that the initial metabolism of acetochlor was principally focused on two reaction sites, the ethoxymethyl side-chain and the chlorine atom. The identification of the major biliary metabolite (B9) as the glucuronide conjugate of *O*-dealkylated acetochlor indicates that this was a preferred biotransformation reaction. Conjugation of acetochlor with GSH via displacement of the chlorine atom was the second preferred biotransformation reaction. In addition to the GSH conjugate of acetochlor (B7), bile also contained the corresponding mercapturic acid (B15). A second minor metabolite was identified as the GSH conjugate of *N*-dealkylated acetochlor (B5). No acetochlor was detected in the bile in rats administered 200 mg/kg bw; therefore, low faecal excretion in bile duct–cannulated rats appears to represent the unabsorbed dose.

In summary, an oral acetochlor dose of 10 or 200 mg/kg bw was rapidly and almost completely absorbed by the rat and was subject to extensive metabolism. The two main biotransformation pathways involved *O*-dealkylation and glucuronidation of the *N*-ethoxymethyl side-chain or the complete cleavage of this moiety and displacement of the chlorine atom from the chloromethyl group by GSH, leading to mercapturic acid formation. The metabolic intermediates were extensively eliminated via bile, with evidence of enterohepatic circulation (Jones, 1990).

Urine samples collected from male and female rats during the first day after a single oral dose of ^{14}C-labelled acetochlor at 10 or 200 mg/kg bw were used to further characterize metabolites by TLC, liquid chromatography, HPLC and gas–liquid chromatography following derivatization. The urine used for isolation and identification of metabolites reported here was obtained from the Hawkins, Kirkpatrick & Dean (1988, 1989a) studies.

Three additional minor urinary metabolites of acetochlor were characterized by gas chromatography–mass spectromety. Their relative proportions were determined by TLC using a linear analyser and are expressed as percentage of the dose in Table 16.

Table 16. Quantification of additional acetochlor metabolites in the urine of rats expressed as percentages of the administered radioactivity after a single oral dose of 10 or 200 mg/kg bw

	% of the dose					
	10 mg/kg bw		200 mg/kg bw		TLC fraction	EU reference no.
Metabolite (trivial name[a])	M	F	M	F		
p-OH-EMA	3.7	3.5	3.4	2.0	U8	71
sec-Amide chloride	0.6	1.2	1.2	1.8	U12a	8
sec-Amide methyl sulfide					U12b	36

bw: body weight; *p*-OH-EMA: *para*-hydroxy-2-ethyl-6-methylaniline; EU: European Union; F: female; M: male; TLC: thin-layer chromatography

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

Source: Prout & Gledhill (1991)

para-Hydroxy-2-ethyl-6-methylaniline (*p*-OH-EMA) (EU reference no. 71) corresponds to TLC fraction J8, and *sec*-amide chloride (EU reference no. 8) and *sec*-amide methyl sulfide (EU reference no. 36) both correspond to TLC fraction J12 (Hawkins, Kirkpatrick & Dean, 1989c).

In summary, three additional minor urinary metabolites of acetochlor in the rat were identified in this study: *p*-OH-EMA (EU reference no. 71), *sec*-amide chloride (EU reference no. 8) and *sec*-amide methyl sulfide (EU reference no. 36). The last metabolite confirms the formation of GSH conjugates and derived products that are excreted in the bile and subsequently reabsorbed from the gastrointestinal tract after cleavage of the C–S bond. The *sec*-amide chloride (EU reference no. 8) is indicative of the *N*-dealkylation pathway, whereas *p*-OH-EMA (EU reference no. 71) is the result of *N*-dealkylation followed by amide hydrolysis and *para*-hydroxylation (Prout & Gledhill, 1991).

In another study, the urinary and plasma metabolite profiles were compared in CD rats and CD-1 mice given single oral doses of ^{14}C-labelled acetochlor. In addition, the dose-dependent metabolism of acetochlor was studied over a wide range of dose levels (10–2000 mg/kg bw). The study design is shown in Table 17.

Table 17. Design of comparative metabolism study in rats and mice

Group	No. of animals	Dose (mg/kg bw)	Dose regimen	Sampling times (h)	Samples for radioactivity determination
1	5 rats 6 mice	200	Gavage (corn oil)	24, 48	Urine, faeces
2	4 rats 10 mice	200	Gavage (PEG 600)	17	Blood (cardiac puncture)
3	8 rats	200	6 months of treatment at 1 750 ppm non-labelled acetochlor in diet followed by single gavage dose of ^{14}C-labelled acetochlor at 200 mg/kg bw 24 h prior to termination	24 h after dosing with ^{14}C-labelled acetochlor	Blood (cardiac puncture)
4	5 rats	10, 200, 1 000, 2 000	Gavage (corn oil)	24	Urine

bw: body weight; PEG: polyethylene glycol; ppm: parts per million
Source: Green et al. (1998)

Urinary metabolites in the rat were found to be derived principally from GSH conjugation at the chloramide chlorine atom, the major metabolite being the mercapturic acid (metabolite F; Table 18). In contrast, the metabolite profile in mouse urine was markedly different from that in rat urine. The major metabolite (metabolite H), which still retained the chlorine atom of the chloramide group, was identified as a glucuronide conjugate of the *N*-methylol group remaining after oxidative *O*-de-ethylation of the *N*-ethoxymethyl side-chain. Glucuronides formed from ring side-chain oxidations were also present. The marked difference between the metabolism of acetochlor in rats and mice was further highlighted by treatment of the urine samples with β-glucuronidase. Whereas this enzyme had little effect on the metabolites in rat urine, the retention times of almost all of the mouse metabolites changed after treatment, demonstrating that these were, in fact, glucuronides.

Acetochlor sulfoxide (*sec*-amide methyl sulfoxide; EU reference no. 46) was the major metabolite in plasma from rats given a single oral acetochlor dose of 200 mg/kg bw. This was also the major plasma metabolite in rats treated for 6 months with acetochlor at 1750 parts per million (ppm) in the diet. A second significant metabolite, which was coincident with the chloramide, was present.

Table 18. Metabolites identified in 0- to 24-hour urine of rats and mice treated with a single oral dose of ^{14}C-labelled acetochlor at 200 mg/kg bw

Metabolite (trivial name[a])	HPLC fraction	EU reference no.
Rats		
Hydroxylated *sec*-amide methyl sulfoxide	A	65
Hydroxylated *sec*-amide methyl sulfone	B+C	66
sec-Amide cysteine	D	67
sec-Amide methyl sulfoxide	E	46
sec-Amide mercapturic acid	F	40
sec-Amide methyl sulfone	G	10
sec-Amide chloride	I	8
Mice		
O-Dealkylated acetochlor *O*-glucuronide	H	52
sec-Amide chloride	I	8

bw: body weight; EU: European Union; HPLC: high-performance liquid chromatography
[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.
Source: Green et al. (1998)

Mouse plasma, in contrast, contained very little acetochlor sulfoxide (EU reference no. 46), the major metabolite being coincident with the chloramide (*sec*-amide chloride; EU reference no. 8). Both the rate of excretion and the metabolism of acetochlor in the rat differed with dose. The rat eliminated almost all radioactivity within 24 hours when the animals were administered low doses (100–200 mg/kg bw) of acetochlor. However, the time of elimination increased to 48 hours when higher doses (1000–2000 mg/kg bw) were administered. The major metabolite over the entire dose range was the *sec*-amide mercapturic acid (EU reference no. 40). However, as the dose level increased, there was a significant increase in the excretion of glucuronic acid conjugates, which is indicative of a saturation of the GSH conjugation pathway.

In summary, the metabolism of acetochlor differed between rats and mice. In rats, acetochlor was eliminated as urinary metabolites derived from GSH conjugates, whereas in mice, it was eliminated as urinary metabolites derived from glucuronide conjugates. Plasma analysed after 17 hours of acetochlor administration confirmed the difference. The major metabolite in plasma of rats was the *sec*-amide methyl sulfoxide (EU reference no. 46). In contrast, the levels of the *sec*-amide methyl sulfoxide (EU reference no. 46) in mouse plasma were extremely low. Significant levels of the *sec*-amide chloride (EU reference no. 8) were found in the plasma of both the rat and mouse. When the dose of acetochlor to rats was increased, the glucuronide conjugates eliminated by urine increased at the same time, indicating a saturation of the GSH conjugation system; therefore, cells would remain unprotected from other free radicals. Rats eliminated almost all acetochlor within 24 hours when the animals were dosed with low levels of acetochlor (10–200 mg/kg bw); however, higher doses (1000–2000 mg/kg bw) were eliminated within 48 hours (Green et al., 1998).

A study was conducted to determine the nature of the acetochlor metabolites excreted in rat bile, the extent of biliary excretion and the relationship of the bile metabolites to the metabolites previously identified in the urine and faeces. Sprague-Dawley rats were dosed with an approximately 1:1 mixture of acetochlor labelled with phenyl-^{14}C and with ^{13}C in the carbon 2 position through different routes, but the dose level was 7 mg/kg bw for all dose groups. The study design is shown in Table 19.

Table 19. Design of study to determine acetochlor metabolites in rat bile

Group	No. of rats of each sex	Dose (mg/kg bw)	Route of administration	Primary samples collected and sampling interval
1	3	7	Intravenous	Bile was collected at 30 min intervals for 6 h
2	2	7	Intragastric injection	Bile was collected at 30 min intervals for 7.5 h
3	3	7	Oral gavage	Bile, urine, faeces; bile was collected at 3, 6, 12, 24, 48 and 72 h
4	2	7	Intravenous	Bile duct cannula of donor rat was inserted into duodenum of acceptor rat; samples were collected by bile duct cannulation of acceptor rat; bile was collected at 30 min intervals for 7 h

bw: body weight
Source: Yalamanchili, Oppenhuizen & Wilson (1985, 1987)

Table 20. Mean excretion of radioactivity in the bile of rats of test groups 1, 2, 3 and 4

	% of administered radioactivity excreted in bile	
Group	**Males**	**Females**
1[a]	60	52
2[b]	7	8
3[c]	72	65
4[d]	17	15

[a] Amount recovered within 6 hours of intravenous dose.
[b] Amount recovered within 7.5 hours of intragastric injection.
[c] Amount recovered within 72 hours of oral gavage.
[d] Amount recovered from recipient rats within 7 hours after intravenous dosing of donor rats.
Source: Yalamanchili, Oppenhuizen & Wilson (1985, 1987)

The excretion of radioactivity in the bile is shown in Table 20. More than 50% of the administered dose was recovered only from those bile duct–cannulated rats administered acetochlor orally and intravenously. The rate of excretion of total radioactivity in the bile of the rats of group 1 followed a biphasic pattern, with half-lives of 0.43 and 0.31 hour for the initial phase (α phase) for males and females, respectively, and half-lives of 2.1 and 2.2 hours for the late phase (β phase) for males and females, respectively.

Preparative HPLC with radioactivity detection separation of the bile samples from the four test groups revealed eight major metabolites. The percentage of identified acetochlor metabolites in the pooled bile samples collected from the four test groups is given in Table 21.

There were no important differences in radioactivity HPLC profiles between the pooled bile samples from male and female animals within the experimental groups. The biliary metabolites that were isolated and identified are presented in Table 22, together with their range of percentage of dose for each sex.

The GSH, cysteinylglycine and cysteine conjugates were not detected in the bile of rats from group 4. This is the group where the bile duct cannula of each donor rat (receiving an intravenous dose) was inserted into the duodenum of an acceptor rat from which the bile was collected for analysis. The most abundant metabolite present in the bile of acceptor rats was *tert*-amide mercapturic acid (EU reference no. 44). This metabolite represented 49.1% and 44.7% of the radioactivity excreted in the bile of male and female animals, respectively. Both the glucuronic acid and GSH conjugates and their metabolites could be detected at 30 minutes after administration of ^{14}C-labelled acetochlor.

Table 21. Percentage of radioactivity excreted in the bile of male and female rats from all test groups representative of the eight major metabolites identified

	% of radioactivity excreted in the bile	
Group	**Males**	**Females**
1	86.6	76.5
2	84.4	77.5
3	75.9	76.3
4	77.0	74.5

Source: Yalamanchili, Oppenhuizen & Wilson (1985, 1987)

Table 22. Metabolites and their percentage of dose range isolated from the bile samples of male and female rats from all test groups

	% of dose		HPLC fraction	EU reference
Metabolite (trivial name[a])	**Males**	**Females**	**(metabolite no.)**	**no.**
sec-Amide glutathione	0.1–3.0	0.1–3.7	A (1)	51
sec-Amide mercapturic acid	0.3–3.7	0.4–3.2	B (2)	40
O-Dealkylated acetochlor *O*-glucuronide	1.1–26.8	1.6–33.3	C (3)	52
Hydroxymethyl acetochlor *O*-glucuronide	0.4–2.5	0.1–1.5	D (4)	53
tert-Amide glutathione	0.6–9.3[b]	0.3–9.4[b]	E (5)	54
tert-Amide mercapturic acid	0.8–15.1	0.9–7.8	F (6)	44
tert-Amide cysteinylglycine	0.6–3.8[b]	0.6–4.4[b]	G (7)	55
tert-Amide cysteine	0.2–1.4[b]	0.4–3.4[b]	H (8)	56

EU: European Union; HPLC: high-performance liquid chromatography

[a] "*sec*-amide": *N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor; "*tert*-amide": *N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) acetamide moiety of acetochlor.

[b] Metabolite was not found in group 4.

Source: Yalamanchili, Oppenhuizen & Wilson (1985, 1987)

Eight major metabolites were identified in groups 1, 2 and 3, and five in group 4. The most abundant metabolite present in the bile of groups 1, 2 and 3 was the *O*-glucuronic acid conjugate of *O*-dealkylated acetochlor (EU reference no. 52). This metabolite is formed by the *O*-dealkylation of the *N*-ethoxymethyl side-chain of acetochlor, yielding the corresponding hydroxymethylene (-N-CH_2-OH) intermediate, which is then conjugated with glucuronic acid. Once this metabolite is excreted in the gastrointestinal tract via the bile, it can be deconjugated by the β-glucuronidase activity of the gut microflora. The hydroxymethylene compound (methylol) is then released again and degrades to *sec*-amide chloride (EU reference no. 8). This gut metabolite can be readily reabsorbed and undergo further liver metabolism. Another glucuronic acid conjugate that was identified is hydroxymethyl acetochlor *O*-glucuronide (EU reference no. 53). Both metabolites belong to the oxidative and glucuronic acid conjugation pathways of the liver.

All the other metabolites are part of the GSH conjugation pathway of the liver. Acetochlor and its *N*-dealkylated metabolite are conjugated with GSH through displacement of the chlorine atom from the chloromethyl group (EU reference nos 51 and 54). The GSH residue is then gradually degraded by gamma-glutamyltranspeptidase (GGT) (formation of cysteinylglycine conjugate, EU reference no. 55), cysteinylglycinase (formation of cysteine conjugate, EU reference no. 56) and *N*-acetyltransferase (formation of mercapturic acid conjugates, EU reference nos 40 and 44). The

GSH conjugates, the mercapturic acid conjugates and all the intermediates are excreted in the gastrointestinal tract via the bile, where the C–S bond is cleaved by C–S lyase (β-lyase) activity of the gut microflora. The thiol compounds can then be reabsorbed and further metabolized by the liver. The most abundant metabolite in the bile of rats from group 4 was *tert*-amide mercapturic acid (EU reference no. 44); the intermediate metabolites of that pathway could not be isolated and characterized.

In summary, a substantial amount of the administered radioactivity is excreted in the bile after 6 hours (60% in males, 52% in females) by the intravenous route and 72 hours (72% in males, 65% in females) by the oral route. There were no sex-related differences in excretion in the bile. Eight metabolites were identified in the bile of rats treated with intravenous, intragastric or single oral gavage doses. Five major metabolites were identified in bile duct–cannulated donor and recipient rats. From the results of the test with the bile duct–cannulated donor and recipient rats, it can be concluded that the metabolites that are excreted in the bile undergo enterohepatic circulation and are partly excreted again in the bile (14–20% of administered radioactivity) after further metabolism in the liver. On the basis of the metabolites that were identified in the bile in this study, it can be concluded that there are two main metabolic pathways leading to biliary excretion in the rat: the glucuronic acid conjugation pathway and the GSH conjugation pathway (Yalamanchili, Oppenhuizen & Wilson, 1985).

(b) Other routes of administration

Urine samples collected during a dermal penetration study of acetochlor with monkeys (Maibach, 1983) were used to identify the major metabolites of acetochlor. Rhesus monkeys were dosed with ^{14}C-labelled acetochlor intravenously, intramuscularly, subcutaneously or topically. For each dose group, the urine samples for each monkey containing the maximum level of ^{14}C radioactivity were prepared by concentration and/or Sep-Pak treatment for a chromatographic evaluation of the urine-contained ^{14}C radioactivity.

Two mercapturic acid metabolites were found to predominate in the histograms generated by the HPLC/liquid scintillation counting analyses in each dose group. These metabolites together accounted for 36–52% of the ^{14}C radioactivity that was profiled. The major metabolite (23–38% of the ^{14}C radioactivity in the samples analysed) was identified by HPLC retention time and mass spectral analysis as 3-[*N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl)acetamido]thio-2-acetylaminopropanoic acid. It is also known as *tert*-amide mercapturic acid conjugate (EU reference no. 44). The second most abundant metabolite (13–16% of the HPLC radioactivity in the samples analysed) was identified by HPLC retention time and mass spectral analysis as 3-[*N*-(2-ethyl-6-methylphenyl)acetamido]thio-2-acetylaminopropanoic acid (*sec*-amide mercapturic acid conjugate; EU reference no. 40). No unchanged acetochlor was found in the urine of any monkey from any dose group, and no other metabolites were found to be present in appreciable quantities relative to the two mercapturic acids.

The metabolic fate of acetochlor in rhesus monkey urine was not significantly altered by the different routes or forms of administration and was very consistent from animal to animal. The rate of metabolite elimination was rapid following the intravenous and intramuscular administrations and was somewhat slower following the subcutaneous and topical applications. However, the distribution of metabolic end-products indicated that the mercapturate conjugates were consistently formed and eliminated and accounted for the majority of the ^{14}C radioactivity in the urine.

In summary, the two predominant metabolites of acetochlor in rhesus monkey urine were mercapturic acid conjugates. The two metabolites were identified to be the mercapturic acid conjugates of acetochlor, *tert*-amide mercapturic acid (EU reference no. 44) and *sec*-amide mercapturic acid (EU reference no. 40). From the time course analysis of urine samples collected from the monkeys treated dermally, it appears that the *tert*-amide mercapturic acid is excreted initially, followed by the *sec*-amide mercapturic acid. The metabolism data obtained in this study demonstrate that the GSH conjugation pathway leading to the formation of mercapturic acid conjugates is by far the predominant route of metabolism of acetochlor in the rhesus monkey (Livingston, 1983).

A study was conducted to characterize acetochlor metabolites in urine following intravenous administration to rhesus monkeys at two different doses (also described in section 1.1 above). In this study, two groups of three male rhesus monkeys were administered ^{14}C-phenyl-labelled acetochlor intravenously, using propylene glycol/ethanol/saline (60:10:30) as the dose vehicle, at a dose level of 0.005 or 0.05 mg/kg bw.

The metabolism of acetochlor was very similar for both groups, as indicated by comparisons of their metabolite profiles in urine. The major metabolites, each of which constituted more than 10% of the metabolite profile for all six monkeys, were the *tert*-amide mercapturic acid (EU reference no. 44) and the *O*-glucuronic acid conjugate of *O*-dealkylated acetochlor (EU reference no. 52). These two metabolites constituted 19.93–31.58% and 12.72–22.76% of the urine metabolite profiles, respectively. No other metabolite peaks exceeded 10% of the metabolite profile. Only one other identified metabolite, the *sec*-amide mercapturic acid (EU reference no. 40), exceeded 5% of the profile for any monkey (Table 23).

Table 23. Average percentage of radioactivity in chromatographic profiles of the three major metabolites identified in the urine of monkeys of each dose group

		Average % of profile (*n* = 3)		
Dose level (mg/kg bw)	**Collection period (h)**	***tert*-Amide mercapturic acid (EU reference no. 44)**	**Glucuronide of acetochlor (EU reference no. 52)**	***sec*-Amide mercapturic acid (EU reference no. 40)**
0.005	0–48	27.26	17.46	6.08
0.05	0–48	25.39	19.15	5.30

bw: body weight; EU: European Union
Source: Kurtzweil (2014)

Two other metabolites, the *sec*-amide chloride (EU reference no. 8) and the *tert*-thioacetic acid (EU reference no. 4), with maximum percentages of profile values of 3.89% and 3.46%, respectively, were also identified. Unchanged acetochlor was not observed in the urine from any of the monkeys. Profiling of metabolites in the faeces was not conducted, because the percentage of the dose recovered was relatively low.

Three other polar peaks eluting at retention times much earlier than those of the identified metabolites, which reached maxima of 6.47% (P1), 6.85% (P2) and 3.77% (P3) of the profiles, were further characterized using acid pressure hydrolysis. The metabolite isolated from peak P1 was characterized as being hydroxylated at the 1-position of the ethyl group attached to the phenyl ring. Peak P2 consisted of at least two metabolites, one of which was consistent with hydroxylation at the 1-position of the ethyl group attached to the phenyl ring. The other metabolite gave two products upon hydrolysis. One product was consistent with a metabolite with hydroxylation at the methyl group attached to the phenyl ring, whereas the retention time of the other product did not correspond to any of the reference standards. Peak P3 also appeared to consist of at least two metabolites. Acid pressure hydrolysis of each isolated peak gave a metabolite with hydroxylation at the methyl group attached to the phenyl ring and a product with a retention time that did not correspond to any of the reference standards.

Acid pressure hydrolysis of metabolites in urine from monkeys in the low-dose group (1M) gave levels of 2-ethyl-6-methylaniline (EMA; EU reference no. 34) ranging from 65.1% to 68.8% of the mixture. By comparison, EMA levels from monkeys in the high-dose group (2M) ranged from 59.5% to 70.2%. The lowest conversion level was observed for monkey 2M1 of the high-dose group, which had the highest level of polar metabolites. In addition, the levels of EMA obtained from acid hydrolysis were 11–14% higher than the summed percentage distributions for the five metabolites

shown in Table 23 from the urine of a particular monkey. This indicates that the conversion of metabolites to EMA primarily accounts for the five identified metabolites.

In summary, acetochlor was extensively metabolized in the rhesus monkey primarily by two metabolic pathways involving either GSH conjugation or cytochrome P450 (CYP) oxidation followed by glucuronic acid conjugation (Kurtzweil, 2014).

The major metabolic pathways of acetochlor in the rat, mouse and rhesus monkey are illustrated in Figs 2, 3 and 4, respectively.

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with acetochlor (including skin and eye irritation and dermal sensitization studies) are summarized in Table 24.

(a) Oral administration

Rats

In an acute oral toxicity study, groups of five Sprague-Dawley rats of each sex received a single oral dose of undiluted acetochlor (purity 96.3%) at 1600, 2172, 2947 or 4000 mg/kg bw.

The incidences of mortality were as follows: 1600 mg/kg bw: 0/5 males and 2/5 females; 2172 mg/kg bw: 2/5 males and 2/5 females; 2947 mg/kg bw: 4/5 males and 5/5 females; and 4000 mg/kg bw: 5/5 males and 5/5 females. All deaths occurred within 2 days after administration. Signs of toxicity included salivation, lethargy, ptosis, perioral wetness and red discoloration around the mouth and fur. Signs of systemic toxicity included ataxia, tremors and convulsions; 5/6 rats with at least one of these clinical signs died. Diarrhoea and the presence of reddish material in the urine occurred sporadically. Most clinical signs of toxicity were observed on the day of dosing and/or the following day. At necropsy, gastrointestinal inflammation, presence of red-brown material in the intestines and discoloured hepatic tissue were observed.

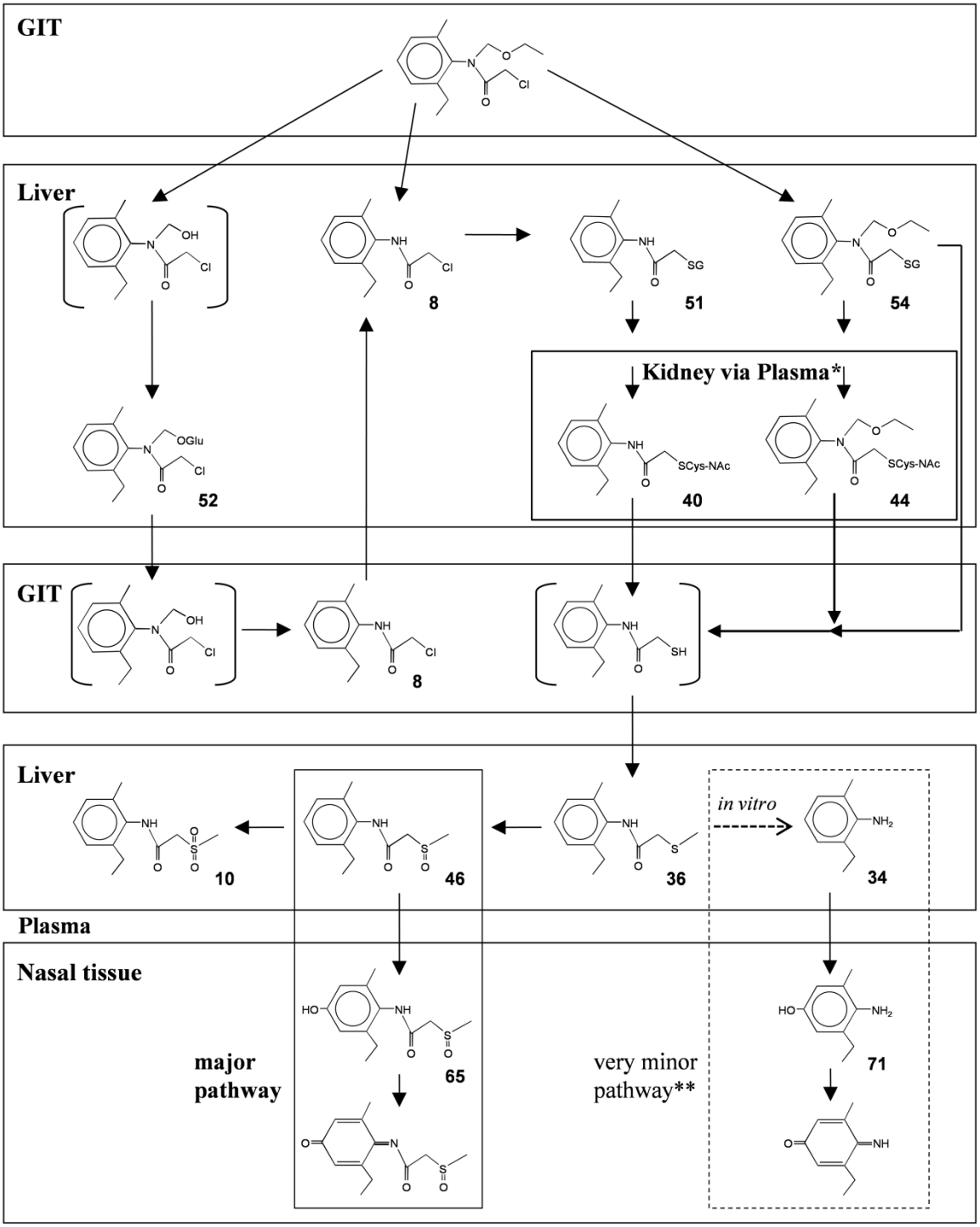
Under the study conditions utilized, the acute oral median lethal dose (LD_{50}) values for acetochlor were 2389 mg/kg bw for males, 1929 mg/kg bw for females and 2148 mg/kg bw for both sexes combined (Branch, 1982b).

In a second acute oral toxicity study, acetochlor (purity 90.5%) suspended in corn oil was administered as a single dose at a constant dose volume of 10 mL/kg bw to groups of five male and five female CD (Sprague-Dawley origin) rats at a dose of 2324, 3000, 3873 or 5000 mg/kg bw.

The incidences of mortality were as follows: 2324 mg/kg bw: 0/5 males and 0/5 females; 3000 mg/kg bw: 1/5 males and 1/5 females; 3873 mg/kg bw: 1/5 males and 2/5 females; and 5000 mg/kg bw: 4/5 males and 4/5 females. All deaths occurred within 6 days of administration. Clinical signs included decreased motor activity, lethargy, piloerection, diarrhoea, hunched posture, salivation and ungroomed appearance. Rats in the lowest-dose group recovered by day 2. At higher dose levels, recovery was not complete until day 12. Necropsy of decedents did not indicate a clear organ-specific toxicological effect. Necropsy of surviving animals on day 15 was unremarkable.

Under the study conditions utilized, the acute oral LD_{50} values for acetochlor were 4238 mg/kg bw for males and 4015 mg/kg bw for females (Cummins, 1986b).

Fig. 2. Major metabolic pathway of acetochlor in the rat[a]



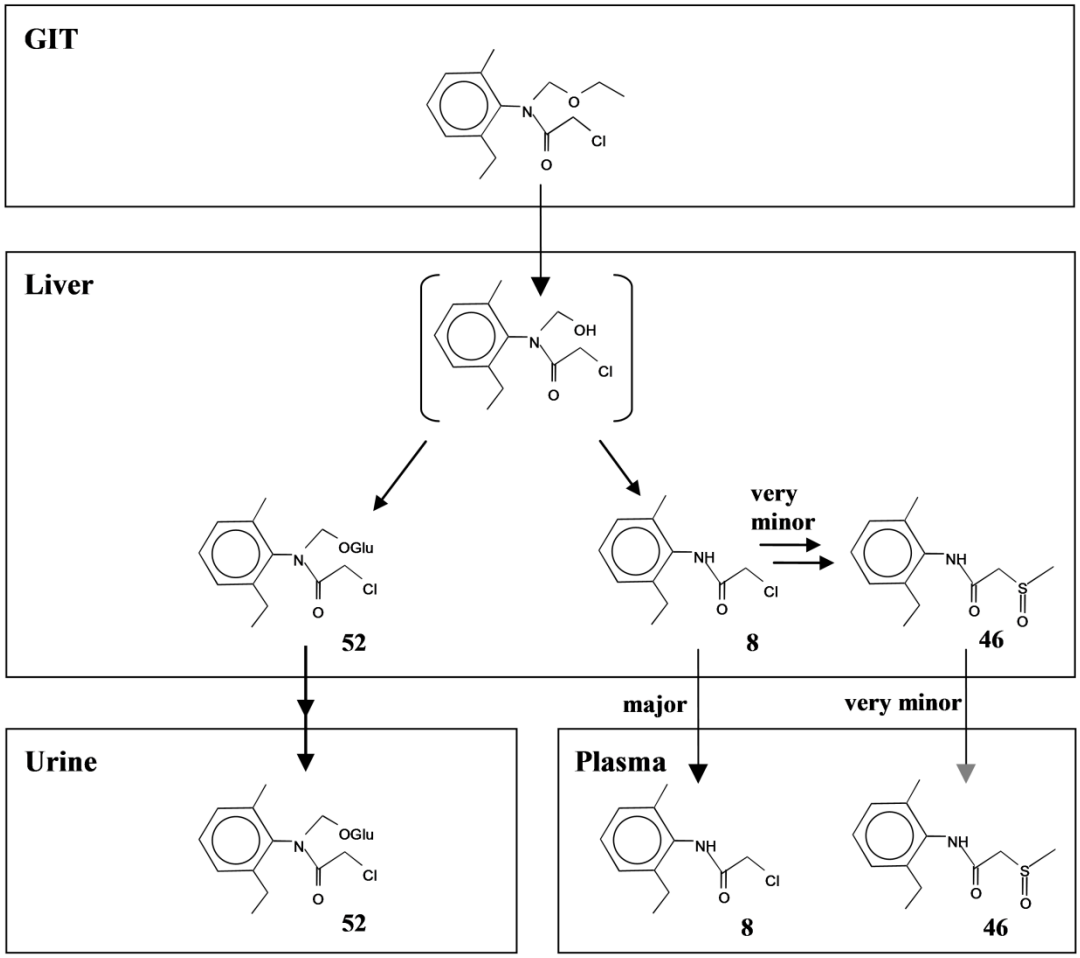
GIT: gastrointestinal tract

[a] This pathway represents a simplified version of a very complex metabolic pathway. A number of minor metabolites, including those formed from *S*-methylation, thioethers, disulfides and those formed from *S*-oxidation and/or hydroxylation of the alkyl group side-chains, are not shown in the figure. All numbers shown in figure are EU reference numbers.

* Kidney not actually analysed, but mercapturate formation can occur in the kidney based on similar compounds and then be excreted in urine.

** Formation of EU reference no. 71 from EU reference no. 36 via EU reference no. 34 was observed in in vitro experiments conducted primarily in the absence of NADPH. However, the direct precursor to EU reference no. 71, EMA (EU reference no. 34), was not identified in plasma in rat metabolism studies. Work from a different in vitro study has also shown that EMA (EU reference no. 34) can be formed directly from EU reference no. 8 (not shown). However, the vast majority of benzoquinoneimine comes from the sulfoxide (EU reference no. 46).

Fig. 3. Major metabolic pathway of acetochlor in the mouse[a]



GIT: gastrointestinal tract

[a] Glucuronides formed following hydroxylation of the alkyl group side-chains were also observed, but are not shown in this figure. All numbers shown in figure are EU reference numbers.

(b) Dermal application

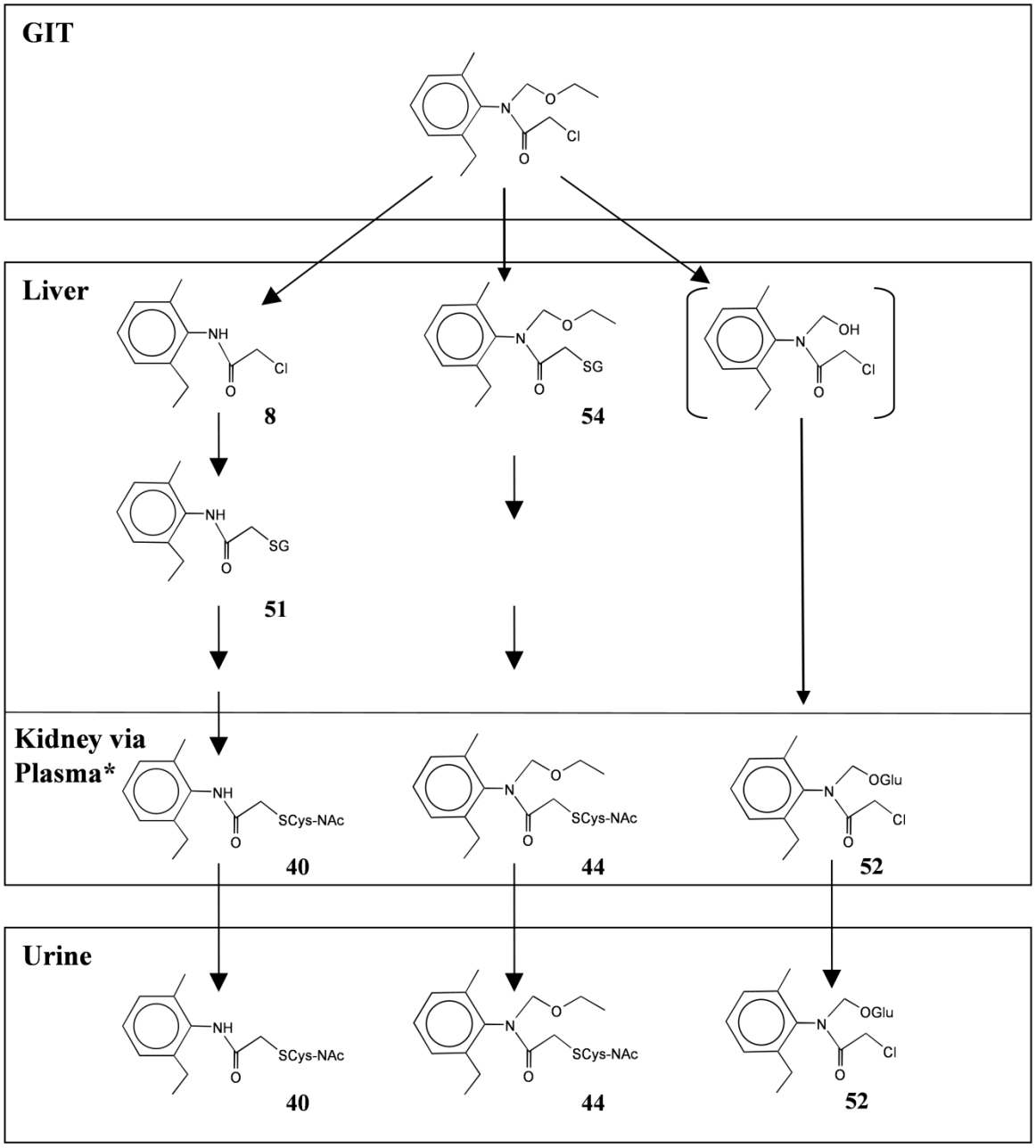
Rabbits

In an acute dermal toxicity study, undiluted acetochlor (purity 96.3%) was applied at 5000 mg/kg bw to the shaved and abraded dorsal skin of five male and five female New Zealand White rabbits, with 24-hour occluded exposure. Because 4/5 males and 4/5 females died, doses of 2500 and 3536 mg/kg bw were applied onto the intact and abraded skin of two groups of four male and four female rabbits. There was no mortality at 2500 mg/kg bw, and at 3536 mg/kg bw, 0/4 males and 1/4 females died. Clinical signs included convulsions, tremors, ataxia, bruxism, salivation, prostration, erythema, oedema and epidermal desquamation of the skin in the exposed area.

Under the study conditions utilized, the acute dermal LD_{50} for the combined sexes was calculated to be 4166 mg/kg bw. Acute dermal LD_{50} values could not be calculated for the separate sexes, but lethality data indicate that both of these values were between 3536 and 5000 mg/kg bw (Branch, 1982a).

In an acute dermal toxicity study in rats, acetochlor (purity 90.5%) was applied at 2.0 mL/kg (= 2.06 g/kg) bw to the shaved dorsal skin of five male and five female Charles River CD rats, with 24-hour occluded exposure.

Fig. 4. Major metabolic pathways of acetochlor in the rhesus monkey[a]



GIT: gastrointestinal tract

[a] A number of other minor metabolites were also observed, but are not shown in this figure. All numbers shown in figure are EU reference numbers.

* Kidney and plasma not actually analysed, but mercapturate formation can occur in the kidney based on similar compounds and then be excreted in urine.

There was no mortality. The only possible signs of systemic toxicity were pigmented orbital secretion in two rats on days 2 and/or 3 and loss of corneal lustre in one male on day 2. Dermal responses at the application site consisted of well-defined erythema (all animals on days 2–4) and exfoliation (days 4–5), with full recovery by day 7. Necropsy at day 15 showed no evidence of any treatment effect.

Under the study conditions utilized, the acute dermal LD_{50} of acetochlor in the rat was greater than 2.06 g/kg bw (Cummins, 1986a).

Table 24. Results of studies of acute toxicity of acetochlor

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	Sprague-Dawley	Male Female	Oral	96.3	2 389 (male) 1 929 (female) 2 148 (combined)	Branch (1982b)
Rat	Sprague-Dawley (CD)	Male Female	Oral	90.5	4 238 (male) 4 015 (female)	Cummins (1986b)
Rabbit	New Zealand White	Male Female	Dermal	96.3	4 166 (combined)	Branch (1982a)
Rat	Sprague-Dawley	Male Female	Dermal	90.5	> 2 060	Cummins (1986a)
Rat	Sprague-Dawley	Male Female	Inhalation	92.5	> 3.0	Bechtel (1988)
Rat	Wistar	Male Female	Inhalation	89.4	3.99 (female) > 4.46 (male)	Brammer (1989)
Rat	Wistar	Male Female	Inhalation	90.4	> 2.07	Duerden & Lewis (1990)
Rabbit	New Zealand White	Male Female	Skin irritation	96.3	Mildly irritating	Branch (1982d)
Rabbit	New Zealand White	Male	Skin irritation	89.4	Severely irritating	Barlow & Ishmael (1989)
Rabbit	New Zealand White	Male Female	Eye irritation	96.3	Minimally irritating	Branch (1982c)
Rabbit	New Zealand White	Female	Eye irritation	89.4	Minimally irritating	Pemberton & Ishmael (1989)
Guinea-pig	Hartley	Male Female	Skin sensitization	96.3	Positive (Buehler test)	Auletta (1983)
Guinea-pig	Dunkin Hartley	Male Female	Skin sensitization	89.4	Positive (maximization test)	Botham & Ishmael (1989
Mouse fibroblast	Mouse (Balb/c 3T3 clone A31)	Not applicable	Phototoxicity	96.1	Positive	Bowen (2014)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

(c) Exposure by inhalation

Rats

In an acute inhalation toxicity study, five male and five female Sprague-Dawley rats were exposed (whole body) for 4 hours to an acetochlor (purity 92.5%) aerosol at a mean analytical concentration of 3.0 mg/L.

There was only one particle size measurement (mass median aerodynamic diameter [MMAD] = 2.1 µm; geometric standard deviation [GSD] = 1.8; 99.6% of the particles were < 10 µm) during the study. There was no mortality. Salivation and perinasal wetness were noted during exposure. The only observation made immediately after exposure was perioral wetness. All rats appeared normal during the post-exposure period. No gross necropsy abnormalities were noted.

Under the study conditions utilized, the 4-hour acute inhalation median lethal concentration (LC_{50}) was greater than 3.0 mg/L in rats (Bechtel, 1988).

In a second acute inhalation toxicity study, a group of five male and five female rats was exposed (nose only) for 4 hours to acetochlor (purity 89.4%) at a concentration of 4.46 mg/L. Additional groups of five females were similarly exposed to concentrations of 1.81 and 3.57 mg/L.

At 4.46 mg/L, four female rats were found dead on day 3. The remaining female and one male were killed in extremis on days 3 and 8, respectively. There were no mortalities in females exposed to 1.81 or 3.57 mg/L. Clinical signs consistent with neurological effects and respiratory tract irritation were apparent in all treated groups during and after exposure.

Under the study conditions utilized, the 4-hour inhalation LC_{50} was calculated to be 3.99 mg/L for female rats and more than 4.46 mg/L for male rats (Brammer, 1989).

In a third acute inhalation toxicity study, five male and five female Wistar rats were exposed (nose only) to acetochlor (purity 90.4%) at an analytical concentration (± standard deviation) of 2.07 (± 0.26) mg/L. The MMAD was 1.27 µm, and the GSD was 1.50. There was no mortality. All rats showed clinical signs, which included activity decrease and hunched posture, as well as some signs (lacrimation and abnormal respiratory noise) consistent with respiratory tract irritation.

Under the study conditions utilized, the 4-hour inhalation LC_{50} was greater than 2.07 mg/L in rats (Duerden & Lewis, 1990).

(d) Dermal irritation

In a primary skin irritation study with three male and three female New Zealand White rabbits, 0.5 mL aliquots of acetochlor (purity 96.3%) were applied to each of four sites (two intact, two abraded) per animal, with 24-hour occluded exposure. Skin reactions were scored at approximately 1 hour, 48 hours and 5 days after removal of the patch.

The overall mean scores for intact skin were 0.08 for erythema and 0.00 for oedema at a 24-hour exposure time.

Under the study conditions utilized, acetochlor was mildly irritating to the skin of rabbits (Branch, 1982d).

In a second primary skin irritation study, 0.5 mL samples of undiluted acetochlor (purity 89.4%) were applied to a 2.5 cm × 2.5 cm site on the left flank of each of six male New Zealand White rabbits. Exposure was for 4 hours.

At 30–60 minutes after removal of the dressing, there was severe (grade 4) erythema and slight (grade 2) to severe oedema (grade 4) at all six sites. On day 3, two sites scored 4 for erythema,

and three sites scored 4 for oedema. The primary dermal irritation index (mean of scores for 30–60 minutes and days 1, 2 and 3) was 6.13. On day 7, two sites scored 4 for erythema, whereas all six sites scored 0 for oedema. On day 21, one site scored 3 for erythema; the other five scored 0. One of six animals still showed grade 2 erythema on day 29. Signs of irritation persisting 29 days after application included thickening of the skin and increased hair growth on the application site. Histopathological examination revealed a marked increase in the number of hair follicles. One animal also showed subepithelial fibrosis.

Under the study conditions utilized, acetochlor was severely irritating to rabbit skin (Barlow & Ishmael, 1989).

The submitter stated that the test material used in the Branch (1982d) study is more representative of the technical material currently being produced.

(e) Ocular irritation

Rabbits

In a primary eye irritation study, 0.1 mL of acetochlor (96.3%) was instilled into the conjunctival sac of the right eye of each of nine New Zealand White rabbits. Three eyes (from one male and two females) were washed out after 25 seconds, and the eyes of the other six animals (three of each sex) remained unwashed after exposure.

Only conjunctival effects (which included redness, chemosis and discharge; however, the report does not break down the effects by these categories) were observed in rabbits with unwashed eyes. The mean maximum total score (at 24 hours, with a maximum possible score of 110) was 1.67, with one rabbit scoring 4 at 24 hours. All scores were 0 by day 4.

Under the study conditions utilized, acetochlor was minimally irritating to the eyes of rabbits (Branch, 1982c).

In a second primary eye irritation study, 0.01 mL of undiluted acetochlor (89.4%) was instilled in the eye of one female New Zealand White rabbit. As the rabbit showed a slight initial pain reaction and no irritation, this was followed by the instillation of 0.1 mL of undiluted acetochlor (purity 89.4%) into the eye of a second rabbit before the study proceeded to a full group of six rabbits.

There were no effects on the cornea or iris. At 1 hour, all eyes showed slight (score of 1) conjunctival redness, three eyes showed slight (score of 1) chemosis, and all eyes showed severe (score of 3) discharge. At 24 hours, one eye showed slight redness; the mean irritation score at 24 hours was 0.06 (maximum 110).

Under the study conditions utilized, acetochlor was minimally irritating to the eyes of rabbits (Pemberton & Ishmael, 1989).

(f) Dermal sensitization

In a dermal sensitization study, a group of 10 Hartley albino guinea-pigs was evaluated in a nine-induction Buehler protocol. A group of five males and five females was exposed to 0.2 mL of undiluted acetochlor (purity 96.3%) for 6 hours/day, 3 days/week, for 3 weeks. Two weeks after the last induction dose, 0.2 mL of undiluted acetochlor was applied to previously untreated areas on the guinea-pigs that received the induction doses and to three male and three female naive animals.

All 10 previously induced animals showed positive responses at 24 and 48 hours; 1/6 and 0/6 naive animals showed a positive response at 24 hours and 48 hours, respectively.

Under the study conditions utilized, acetochlor was positive as a dermal sensitizer (Auletta, 1983).

In a second dermal sensitization study, acetochlor (purity 89.4%) was evaluated in a guinea-pig maximization test. Dose levels used for induction and challenge were based on the results of a preliminary irritation study. For induction, the following three intradermal injections were made on each side of the midline of the scapular region: (1) A 1:1 mixture of Freund's Complete Adjuvant (FCA) + corn oil; (2) 10% (weight per volume [w/v]) acetochlor in corn oil; and (3) 10% (w/v) acetochlor in a 1:1 mixture of FCA + corn oil. One week later, there was topical exposure to the undiluted test substance for 48 hours under occlusive dressing. Challenge was 2 weeks later to 0.05–0.1 mL of undiluted acetochlor (shorn left flank) and to a 30% (w/v) preparation of acetochlor in corn oil (shorn right flank). Sites exposed to undiluted acetochlor showed an extreme response (18/19 positive versus 0/10 controls), whereas sites exposed to the 30% (w/v) preparation of acetochlor in corn oil showed a strong response (erythema in 13/19 versus 0/10 controls).

Under the study conditions utilized, acetochlor was positive as a dermal sensitizer (Botham & Ishmael, 1989).

(g) Phototoxicity

Acetochlor was assayed for phototoxicity to Balb/c 3T3 fibroblast cells using the neutral red uptake assay.

Based on the results of this assay, acetochlor was considered a probable phototoxicant in this in vitro test system, when tested up to the limit of toxicity, according to OECD Test Guideline 432 (Bowen, 2014).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 91-day dietary toxicity study, acetochlor (purity 91.3%) was administered in the diet to 20 Swiss albino CD-1 mice of each sex per group at a dose level of 0, 800, 2000 or 6000 ppm (equivalent to 0, 120, 300 and 900 mg/kg bw per day, respectively) for 91 days. Homogeneity and stability of diets at room temperature were measured. The test substance intake was not reported. Clinical chemistry and haematological parameters were not evaluated. Urine analysis and ophthalmological examination were not performed.

No clinical abnormalities were observed throughout the course of the study. One control female mouse was found dead after 48 days on test. One high-dose female mouse was killed after 7 days on test, and one high-dose female mouse died after 12 days on test. Statistically significant decreases in feed consumption were observed in all dose groups at various intervals, but these were not considered to be compound related because the mean daily consumption of treated mice was similar to that of the untreated mice. The feed efficiency in both sexes at the various dose levels was not remarkably different from that of the control groups. In the males and females, body weights of mice in the low-dose group were comparable with those of controls. In males, a statistically significant decrease in body weight was observed in the mid-dose group compared with the control group at weeks 10 and 12, and a statistically significant decrease was observed in the high-dose group from week 1 throughout the remainder of the study. In the females, no statistically significant decrease in body weight was observed in the mid-dose group throughout the study duration. A statistically significant decrease in body weight was observed in the high-dose females from weeks 5 through 13. The changes in body weight at the high dose are considered to be test substance related.

Statistically significant changes in absolute mean organ weights included a decrease in brain weights in males at the high dose, an increase in liver weights in males at the middle and high doses and in females at the low and middle doses, an increase in kidney weights in males at the low and middle doses and a decrease in kidney weights in females at the high dose, and a decrease in gonad weights in females at the high dose. Statistically significant changes in relative mean organ weights included an increase in relative brain weights in males at the high dose, an increase in relative heart

weights in males at the middle and high doses, an increase in relative liver weights in males at the middle and high doses and in females at all dose levels (not dose related), and an increase in relative kidney weights in males at all dose levels and in females only at the middle dose level.

No test substance–related gross or microscopic pathological findings were noted in any of the mice that died or were killed during the study.

Based on the results of the 91-day dietary toxicity study in mice, the no-observed-adverse-effect level (NOAEL) was 2000 ppm (equivalent to 300 mg/kg bw per day), based on decreased body weights observed at 6000 ppm (equivalent to 900 mg/kg bw per day) (Ahmed, 1981a).

Rats

In a 4-week oral toxicity study, acetochlor (purity not reported) was administered in the diet to 10 CD (Sprague-Dawley-derived) rats of each sex per group at a dose level of 0, 300, 600, 1200, 2400, 4800 or 9600 ppm (equal to 0, 33.3, 67.7, 132, 267, 519 and 1012 mg/kg bw per day for males and 0, 35.2, 69.3, 139, 279, 539 and 1081 mg/kg bw per day for females, respectively) for 29 days.

Signs of reaction to treatment comprised piloerection and loss of skin elasticity, mainly observed at the two highest dose levels. One male of the highest-dose group was killed in extremis on day 12 following sustained weight loss; this death was attributed to a congenital defect of the brain unrelated to treatment. Five animals died during the blood sampling in week 4 of treatment. Two of the deaths at the highest dose may be considered to be indirectly associated with treatment. Changes in body weight, feed consumption, haematology and blood biochemistry are summarized in Tables 25 and 26.

Table 25. Consumption, body weight, haematology and blood biochemistry data for male rats

Significant findings	0 ppm	300 ppm	600 ppm	1 200 ppm	2 400 ppm	4 800 ppm	9 600 ppm
Feed consumption (% of control)	100	97	100	97	90	82	59
Water consumption (% of control)	100	97	106	106	98	89	80
Body weight gain (0–28 days, g)	198	179*	182*	183*	149**	125**	40**
Packed cell volume (%)	47	47	46	47	46	45*	45**
Haemoglobin (g%)	15.9	16.2	15.5	16.0	15.9	15.4	15.1**
MCV (μm^3)	63	62	62	63	61	62	59**
MCH (pg)	22	22	21	21	21	21	20**
Prothrombin time (s)	14	14	13.6	13.5*	13.3**	13.3**	12.9**
GGT (IU/L)	2	3	2	2	3	7**	30**
Plasma butyrylcholinesterase (IU/L)	486	555	543	490	448	523	840**
Plasma acetylcholinesterase (IU/L)	575	606	580	566	521	557	744**

GGT: gamma-glutamyltranspeptidase; IU: International Units; MCH: mean corpuscular haemoglobin; MCV: mean cell volume; ppm: parts per million; *: $P < 0.05$, **: $P < 0.01$
Source: Broadmeadow (1985)

Table 26. Consumption, body weight, haematology and blood biochemistry data for female rats

Significant findings	0 ppm	300 ppm	600 ppm	1 200 ppm	2 400 ppm	4 800 ppm	9 600 ppm
Feed consumption (% of control)	100	104	102	102	97	92	75
Water consumption (% of control)	100	108	105	108	97	111	109
Body weight gain (0–28 days, g)	87	88	85	81	67**	59**	29**
Packed cell volume (%)	48	48	47	49	48	47	45**
Haemoglobin (g%)	16.1	15.8	15.8	16.2	16.0	15.7	14.8**
MCHC (%)	34	33	33	33	33	34	33**
MCV (μm^3)	63	63	63	63	63	65	61*
MCH (pg)	21	21	21	21	21	22	20**
WBC (1 000/mm^3)	10.5	10.8	11.1	12.1	12.2	10.6	15.6**
Prothrombin time (s)	14.1	13.9	13.8	14.0	13.7	13.4**	13.0**
ALP (IU/L)	95	92	94	87	99	107	117*
GGT (IU/L)	3	3	3	3	4	11**	26**
Plasma butyrylcholinesterase (IU/L)	3 135	3 166	2 485	2 960	2 248**	2 081**	1 886**
Plasma acetylcholinesterase (IU/L)	1 858	1 872	1 589	1 592	1 368**	1 231**	1 287**
RBC acetylcholinesterase (IU/L)	2 218	1 938**	1 946**	2 185	1 755**	2 268	2 023*

ALP: alkaline phosphatase; GGT: gamma-glutamyltranspeptidase; IU: International Units; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean cell volume; ppm: parts per million; RBC: red blood cells; WBC: white blood cells; *: $P < 0.05$, **: $P < 0.01$
Source: Broadmeadow (1985)

Decreased body weight gain was noted in both sexes at 2400 ppm and above. Slight but statistically significant decreases in weight gain were also noted in males at 300, 600 and 1200 ppm, but were not dose related. A dose-related shortening of prothrombin time was recorded in males receiving 1200 ppm or above and in females receiving 4800 ppm or above. A marked elevation of GGT activity was observed in all animals treated with 4800 ppm or above. Plasma butyrylcholinesterase and acetylcholinesterase activities were lower than control values in females treated at 2400 ppm or above and higher than control values in males treated with 9600 ppm. Red blood cell acetylcholinesterase activity tended to be lower than control values in females, but these differences were not dose related and were considered not to be attributable to treatment. Brain cholinesterase activity was unaffected. There was a dose-related increase in relative liver and kidney weights in males and females at 2400 ppm and above. Statistically significant changes in spleen and testes weights in males and adrenal weights in females were also noted at 4800 and/or 9600 ppm. No macroscopic pathological changes were detected in rats killed after 4 weeks of treatment that could be attributed to the administration of acetochlor.

In conclusion, the results from this range-finding study indicate that the liver is a target organ of acetochlor. The NOAEL was 600 ppm (equal to 67.7 mg/kg bw per day), based on slight decreases in body weight gain and prothrombin time observed at 1200 ppm (equal to 132 mg/kg bw per day) (Broadmeadow, 1985).

In a 91-day oral toxicity study, acetochlor (purity 91.3%) was administered in the diet to 30 CD (Sprague-Dawley-derived) rats of each sex per group at a dose level of 0, 800, 2000 or 6000 ppm (equal to 0, 53.2, 134 and 460 mg/kg bw per day for males and 0, 69.3, 173 and 530 mg/kg bw per day for females, respectively).

Table 27. Mean body weight data in 91-day study in rats

Dietary concentration (ppm)	Body weight (g)												
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**	**Week 8**	**Week 9**	**Week 10**	**Week 11**	**Week 12**	**Week 13**
Males													
0	181	225	272	310	344	365	376	398	417	433	444	460	470
800	174	215*	261*	297*	327*	353	355	370*	383*	402*	415*	431*	439*
2 000	167*	211*	251*	288*	318*	338*	348*	364*	373*	387*	403*	420*	427*
6 000	140*	169*	217*	247*	267*	286*	300*	313*	324*	343*	354*	363*	375*
Females													
0	137	156	176	191	202	213	220	228	234	241	246	253	257
800	135	151	167*	183*	193	202*	208	216	220*	226*	231*	236*	240*
2 000	135	151	168*	180*	195	201*	204*	214*	218*	224*	228*	233*	236*
6 000	123*	140*	162*	175*	185*	191*	193*	199*	204*	207*	214*	216*	218*

ppm: parts per million; * $P \leq 0.05$ (Student's *t*-test)
Source: Ahmed (1980a)

No abnormal clinical findings were observed and no animals died during the course of the study. Decreased feed consumption was noted at all dose levels. Student *t*-test analysis of the data showed that body weight was statistically significantly decreased for both sexes in all groups at several time points (Table 27). However, use of another statistical analysis, Dunnett's test, changes the interpretation of these data, especially at the low dose level in females, as the significance becomes limited only to weeks 6 and 12 compared with weeks 3, 4, 6 and 9 through 13 using Student's *t*-test.

Treatment-related changes in body weight, feed consumption, haematology, blood biochemistry and organ weights are summarized in Table 28.

A number of changes in haematological, clinical chemistry and urine analysis parameters were observed in all groups, but were not considered toxicologically significant, as the changes were small in magnitude and there was no underlying histopathology. Relative liver weight was increased by 18% in males and females at 6000 ppm, but remained without associated histopathological findings. Necropsy did not reveal any treatment-related lesions.

Based on the results of the 91-day toxicity study in rats, a NOAEL could not be identified, as effects were observed at all doses. The lowest-observed-adverse-effect level (LOAEL) was 800 ppm (equal to 53.2 mg/kg bw per day), based on marginally decreased feed consumption and body weight gain observed at the lowest dose tested (Ahmed, 1980a).

In a second 13-week oral toxicity study, acetochlor (purity 90.5%) was administered in the diet to 10 CD (Sprague-Dawley-derived) rats of each sex per group at a dose level of 0, 20, 200 or 2000 ppm (equal to 0, 1.60, 16.1 and 161 mg/kg bw per day for males and 0, 1.92, 18.8 and 191 mg/kg bw per day for females, respectively) for 13 weeks.

There were no treatment-related mortalities or clinical signs of toxicity. Two females (one in the control group and one in the 20 ppm group) died during routine blood sampling. Treatment-related changes in body weight gain, feed consumption, some blood biochemistry parameters and organ weights are summarized in Table 29. There was no effect on survival, and there were no treatment-related haematology, necropsy or histopathology findings. Ophthalmoscopic examination after 12 weeks of treatment did not reveal any treatment-related ocular lesions. Urine analysis parameters were not altered by treatment. The body weight gains of rats receiving 2000 ppm were significantly lower

Table 28. Body weight, haematology and blood biochemistry data and gross pathology findings in a 91-day study in rats

	Males				Females			
Significant findings	**0 ppm**	**800 ppm**	**2 000 ppm**	**6 000 ppm**	**0 ppm**	**800 ppm**	**2 000 ppm**	**6 000 ppm**
Body weight, 91 days (g)	470	439*	427*	375*	257	240*	236*	218*
Feed consumption, 91 days (g/week)	169	169	161	153*	124	126	118	110*
Haemoglobin, 91 days (g/dL)	14.4	14.6	14.5	13.8	14.6	14.0	14.6	13.5*
ALP, 91 days (IU/L)	150	141	114*	103*	67	87	53	53
ALT, 91 days (IU/L)	48	42	31*	45	46	31*	37	30*
Cholesterol, 91 days (mg/dL)	88	99	98	113*	99	106	99	104
Potassium, 91 days (mEq/L)	5.6	5.8	5.4	5.5	5.6	5.9	6.0*	6.0*
Total bilirubin, 91 days (mg/dL)	0.5	0.4	0.6	0.6*	0.6	0.5*	0.6	0.6
Albumin, 91 days (g/dL)	4.7	4.6	4.4*	4.2*	4.5	4.3	4.2	4.4
LDH, 91 days (IU/L)	392	412	410	426*	517	534	480	475
Total protein (g/dL)	7.2	7.1	6.8*	6.7*	7.2	7.1	6.9	6.7*
Absolute brain weight, 91 days (g)	2.11	2.03	2.01*	1.94*	1.86	1.89	1.83	1.88
Absolute heart weight, 91 days (g)	1.42	1.38	1.36	1.20*	0.85	0.83	0.80	0.76*
Relative brain weight, 91 days	0.005	0.005	0.005	0.006*	0.008	0.009*	0.008	0.009*
Relative liver weight, 91 days	0.033	0.030	0.033	0.039*	0.030	0.030	0.030	0.038*
Relative kidney weight, 91 days	0.007	0.007	0.008	0.009*	0.007	0.008*	0.008	0.008*
Relative gonad weight, 91 days	0.008	0.008	0.008	0.010*	0.000	0.001*	0.001*	0.001*

ALP: alkaline phosphatase; ALT: alanine transaminase; Eq: equivalents; IU: International Units; LDH: lactate dehydrogenase; *: $P < 0.05$ (Dunnett's test)
Source: Ahmed (1980a)

than those of controls during the first half of the treatment period ($P < 0.001$). Males receiving 200 ppm were similarly affected ($P < 0.05$) during this period. After 13 weeks of treatment, the body weight gains of males and females receiving 2000 ppm were, respectively, 14% and 23% lower than those of controls, and the body weight gains of males receiving 200 ppm were 7% lower than those of controls. The decreased body weights of males at 200 ppm were not considered to be adverse.

Male and female rats fed 2000 ppm acetochlor exhibited slightly increased plasma urea and cholesterol concentrations. Marginally lower plasma potassium and calcium concentrations were observed in females at 2000 ppm. The changes in clinical chemistry parameters in males and females at 2000 ppm were not considered to be adverse, as the magnitude of change was marginal and not associated with any histopathological findings. Among females receiving 2000 ppm, plasma butyrylcholinesterase and plasma acetylcholinesterase activities were low in comparison with contemporary control values. Among male rats treated at 2000 ppm, the plasma cholinesterase activities were 86% (butyryl) or 89% (acetyl) of those of control males, but the intergroup differences were not statistically significant ($P > 0.05$). The level of activity of erythrocyte acetylcholinesterase

Table 29. Body weight, blood biochemistry and gross pathology findings in a 13-week study in rats

	Males				Females			
Significant findings	**0 ppm**	**20 ppm**	**200 ppm**	**2 000 ppm**	**0 ppm**	**20 ppm**	**200 ppm**	**2 000 ppm**
Body weight gain, 0–7 weeks (g)	283	288	259*	234**	128	123	138	98**
Body weight gain, 7–13 weeks (g)	63	64	62	61	20	15	23	16
Body weight gain, 0–13 weeks (g)	345	353	321	295**	147	138	161	113**
Feed consumption, 1–7 weeks (g/week)	192	198	188	176*	145	142	147	131**
Feed consumption, 8–13 weeks (g/week)	192	192	182	174*	141	137	145	124**
Feed consumption, 1–13 weeks (g/week)	192	195	185	175**	143	139	146	128**
Urea, 13 weeks (mg/L)	36	37	35	52**	36	39	33	46*
Cholesterol, 13 weeks (mg/L)	63	68	71	83	73	69	77	88**
Potassium, 13 weeks (mEq/L)	3.6	3.5	3.7	3.8	3.6	3.5	3.6	3.3*
Calcium, 13 weeks (mEq/L)	5.0	5.0	5.0	5.1	5.2	5.2	5.2	5.1*
Plasma acetylcholinesterase, 13 weeks (IU/L)	873	810	823	773	4 288	4 608	3 898	3 277*
Plasma butyrylcholinesterase, 13 weeks (IU/L)	1 267	1 121	1 053*	1 084	4 389	4 826	4 299	3 271**
Relative liver weight, 13 weeks (%)	3.70	3.77	3.89	4.42**	3.88	3.88	3.96	4.51**
Relative kidney weight, 13 weeks (%)	0.756	0.780	0.745	0.868**	0.823	0.836	0.800	0.879

Eq: equivalents; IU: International Units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Broadmeadow (1986a)

was unaffected. The brain acetylcholinesterase activity of males receiving 2000 ppm was significantly lower than that of control males ($P < 0.001$). The brain cholinesterase activity at 2000 ppm in females was unaffected.

At the high dose level, statistically significant increases in relative liver and brain weights were observed in both sexes, a statistically significant increase in relative kidney weight was observed only in males and a statistically significant increase in relative spleen weight was observed only in females. There was no evidence of any treatment-related histopathological effects.

Based on the results of the 13-week dietary toxicity study in rats, the NOAEL was 200 ppm (equal to 16.1 mg/kg bw per day), based on statistically significant decreases in feed consumption and body weight gain (14% in males and 23% in females) observed at 2000 ppm (equal to 161 mg/kg bw per day) (Broadmeadow, 1986a).

Dogs

Acetochlor (purity 90.5%) was administered orally in gelatine capsules to groups of four male and four female Beagle dogs at a dose level of 0, 2, 10 or 60 mg/kg bw per day for 91 days. A similarly constituted control group received empty gelatine capsules only.

There was no mortality. Clinical signs of reaction to treatment were confined to dogs receiving 60 mg/kg bw per day and comprised diarrhoea, mucus in the faeces, salivation, emesis and vocalization during defecation. The incidence of these signs was higher in females than in males.

Clinical, haematology, blood biochemistry and gross pathology parameters for which treatment-related changes were observed are reported in Table 30.

Table 30. Clinical toxicology, clinical biology and gross pathology findings in a 91-day study in dogs

	Males				Females			
Significant findings	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**60 mg/kg bw per day**	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**60 mg/kg bw per day**
Body weight change, 1–13 weeks (kg)	2.5	2.7	2.4	1.7*	2.0	2.4	1.8	1.2*
Haematocrit, 12 weeks (%)	43	44	46	44	47	46	47	41**
Haemoglobin, 12 weeks (g/dL)	15.3	15.3	16.4	15.6	16.3	16.3	16.8	14.8**
RBC, 12 weeks ($\times 10^6$/mm^3)	6.40	6.43	6.87	6.43	7.03	6.73	6.99	6.08*
Glucose, 6 weeks (g/dL)	103	101	98	91**	99	98	100	93
Glucose, 12 weeks (g/dL)	106	106	101	92**	104	102	97	91*
ALT, 12 weeks (IU/L)	54	44	40	131*	45	36	42	92**
Relative liver weight, 13 weeks (%)	3.0	3.0	3.0	3.4*	2.9	3.1	3.1	3.4*

ALT: alanine transaminase; bw: body weight; IU: International Units; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Broadmeadow (1986b)

Low feed consumption was recorded for dogs of all groups, associated with feed withdrawal before routine clinical pathology investigations, and was more pronounced in dogs treated at 60 mg/kg bw per day. In the high-dose group, body weight gain was significantly lower than in control animals from week 2 to the end of the treatment. Ophthalmoscopic examinations did not reveal any treatment-related lesions. Urine analysis and examination of faeces for occult blood did not indicate any changes that were attributable to treatment. Haematocrit, haemoglobin concentration and erythrocyte count were reduced compared with control values in females dosed at 60 mg/kg bw per day after 12 weeks of treatment. Reduced blood glucose concentrations after 6 (males only) and 12 weeks of treatment were seen in animals dosed at 60 mg/kg bw per day when compared with controls. Plasma alanine transaminase (ALT) activity was increased slightly in the high-dose animals after 12 weeks. Other statistically significant differences from control values were not considered to be dose related or were minor in nature. The brain cholinesterase activity was not affected by treatment. Increased liver weights (relative to body weights) were observed in dogs receiving 60 mg/kg bw per day. Necropsy and histopathological examination did not reveal any treatment-related lesions.

Based on the results of the 91-day oral toxicity study in dogs, the NOAEL was 10 mg/kg bw per day, based on decreased body weight gain, clinical signs, increased relative liver weights, increased serum ALT activity and decreased blood glucose concentration in both sexes and reduced haemoglobin, haematocrit and erythrocyte counts in females observed at 60 mg/kg bw per day (Broadmeadow, 1986b).

Acetochlor (purity 91.3%) was administered orally to groups of six male and six female Beagle dogs in gelatine capsules for 119 days at a final dose level of 0, 25, 75 or 200 mg/kg bw per day. As these dogs exhibited vomiting at higher doses in an earlier pilot study, it was decided to condition the dogs at the start of the study for 3 weeks as follows: the low-dose group received 25

mg/kg bw per day for the duration of the study; the mid-dose group (75 mg/kg bw per day) received 25 mg/kg bw per day during the first week, 50 mg/kg bw per day during the second week and 75 mg/kg bw per day during the third through the 17th weeks; and the high-dose group (200 mg/kg bw per day) received 50 mg/kg bw per day during the first week, 100 mg/kg bw per day during the second week and 200 mg/kg bw per day during the third through the 17th weeks. Ophthalmoscopy was not performed.

Twelve dogs died or were killed in a moribund condition during the study, as follows: one male in the 75 mg/kg bw per day dose group and five males and six females in the 200 mg/kg bw per day group. Severe clinical signs, such as emaciation, loss of appetite, bloody diarrhoea, proteinuria, haematuria, decreased body weight and a very high mortality rate, indicated that the dose level of 200 mg/kg bw per day was too high for a meaningful toxicological evaluation. The mean feed consumption of the 25 and 75 mg/kg bw per day groups was slightly reduced (in a dose-related manner) in males compared with the controls from week 6 through to the end of the study. A slight decrease in mean feed consumption was also observed in the 75 mg/kg bw per day female group. Statistical significance was not achieved for reduced feed consumption at 25 or 75 mg/kg bw per day. The mean body weights for the dogs receiving 25 or 75 mg/kg bw per day did not show any statistically significant differences compared with the controls.

Many of the haematology and blood biochemistry parameters were statistically significantly different from control values (Table 31). The blood biochemistry parameter that was most frequently higher than the historical control range of the laboratory was ALT. ALT activities were increased by approximately 2.3-fold in males at 25 and 75 mg/kg bw per day and 2.2-fold in females at 75 mg/kg bw per day after 119 days of treatment. Relative liver weight was increased by 26% in males at 75 mg/kg bw per day and by 27% and 22% in females at 25 and 75 mg/kg bw per day, respectively. The increases in serum ALT activity and relative liver weight were not accompanied by any histopathological findings. The dose-dependent increase in relative adrenal weight in females also remained without any histopathological correlate. The histopathological findings at dose levels greater than 25 mg/kg bw per day were liver atrophy, fatty infiltration in renal tubules and hypocellularity of the bone marrow.

Based on the results of the 119-day oral toxicity study in dogs, a NOAEL could not be identified, as effects were observed at all doses. The LOAEL was 25 mg/kg bw per day, based on decreased red blood cell counts, decreased haematocrit, increased alkaline phosphatase activity, increased ALT activity and increased relative adrenal and liver weights in females (Ahmed, 1980b).

Acetochlor (purity 94.5%) was administered orally to groups of six male and six female Beagle dogs in gelatine capsules for 12 months at a dose level of 0, 4, 12 or 40 mg/kg bw per day.

All dogs survived the duration of the study, and no compound-related clinical signs were observed in any of the dogs. Decreases in feed consumption between 11% and 24% were observed in males receiving 40 mg/kg bw per day beginning in week 5 of the treatment. Absolute body weights of males and females at 40 mg/kg bw per day at month 12 were decreased by 13% and 17%, respectively, relative to the controls. Sporadic changes in clinical chemistry parameters were not considered to be of any biological significance, because most of them were borderline values, and many disappeared by the end of the study. No abnormal gross pathology findings were observed at terminal sacrifice. A statistically significant increase in absolute liver (18%) and kidney weights (18%) and a decrease in the testes to body weight ratio (60%) were observed in males receiving 40 mg/kg bw per day. Females at this dose level showed an increase in relative liver and kidney weights of 20% and 12%, respectively, but without statistical significance. A moderate diffuse and mild multifocal periportal fatty infiltration of the liver was observed in one male in each of the 12 and 40 mg/kg bw per day dose groups, respectively. Testicular atrophy was observed in all high-dose males.

The NOAEL in the 1-year toxicity study in dogs was 12 mg/kg bw per day, based on decreased body weights, decreased feed consumption, testicular atrophy, increased absolute liver and

Table 31. Clinical toxicology, haematology, blood biochemistry and gross pathology findings in a 119-day study in dogs

	Males				Females			
Significant findings	**0 mg/kg bw per day**	**25 mg/kg bw per day**	**75 mg/kg bw per day**	**200 mg/kg bw per day**	**0 mg/kg bw per day**	**25 mg/kg bw per day**	**75 mg/kg bw per day**	**200 mg/kg bw per day**
Survival, 17 weeks (%)	100	100	83	17	100	100	100	0
Body weight, 7 weeks (kg)	10.2	9.5	9.1	7.3*	8.8	8.7	7.8	6.3
Body weight, 17 weeks (kg)	10.9	10.1	9.5	–	9.4	9.4	8.0	–
Feed consumption, 7 weeks (g/day)	312	298	275	87*	256	283	223	85*
Feed consumption, 17 weeks (g/day)	307	287	295	–	252	285	230	–
RBC, 17 weeks ($\times 10^6/mm^3$)	7.00	6.83	6.32*	–	7.09	6.45*	6.50	–
Haemoglobin, 17 weeks (g/dL)	14.9	14.4	13.4*	–	15.6	13.5	14.5	–
Haematocrit, 17 weeks (pc/dL)	43.7	42.3	40.0*	–	44.7	39.8*	41.7	–
Glucose, 17 weeks (g/dL)	115	104*	102*	–	106	112	111	–
Globulin, 8 weeks (g/dL)	3.23	2.71*	2.74*	–	2.39	2.89	2.89	1.97
Globulin, 17 weeks (g/dL)	4.11	3.70*	3.64*	–	3.96	3.76	3.80	–
ALP, 8 weeks (IU/L)	95	99	101	–	88	152*	121*	112
ALP, 17 weeks (IU/L)	70	91	128	–	71	117	122*	–
ALT, 17 weeks (IU/L)	61	118*	200*	–	72	137	227*	–
Total protein, 8 weeks (g/dL)	6.73	5.85*	5.53*	–	6.50	6.22	6.03	4.45*
Total protein, 17 weeks (g/dL)	7.83	7.41	7.34*	–	7.80	7.54	7.49	–
LDH, 8 weeks (IU/L)	108	112	146*	–	161	153	135*	135*
LDH, 17 weeks (IU/L)	134	130	184*	–	162	143	137	–
Potassium, 17 weeks (mEq/L)	4.9	4.6	4.4*	–	4.6	4.5	4.7	–
BUN, 8 weeks (mg/dL)	15.4	17.1	19.1*	–	17.9	16.5	15.6	16.8
BUN, 17 weeks (mg/dL)	11.1	12.8	17.5*	–	16.9	14.4	13.8	–
Relative adrenal weight, 91 days	0.104	0.110	0.127	–	0.120	0.154*	0.168*	–
Relative liver weight, 91 days	27.18	29.61	34.38*	–	26.25	33.41*	31.95*	–
Relative spleen weight, 91 days	3.47	4.71	4.63	–	3.24	4.16	4.35*	–

ALP: alkaline phosphatase; ALT: alanine transaminase; BUN: blood urea nitrogen; bw: body weight; Eq: equivalents; IU: International Units; LDH: lactate dehydrogenase; pc: packed cells; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ahmed (1980b)

kidney weights and decreased relative testes weight observed at 40 mg/kg bw per day (Ahmed, 1981b).

In a second study, acetochlor was administered orally to groups of five male and five female Beagle dogs in gelatine capsules for 52 weeks at a dose level of 0, 2, 10 or 50 mg/kg bw per day.

Two males and four females from the 50 mg/kg bw per day group were killed between weeks 39 and 51 as a result of excessive toxicity (above the maximum tolerated dose [MTD]). Clinical signs at 50 mg/kg bw per day included progressive body weight loss, dehydration, pallor, emaciation, salivation, swaying or shaking of the head and body, and stiffness and rigidity of the hindlimbs, resulting in incoordination, ataxia and/or abnormal gait, hopping and flexor reflexes and exaggerated tonic neck reflex. Salivation was occasionally seen in dogs from all groups, including controls, but was more frequently observed in dogs treated at 10 and 50 mg/kg bw per day. There were no observations of ataxia or abnormal gait in animals given 2 or 10 mg/kg bw per day. There were no treatment-related changes seen during the ophthalmoscopy, haematology, faecal occult blood or brain cholinesterase determinations. High urinary volumes, associated with low specific gravities, were recorded in dogs dosed with 50 mg/kg bw per day.

A 23–28% decrease in feed consumption was observed for females dosed with 50 mg/kg bw per day during the last 39 weeks of the study. At 10 mg/kg bw per day, a 13% decrease in feed intake was observed for females during the last 13 weeks of the study. The mean overall weight gains of females receiving 10 mg/kg bw per day (19.4% decrease compared with controls) and of males and females receiving 50 mg/kg bw per day were lower than those of their respective controls. Mean weight gains of males receiving 2 or 10 mg/kg bw per day were also low, but there was no dose–response relationship. Statistical significance for the decrease in feed intake and body weight gains was achieved only at 50 mg/kg bw per day in males and females.

Changes in biochemical parameters are shown in Table 32. High plasma ALT activities were recorded for animals treated at 10 or 50 mg/kg bw per day and were associated with high ornithine carbamyl transferase, alkaline phosphatase, GGT and/or aspartate transaminase (AST) activities. Other changes comprised high urea and creatinine concentrations after 24 and 50 weeks in animals receiving 50 mg/kg bw per day, low glucose and high cholesterol concentrations in animals from all treated groups and low triglyceride concentrations in animals treated at 10 or 50 mg/kg bw per day. At week 12, total protein concentrations were low, and at week 24, albumin concentrations were high, in females receiving 50 mg/kg bw per day.

Macroscopic examination at necropsy revealed abnormally shaped kidneys that had pale or mottled surfaces and were occasionally cystic in dogs treated at 50 mg/kg bw per day (Table 33). Histopathological examination revealed changes in brain, kidneys and testis in animals treated at 50 mg/kg bw per day. Changes in the brain were confined to the cerebellum: degeneration of the granular layer in the deeper parts of the vermis and depletion of the Purkinje cells in adjacent areas. In the kidney, there was evidence of renal toxicity, seen as hyperplasia of the collecting ducts and of the transitional epithelium of the papilla; in addition, there was cortical atrophy with fibrosis and scarring and dilatation of the Bowman's space. The cortical and collecting duct changes were considered to be potentially fatal if treatment had continued. In the testis, there was degeneration of the seminiferous tubules, maturation arrest and spermatic giant cells. Hypospermia was also noted in the epididymides. Microscopic findings at 10 mg/kg bw per day, confined to males, were interstitial nephritis and chronic vasculitis and seminiferous tubule degeneration. These changes were generally less severe than at the high dose. No treatment-related microscopic findings were observed in females receiving 10 mg/kg bw per day or in animals of either sex receiving 2 mg/kg bw per day. Fatty infiltration of the liver was not observed in this study.

The NOAEL in the 52-week toxicity study in dogs was 2 mg/kg bw per day, based on a decrease in feed consumption and body weight gain in females and changes in kidneys (interstitial nephritis) and tubular degeneration in testes in males observed at 10 mg/kg bw per day (Broadmeadow, 1989b).

Slides from the testes and epididymis of male dogs from this 52-week oral toxicity study (Broadmeadow, 1989b) were re-examined microscopically by a consulting pathologist. All of the high-dose males exhibited a treatment-related degeneration of germ cells and associated reduction of sperm in the epididymis. Germ cell degeneration was particularly prominent in the spermatid population, and especially in the elongating and elongated spermatids, which are the final maturation

phases of development. Despite the depletion and degeneration of germ cells within the seminiferous epithelium, active spermatogenesis was still ongoing in most tubules. One animal in the mid-dose group also showed partial germ cell depletion, focal tubular atrophy and increased germ cell degeneration, but this was associated with multifocal tubular necrosis with a lymphocytic infiltrate (lymphocytic orchitis). The epididymis of this animal was devoid of sperm and had multifocal lymphocytic aggregates in the interstitial tissue. The findings are consistent with lymphocytic orchitis and epididymitis, which is a condition of autoimmune etiology occasionally seen in Beagle dogs. As these findings show a different profile of changes than was seen in animals in the high-dose group, this single occurrence is not considered to be related to treatment with the test material (Creasy, 2003).

Table 32. Selected blood biochemistry parameters (selected time points) in a 52-week study in dogs

		Males				**Females**			
Parameter	**Week**	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**50 mg/kg bw per day[a]**	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**50 mg/kg bw per day[a]**
ALT (IU/L)	12	34	34	45	147**	29	36	35	115**
	24	31	29	37	86**	22	25	29	40**
	50	61	37	104	179*	34	41	183**	45
Ornithine carbamyl transferase (IU/L)	12	6.0	6.3	8.9	19.0**	5.3	5.4	4.8	12.3
	24	4.8	6.7	3.4	10.9**	7.2	6.4	5.8	6.9
	50	7.9	9.3	39.0	56.9	9.5	10.6	21.1*	9.8
GGT (IU/L)	12	3	4	4**	6**	4	4	3	4
	24	4	5	4	5*	5	4	5	6
	50	4	4	4	7*	5	6	6	7
Urea (mg%)	24	26	25	25	41**	28	30	26	43
	50	30	29	30	54**	30	27	30	58*
Creatinine (mg%)	24	0.8	0.9	0.8	1.0	0.9	0.9	0.8	1.2*
	50	1.2	1.1	1.1	1.8**	1.2	1.1	1.1	2.2*
Glucose (mg%)	12	105	92*	99	94*	105	96	93	91*
	24	121	113	113	104**	110	110	102*	95**
	50	109	112	112	105	123	113**	112**	109**
Cholesterol (mg%)	12	116	129	141	146*	133	136	145	118
	24	115	134	146	161**	132	155	140	164
	50	121	131	123	164*	149	143	174	170
Triglyceride (mg%)	12	35	36	33	51**	32	36	43*	44*
	24	22	22	27	35**	31	29	35	35
	50	39	44	49	65**	40	41	54	38
Protein (g%)	12	5.8	6.1	5.8	5.7	5.9	5.8	5.7	5.4**
Albumin (g%)	24	3.4	3.3	3.3	3.5	3.1	3.5	3.1	3.5*

ALT: alanine transaminase; bw: body weight; GGT: gamma-glutamyltranspeptidase; IU: International Units; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)

[a] Three males and one female surviving at week 50.

Source: Broadmeadow (1989b)

Table 33. Significant histopathological findings in a 52-week study in dogs

		Males (*n* = 5)				Females (*n* = 5)			
Tissue	**Observation**	**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**
Brain	Degeneration of granular layer in vermis	0	0	0	4	0	0	0	3
	Depletion of Purkinje cells in vermis	0	0	0	4	0	0	0	2
Kidney	Interstitial nephritis	0	0	2	5	1	0	0	4
	Collecting duct hyperplasia	0	0	0	5	0	0	0	5
	Chronic vasculitis	0	0	3	4	0	0	0	5
	Cortical fibrosis	0	0	0	4	0	0	0	5
	Dilatation of Bowman's space	0	0	0	4	0	0	0	4
	Cortical atrophy	0	0	0	4	0	0	0	4
	Transitional cell hyperplasia	0	0	0	5	0	0	0	5
Testes	Tubular degeneration	0	0	4	5	–	–	–	–
	Maturation arrest	0	0	0	5	–	–	–	–
	Spermatid giant cell	0	1	0	4	–	–	–	–

bw: body weight
Source: Broadmeadow (1989b)

A Pathology Working Group (PWG) was convened in 2014 to review the testes from four dog studies conducted with acetochlor and to assess the potential relationship to treatment of any changes that were present. The epididymis and prostate were also examined, when present, to aid in the interpretation of the findings in the testes.

The PWG concluded that degenerative changes were observed in the seminiferous tubules of the testes of dogs given acetochlor at extremely high doses (200 mg/kg bw per day; Ahmed, 1980a) in subchronic toxicity studies conducted for up to 119 days. At 200 mg/kg bw per day, excessive toxicity (including emaciation, decreased body weights and a high rate of mortality) was observed. In these short-term studies, the NOAEL with regard to testicular histopathology was identified as 75 mg/kg bw per day.

In 1-year toxicity studies conducted in dogs, degenerative changes were present in the seminiferous tubules of the testes of all dogs at the highest doses tested, 40 mg/kg bw per day in the 12-month study (Ahmed, 1981b) and 50 mg/kg bw per day in the 52-week study (Broadmeadow, 1989b). The NOAELs with regard to testicular histopathology in the 1-year toxicity studies were 12 mg/kg bw per day in the 12-month study (Ahmed, 1981b) and 10 mg/kg bw per day in the 52-week study (Broadmeadow, 1989b).

A definitive pathogenesis of the degenerative changes observed in the seminiferous tubules could not be determined. No microscopic changes were observed in the prostate sections from the 119-day study (Ahmed, 1980b) and 12-month study (Ahmed, 1981b) that were available for examination by the PWG. Additionally, there were no treatment-related changes in the prostate weights or histopathology of the prostate for any of the studies in the final study reports. As the

prostate is very sensitive to hormonal disturbances, this provides additional support for the hypothesis that the treatment-related effect observed in the testes (tubular degeneration) was a direct testicular effect rather than a hormonally mediated effect (Hardisty, 2014).

Available toxicity studies showed testicular changes when acetochlor is administered at high doses in the dog, but not in the rat. Owing to the concern regarding the potential of chemical exposure to cause endocrine disruption, the data submitter consulted an independent scientific opinion from an expert. The expert concluded that based on the profile of changes seen in the reproductive tract of male dogs dosed with acetochlor, the testicular lesions are more likely to be due to a direct effect on the seminiferous epithelium than to any effect on the endocrine axis (Creasy, 2015).

(b) Dermal application

Rats

In a 21-day dermal toxicity study, groups of five male and five female Wistar rats were exposed to acetochlor (purity 89.4%) at 0, 0.1, 1, 10 or 100 mg/kg bw per day in olive oil applied onto the skin under occlusive dressing for 6 hours/day, 5 days/week, for 3 weeks. The control group received olive oil only. The dose range selected was based on the findings from preliminary dose range–finding studies.

There were no deaths and no clear effects on clinical condition. Upward curvature of the spine, not dose related, was observed in the test groups. Transient slight to moderate skin irritation was seen on the sites of application, but this was not dose related and was also observed in the male control group. Males treated at 100 mg/kg bw per day had slightly lower body weight gain compared with controls during the second half of the study. The magnitude of body weight change was small, and statistical significance was not achieved; therefore, it was not considered to be toxicologically significant. In females, this effect appeared in animals treated at 1 and 10 mg/kg bw per day, but not at the high dose level. There were no treatment-related effects on feed consumption. There were no treatment-related effects on haematology, clinical biochemistry, organ weights or pathology.

Based on the results of the 21-day dermal toxicity study in rats, the systemic toxicity NOAEL was 100 mg/kg bw per day, the highest dose tested (Leah, 1989).

Rabbits

Acetochlor (purity 94.5%) was applied dermally to groups of 10 male and 10 female New Zealand White rabbits, one half with intact skin and one half with abraded skin, 6 hours/day, 5 days/week, for 3 consecutive weeks at a dose level of 0, 100, 400 or 1200 mg/kg bw per day. At the end of the study, blood samples were collected from five animals of each sex per group for haematology and blood biochemistry evaluations. At termination, gross necropsy was performed, and organ weights were recorded. A select number of tissues were subjected to histopathological examination.

Eight male and seven female rabbits treated at 1200 mg/kg bw per day died between day 7 and day 19 of the study period. The clinical effects observed at the high dose level were anorexia, respiratory congestion, laboured breathing, ataxia, hypoactivity, body rigid, tonic convulsions, decreased limb tone, bradypnoea, impaired righting reflex, emaciation and hypothermia. No systemic toxicity was observed in the low- and mid-dose rabbits. Dermal irritation (including erythema, oedema, desquamation, coriaceousness, fissuring, atonia, intradermal haemorrhage, blanching, eschar and exfoliation) and associated histopathological changes of the skin were observed at all dose levels.

In the 21-day dermal toxicity study in rabbits, the systemic toxicity NOAEL was 400 mg/kg bw per day, based on mortality observed at 1200 mg/kg bw per day (Johnson, 1981).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an oral carcinogenicity study, acetochlor (purity 94.5%) was administered to 50 Swiss-bred CD-1 albino mice of each sex per dose in the diet for 23 months at a dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 75, 227 and 862 mg/kg bw per day for males and 0, 95, 280 and 1084 mg/kg bw per day for females, respectively). Additional groups of 10 mice of each sex per dose were administered the same diets for 12 months and killed for a 1-year interim evaluation.

No unusual clinical signs were observed. Survival in males at termination was 60%, 50%, 50% and 26% at 0, 500, 1500 and 5000 ppm, respectively. In females, survival was reduced during the last months of the study at 5000 ppm and to a lesser extent at 1500 ppm (at termination, 62%, 50%, 34% and 26% at 0, 500, 1500 and 5000 ppm, respectively). Body weights for 5000 ppm males were 16% less than those of controls at week 53 and 18% less than those of controls at termination. Body weights for 1500 ppm males were 5% less than those of controls at week 53 and 2% less than those of controls at termination. Body weights for 5000 ppm females were 12% less than those of controls at week 53 and 10% less than those of controls at termination. Body weights of males and females at 500 ppm were comparable with those of controls (Table 34). A slight and transient anaemia in males and more pronounced and persistent anaemia in females at the middle and high dose levels were compound related. Other haematology changes were an increase in white blood cell counts in males at the high dose level and an increase in platelet counts in females at the middle and high dose levels. A slight and transient increase in serum liver enzymes was observed at the high dose level in males for ALT and in females for alkaline phosphatase, effects indicative of liver damage. Urine analysis parameters for treated groups were comparable with those of the control group.

Table 34. Body weights and organ weights of mice at 12 and 24 months

	Males				**Females**			
Significant findings	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
Body weight, 12 months (g)	36	32	28*	30*	27	26	27	22*
Body weight, 23 months (g)	35.50	35.88	34.72	29.29*	31.55	32.04	31.75	28.27*
Absolute liver weight, 12 months (g)	1.49	1.58	1.44	1.68	1.30	1.45	1.62*	1.53*
Absolute liver weight, 23 months (g)	1.62	2.10*	1.96*	2.52*	1.76	1.72	1.62	1.92
Absolute kidney weight, 12 months (g)	0.73	0.89*	0.78	0.81	0.46	0.54	0.55	0.41
Absolute kidney weight, 23 months (g)	0.73	1.06*	1.08*	0.87*	0.55	0.64*	0.52	0.60
Relative liver weight, 12 months	41.96	49.64*	52.17*	56.32*	48.86	55.65*	60.34*	70.99*
Relative liver weight, 23 months	45.51	58.46*	56.27*	87.09*	54.36	53.89	50.87	65.60
Relative kidney weight, 12 months	20.71	27.76*	28.00*	26.98*	17.40	20.70*	20.50*	19.17
Relative kidney weight, 23 months	21.35	29.70*	31.03*	29.66*	17.33	19.98	16.28	20.75*

ppm: parts per million; *: $P \leq 0.05$
Source: Ahmed (1983b)

At 500 ppm and above, statistically significantly increased absolute/relative kidney weights were observed in males at the interim and terminal sacrifices. Statistically significantly increased absolute/relative liver weights were observed at all dose levels in males at the interim and terminal sacrifices, with females showing significant increases only at the interim sacrifice time (Table 34).

Gross observations at necropsy included urinary tract lesions for males (all dose groups for scheduled sacrifice, high-dose group for animals that died or were killed in moribund condition) and females (mid- and high-dose groups for scheduled sacrifice and high-dose group for females that died or were killed in moribund condition), digestive tract (primarily liver) masses for males (mid- and high-dose groups for scheduled sacrifice animals), pulmonary masses for females (all dose groups for scheduled sacrifice animals and animals that died or were killed in moribund condition) and reproductive tract masses for females (high-dose group for scheduled sacrifice). A variety of other lesions and masses were observed, but were not considered treatment related. Non-neoplastic lesions likely to be treatment related are shown in Table 35. A positive trend was observed for interstitial nephritis for males and females; the incidences for the high-dose males and females were significantly increased compared with those of controls. A similar trend was also obtained for retinal degeneration in the females, although the incidence of this lesion in the high-dose group was not statistically significantly different from that of the control group.

Table 35. Non-neoplastic histopathology lesions in mice

		Males				**Females**			
Tissue	**Observation**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
Eyes	*No. of tissues examined*	60	60	60	59	60	60	60	59
	Retinal degeneration	4	3	6	3	2††	3	1	8
Kidneys	*No. of tissues examined*	60	60	60	60	60	60	60	59
	Interstitial nephritis	30††	35	42	50**	31††	33	31	45**

ppm: parts per million; **: $P \leq 0.01$ (Fisher's exact test); ††: $P \leq 0.01$ (Cochran-Armitage trend test)
Source: Ahmed (1983b)

Some of the pathology results from this study were subsequently subjected to a comprehensive re-evaluation and/or peer review (Hardisty, 1997a,b,c). Increased incidences of histiocytic sarcomas were noted in all of the treated female groups relative to the controls, although the increase at 500 ppm was not statistically significant, and there was no clear dose–response relationship (Table 36). In female mice with histiocytic sarcoma, the uterus as well as the liver are the most commonly involved organs. Histiocytic sarcomas are rare in mice until 12 months of age and are slightly more common in females than in males. Published data concerning the incidence of histiocytic sarcomas are variable (Table 37). Because of the highly variable incidence of this tumour in untreated female control mice, the absence of precursor lesions and the lack of a clear dose–response relationship, the PWG concluded that "the marginal increase in the incidence of histiocytic sarcoma in these groups is most likely due to normal variation not associated with the dietary administration of acetochlor and [is] of no biological significance".

The incidences of lung tumours (alveolar/bronchiolar adenomas and/or carcinomas) in all of the treated female groups were greater than the incidences in the concurrent controls. The differences from controls were generally statistically significant, but no dose–response relationship was apparent (Table 36). In addition, the incidences of lung tumours in all of the treated female groups in this study were below the upper range of the incidence of spontaneous primary lung tumours reported in the literature (Tables 38 and 39), whereas the incidence in the concurrent control group was unusually low. Furthermore, there was no evidence of an increase in non-neoplastic proliferative changes

Table 36. Neoplastic histopathology lesions in mice fed acetochlor for 23 months (re-evaluation)

		Males				Females			
Tissue	**Observation**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
Liver[a]	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Hepatocellular adenoma	8	7	10	19*	2	0	1	5
	Hepatocellular carcinoma	4	4	4	9	0	0	0	2
	Hepatocellular adenoma/carcinoma	12	10	14	26**	2	0	1	7
Lung[b]	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Alveolar/bronchiolar adenoma	7	10	11	5	1	7*	9**	7*
	Alveolar/bronchiolar carcinoma	6	3	3	3	0	4	1	6**
	Alveolar/bronchiolar adenoma/carcinoma	12	13	14	8	1	10**	10**	11**
Uterus, liver[c]	*No. of tissues examined*	–	–	–	–	50	50	50	50
	Histiocytic sarcoma	–	–	–	–	0	3	7**	6*

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test)
[a] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997a).
[b] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997b).
[c] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997c).

Table 37. Historical control data for histiocytic sarcomas in female CD-1 mice

				Incidence (%)	
Source	**Time period**	**Study duration**	**No. of studies**	**Range**	**Mean**
Charles River (1995)	1981–1991	18 months	12	0–10.0	2.2
		21 months	3	0–6.0	3.8
		24 months	8	0–10.0	3.8
Charles River (2005)	1987–2000	78 weeks	25	0–15.0	2.7
		91–104 weeks	29	0–18.3	6.5
Inveresk Research (1996)	1990–1993	18 months	5	0–2.0	0.8
		24 months	8	0–10.0	4.0

(hyperplasia) or in tumour multiplicity. Therefore, the PWG concluded that the "apparent increased incidence of lung tumours in treated female mice is a spurious finding resulting from the abnormally low incidence of primary lung tumours in the control group and is of no biological significance" (Hardisty, 1997b). There was an increased incidence of hepatic carcinomas in males and females at the high dose. The incidence of hepatocellular carcinoma and hepatocellular adenoma combined was statistically significantly increased only in males given 5000 ppm acetochlor when compared with the control group. Examination of the mortality and body weight data indicated that 5000 ppm acetochlor in the diet for 23 months exceeded the MTD for male and female CD-1 mice. No significant differences in the incidence of hepatocellular neoplasms were present when acetochlor was given at 1500 ppm and below. The PWG concluded that these liver tumours were not appropriate for consideration in human risk assessment, as the increase occurred only at a dose level with high toxicity (Hardisty, 1997a).

Table 38. Historical control incidence data for lung adenomas and carcinomas in female CD-1 mice

Source	Approximate time period	Study duration	No. of studies	Incidence (%)					
				Adenoma		Carcinoma		Total[a]	
				Range	Mean	Range	Mean	Range	Mean
Maita et al. (1988)	1978–1983	24 months	11	na	14.5	na	12.1	17.5–38.8	26.6
Charles River (1995)	1981–1991	18 months	12	0–15.4	6.5	0–9.6	4.0	(3.3–20.0)	(10.5)
		21 months	6	0–10.0	6.2	0–10.0	3.1	(6.0–14.0)	(9.4)
		24 months	11	4.0–18.4	9.8	0–13.5	6.6	(12.0–23.1)	(16.4)
Charles River (2005)	1987–2000	78 weeks	25	0–16.0	7.7	0–12.0	4.3	(1.7–25.7)	(12.0)
		91–104 weeks	29	0–26.7	10.9	0–18.4	4.8	(0–35.0)	(15.7)
Life Sciences Research (1989)	1985–1988	18 months	13	0–9.6	5.4	0–9.6	4.4	(3.8–19.2)	(9.8)
Inveresk Research (1996)	1990–1993	18 months	5	0–16.0	7.6	2–10.0	4.0	(6.0–18.0)	(11.6)
		24 months	8	0–14.0	8.5	2–10.0	6.5	(12.0–20.0)	(15.0)

na: not available

[a] Only Maita et al. (1988) provided values for the total incidence of adenomas and/or carcinomas combined. The combined incidence from the other sources was estimated by adding the separate values for adenomas and carcinomas, and these estimates are enclosed in parentheses. However, this may be a slight overestimate, as a few animals are likely to have both an adenoma and a carcinoma.

Table 39. Historical control incidence data for lung adenomas and carcinomas in male CD-1 mice

Source	Approximate time period	Study duration	No. of studies	Incidence (%)					
				Adenoma		Carcinoma		Total[a]	
				Range	Mean	Range	Mean	Range	Mean
Maita et al. (1988)	1978–1983	24 months	11	na	14.6	na	18.9	17.5–43.5	33.4
Charles River (1995)	1981–1991	18 months	12	1.9–12.0	7.5	0–21.2	5.8	(3.8–23.3)	(13.3)
		21 months	7	0–26.0	11.6	0–16.7	4.9	(4.0–30.0)	(16.5)
		24 months	10	9.7–28	17.9	1.9–20.0	11.1	(15.7–38.0)	(29.0)
Charles River (2005)	1987–2000	78 weeks	25	0–26.0	11.7	0–23.2	5.2	(0–44.9)	(16.9)
		97–104 weeks	29	3.3–42.0	16.3	0–26.6	9.4	(8.3–54.0)	(25.7)
Life Sciences Research (1989)	1985–1988	18 months	13	4.0–17.3	10.6	3.3–12.0	8.6	(15.0–28.8)	(19.2)
Inveresk Research (1996)	1990–1993	18 months	5	0–16.0	12.8	1–8.0	3.6	(8.0–22.0)	(16.4)
		24 months	8	16.0–28.0	20.5	2.0–22.0	13.0	(26.0–38.0)	(33.5)

na: not available

[a] Only Maita et al. (1988) provided values for the total incidence of adenomas and/or carcinomas combined. The combined incidence from the other sources was estimated by adding the separate values for adenomas and carcinomas, and these estimates are enclosed in parentheses. However, this may be a slight overestimate, as a few animals are likely to have both an adenoma and a carcinoma.

An expert histopathological evaluation of the kidneys of CD-1 mice from a 2-year mouse study was conducted to review the classification of renal neoplasms encountered in both males and females in the study, determine whether any lesions representing precursor stages on the pathway to neoplasia were present in the treated groups of female mice and grade the spontaneous nephropathy in the female groups. The renal tubule tumours encountered in the male mouse groups were confirmed by the independent expert. The renal tumours in the male mice were clearly not dose related, as the group distribution was two, one, three and two for the 0, 500, 1500 and 5000 ppm groups, respectively. The tumour count in the female groups was confined to the 5000 ppm dose group and included two adenomas and two non-epithelial neoplasms of uncertain identity. Acetochlor exacerbated the severity of spontaneous nephropathy in the 5000 ppm females and, to a lesser extent, at the 1500 ppm dose. According to the expert, the presence of two adenomas in the 5000 ppm females does not represent a statistically significant finding. An incidence of 4% is lower than the historical control range (usually 10–15%) observed in rodent studies when a chemical is causally associated with the induction of renal tubule tumours in groups of 50 animals. The absence of any foci of atypical tubule hyperplasia, a preneoplastic lesion, in the high-dose group provides further support against the female adenomas being treatment related. Therefore, the expert considered the two adenomas in the high-dose females to represent biological variation and to be incidental to treatment (Hard, 2001).

In addition, a PWG review was performed to provide an expert histopathological evaluation of the kidney of mice used in the carcinogenicity bioassay. Specifically, the scope of work included a review of the classification of renal neoplasms encountered in the study and the determination of whether any lesions representing precursors of neoplasia were present in groups of female CD-1 mice.

The PWG concluded that the two renal adenomas observed in two 5000 ppm dose female mice in the long-term feeding study with acetochlor were not related to the treatment. They confirmed the diagnosis of two renal adenomas, but concluded that two other tumours were undifferentiated sarcomas of possibly lymphoreticular origin (Hardisty, 2001b).

In conclusion, no NOAEL could be identified, as effects were observed at all doses. The LOAEL was 500 ppm (equal to 75 mg/kg bw per day), based on increased absolute and relative kidney (both sexes) and liver weights (males only), a dose-related increase in interstitial nephritis in both sexes and retinal degeneration in females (positive trend). A treatment-related increase in the incidence of liver tumours was noted in males at 5000 ppm; however, the high dose was considered excessive, exceeding the MTD. In addition, statistically significant increases in the incidence of lung tumours in females of all dose groups and in the incidence of histiocytic sarcomas in females of the mid- and high-dose groups were observed, but were not considered to be treatment related (Ahmed, 1983b).

In a 78-week feeding study, acetochlor (purity 90.5%) was administered to 50 CD-1 mice of each sex per dose in the diet at a dose level of 0, 10, 100 or 1000 ppm (equal to 0, 1.1, 11 and 116 mg/kg bw per day for males and 0, 1.4, 13 and 135 mg/kg bw per day for females, respectively). Additional groups of 10 CD-1 mice of each sex per dose were administered the test material at the above dose levels for 52 weeks for interim sacrifice at 1 year. Urine analysis and clinical chemistry parameters were not evaluated in this study.

There were no treatment-related effects on survival, feed consumption, body weight or clinical observations. At the 50-week interval, packed cell volume, haemoglobin and erythrocyte count were slightly reduced (7–9%) in males and females receiving 1000 ppm. After 77 weeks of treatment, low erythrocyte parameters were recorded for males receiving 100 or 1000 ppm.

At 10 ppm and above, males showed statistically significant increases in absolute kidney weights; however, these findings at 10 ppm were not considered clearly adverse in the absence of other renal effects. After 78 weeks, absolute and relative kidney weights for males receiving 100 or 1000 ppm were higher than those of the controls. After 78 weeks of treatment, absolute and relative liver weights were higher for all groups of treated males compared with controls (Table 40). There

Table 40. Selected terminal organ weights in mice treated with acetochlor in the diet for 78 weeks

	Males				Females			
Organ	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**
Terminal body weight (g)	47.0	48.3	46.1	45.3	37.4	36.4	38.1	36.7
Kidney								
Absolute (g)	0.85	0.92*	0.96**	1.12***	0.53	0.56	0.55	0.49*
Relative (%)	1.83	1.93	2.12*	2.50***	1.48	1.56	1.46	1.36
Liver								
Absolute (g)	2.4	2.9*	2.6	2.8	1.8	1.8	1.8	1.9
Relative (%)	5.07	5.96*	5.72	6.27*	4.90	5.01	4.77	5.18
Adrenals								
Absolute (mg)	3.0	3.0	3.0	3.0	7.0	7.0	7.0	7.0
Relative (%)	5.7	6.0	5.9	6.5	18.2	18.7	18.6	18.8
Brain								
Absolute (g)	0.52	0.51	0.51	0.51	0.52	0.53	0.52	0.50*
Relative (%)	1.12	1.08	1.12	1.14	1.45	1.50	1.41	1.39

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Amyes (1989)

were no macroscopic changes that were considered to be related to treatment, among either those animals killed or dying during the treatment period or those killed after 52 or 78 weeks of treatment. There were no non-neoplastic or neoplastic changes that were attributable to treatment in mice killed after 52 weeks of treatment. Increased kidney weights and increased incidences of tubular basophilia, interstitial fibrosis, cortical mineralization and hyaline casts were noted in males at 1000 ppm and were considered to be evidence of chronic renal nephropathy (Table 41). At the same dose level, only an increase in dilatation of cortical tubules was noted in females. A significantly increased incidence of renal tubular basophilia was also noted in males at 10 and 100 ppm. However, with the exception of two animals at 100 ppm, tubular basophilia was classified as minimal or slight in all animals, including those in the 1000 ppm group, and neither the severity nor incidence of this finding exhibited a clear dose–response relationship. Slightly increased kidney weights and an increased incidence of renal tubular basophilia in males at 10 and 100 ppm were not considered to be adverse owing to minimal severity, lack of a clear dose–response relationship, no associated histopathological findings at 10 and 100 ppm, lack of similar effects in females or in either sex at higher dose levels in a study of longer duration, and lack of corroborative renal findings.

In the lung, bronchiolar hyperplasia was observed in males at the two highest doses and in females at the low dose. It could be related to the lung tumours observed; however, there was a lack of a clear dose–response relationship. After 13, 24 or 50 weeks of treatment, the incidences of ophthalmoscopic findings for controls were similar to those for animals receiving 1000 ppm. After 76 weeks of treatment, the incidence of vacuoles in the anterior polar region of the lens was higher among females receiving 1000 ppm than among their controls ($P \leq 0.01$). As a result, the examination was extended to include all animals receiving 10 or 100 ppm. The incidences in these groups were similar to those of the controls.

There was a statistically significantly higher incidence of pulmonary adenomas in female mice at 1000 ppm compared with controls ($P < 0.05$; Table 42). No increase in the incidence of pulmonary carcinomas was seen in females. There were also higher incidences of this tumour in

Table 41. Non-neoplastic histopathological lesions in mice after 78 weeks of treatment

		Males				Females			
Tissue	**Observations**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**
Eye lens	*No. of tissues examined*	41	39	37	34	36	42	38	37
	Anterior polar vacuoles	10	11	12	9	7	10	12	20**
	Anterior polar opacities	2	4	1	1	5	4	0	1
Lungs	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Bronchiolar hyperplasia	6	6	18**	15*	8	19*	17	11
Kidneys	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Tubular basophilia	3	13*	11*	19**	7	7	5	9
	Interstitial fibrosis	8	8	14	26**	8	13	7	8
	Cortical mineralization	13	14	11	26*	1	0	0	4
	Dilatation of cortical tubules	9	11	9	15	2	8	7	12**
	Hyaline casts	9	8	13	22**	8	6	8	8

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test)
Source: Amyes (1989)

Table 42. Neoplastic histopathological lesions in the 78-week carcinogenicity study in mice[a]

		Males				Females			
Tissue	**Observation**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**
Liver	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Adenoma	6	9	5	6	0	2	0	1
	Carcinoma	0	2	3	3	1	0	0	0
	Adenoma/carcinoma combined	6	11	8	9	1	2	0	1
Lung	*No. of tissues examined*	50	50	50	50	50	50	50	50
	Alveolar/bronchiolar adenoma	9	5	11	16	4	4	5	9
	Alveolar/bronchiolar carcinoma	3	3	3	4	1	0	2	2
	Adenoma/carcinoma combined	11	8	13	18	5	4	7	11
	% incidence of adenoma/carcinoma combined	22	16	26	36	10	8	14	22
Uterus, liver	*No. of tissues examined*	–	–	–	–	50	50	50	50
	Histiocytic sarcomas	–	–	–	–	2	1	0	5

ppm: parts per million
[a] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997b).

females at 10 and 100 ppm and in males at 100 and 1000 ppm, but these incidences were not statistically significant. A positive trend with dose was observed for pulmonary adenoma in males ($P < 0.01$) and females ($P < 0.05$). These lung tumours were re-evaluated by the PWG, which concluded that the apparent increase in incidence with treatment is of no biological significance because of the lack of a dose–response relationship, the absence of precursor proliferative changes, the lack of an increase in tumour multiplicity with increasing dose and the comparable incidence of

lung tumours in the treated groups with published incidences of primary lung tumours in untreated control male and female CD-1 mice (Hardisty, 1997b).

In conclusion, the NOAEL in the 78-week dietary study in mice was 10 ppm (equal to 1.1 mg/kg bw per day), based on slight anaemia and increased incidences of bronchiolar hyperplasia and interstitial fibrosis in the kidney in males observed at 100 ppm (equal to 11 mg/kg bw per day). The NOAEL for carcinogenicity was 1000 ppm (equal to 116 mg/kg bw per day), the highest dose tested (Amyes, 1989).

A PWG review was performed on the lungs from all animals from two long-term mouse feeding studies with acetochlor to determine the incidence of pulmonary neoplasms using currently accepted nomenclature and diagnostic criteria and to discuss the relevance, if any, of these tumours for human risk assessment. The results of the PWG review of primary lung tumours from male and female mice in these two studies indicate that the apparent increase in incidence with treatment is of no biological significance. This conclusion is based on the lack of dose–response relationship, the absence of precursor proliferative changes, the lack of an increase in tumour multiplicity with increasing dose and the comparable incidence of lung tumours in treated groups with published incidences of primary lung tumours in untreated control male and female CD-1 mice (Hardisty, 1997b).

A PWG review was performed to confirm the incidence of histiocytic sarcoma of the uterus in female mice from two long-term feeding studies with acetochlor and to discuss the relevance, if any, of these tumours for human risk assessment. The overall weight of evidence indicated that the higher incidence of histiocytic sarcoma observed in female mice given 1000, 1500 and 5000 ppm acetochlor was not clearly related to dietary administration of acetochlor. The lack of increasing incidence with increasing dose, the absence of precursor lesions and the variable incidence known to occur in untreated control female mice are consistent with the interpretation that the marginal increase in the incidence of histiocytic sarcoma in these groups is most likely due to normal variation not associated with the dietary administration of acetochlor and is of no biological significance (Hardisty, 1997c).

The incidences of lung tumours and histiocytic sarcomas observed in the two dietary mouse carcinogenicity studies conducted with acetochlor were re-evaluated by a Scientific Advisory Group organized by the data submitter. The conclusion of the Scientific Advisory Group is that neither the lung tumour incidence nor the incidence of histiocytic sarcomas noted in the two mouse studies is considered an indicator for the carcinogenic potential of acetochlor (Gopinath, 2009).

Rats

In an oral carcinogenicity study, acetochlor (purity 94.5%) was administered to 70 Sprague-Dawley rats of each sex per dose in the diet for 27 months for males and 24 months for females at a dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 22, 69 and 250 mg/kg bw per day for males and 0, 30, 93 and 343 mg/kg bw per day for females, respectively). Ten rats of each sex per dose were killed after 12 months for a 1-year interim evaluation.

Some of the effects at the high dose are not discussed here, as the high dose exceeded the MTD. No ophthalmological changes or clinical signs of toxicity were observed that were considered to be related to treatment. The female rats were killed before the end of the 24th month on test (week 103) because the survivors in at least one female group had decreased to less than 25% of the original number of animals per group. There were enough males in each group, however, to allow the study to proceed to 27 months. The following statistically significant differences were observed in the mean weekly body weight data for the male rats (Table 43): the low-dose group showed a decrease at weeks 9, 12 and 115; and the mid-dose group showed a decrease at weeks 2 through 115. The following statistically significant differences were observed in the mean weekly body weight data for the female rats: the low-dose group showed an increase at week 101; and the mid-dose group showed a decrease at weeks 31, 39, 41, 47 through 53, 75 through 85 and 97. There was a statistically significant decrease in feed consumption by males and females that was considered to be treatment related

Table 43. Survival, body weight and haematology data in a 2-year study in rats

	Males				Females			
Significant findings	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
Survival, termination (%)	32	33	45	25	42	32	43	18
Body weight, start (g)	174.5	170.6	170.4	172.1	147.0	144.6	147.9	146.5
Body weight, termination (g)	745.4	640.9*	618.7*	479.8*	449.5	503.4	431.5	308.1*
Haemoglobin, 18 months (g/dL)	14.2	14.2	15.1	14.8	13.9	14.1	13.3	11.4*
Haematocrit, 18 months (pc%)	41.8	41.3	44.7	43.0	41.2	41.0	40.5	34.8*
WBC, 24 months ($\times 10^3/mm^3$)	36.0	25.4	25.2	24.9	34.0	28.3	26.7	16.9*

pc: packed cells; ppm: parts per million; WBC: white blood cells; *: $P \leq 0.05$ (Dunnett's test)
Source: Ahmed (1983a)

because it occurred at frequent intervals throughout the study. Statistically significant changes in the blood chemistry parameters were not considered to be related to treatment because they were not consistent over time and/or there was no apparent dose–response relationship. No treatment-related effects were observed in urine analysis parameters evaluated during the study.

The mid- and high-dose groups (male and female) showed a dose-related increase in the kidney to body weight ratio at months 12 and 24. Most of the other changes observed were a reflection of the corresponding changes in body weight and were not considered to be treatment related because of the lack of corroborative histopathology.

Several lesions appeared to be increased in the test groups when compared with the control animals. Microscopic lesions were found only during the second year of treatment. With respect to non-tumour histopathology, there was a dose-related increase in polyarteritis in arteries and testes, particularly in dead or moribund male rats, from 12 months to the termination of the study (Table 44). Polyarteritis is a disease process that occurs in rats and is believed to result from an immunopathological mechanism related to the deposition of immune complexes within affected vessels. In females, liver necrosis showed a statistically significant positive trend and statistically significantly increased incidence in the high-dose group. In males, liver necrosis was not observed. This increase in liver necrosis in females was more pronounced in animals either dead or moribund from 12 months to the termination of the study as well as in the high-dose females killed at the end of the study. Cardiac thrombosis and peripheral neuropathy for females showed statistically significant positive trends. This resulted in increased incidences of these findings at the high dose compared with the control group, but the increases were not statistically significant. In lungs, alveolar histiocytosis and interstitial pneumonia in females were dose-relatedly increased. The weights of lungs were not determined in this study. Other findings were fibrosis and gastritis in the stomach and inflammation of the tongue in females at the high dose.

The treatment-related neoplastic histopathological finding are reported in Table 45. To ensure accuracy and consistency of the diagnoses, some of the pathology results were subsequently subjected to a re-evaluation and/or peer review (Hardisty, 1997a; Ribelin, 1987).

Treatment-related neoplastic findings were noted in the liver, nasal turbinate mucosa and thyroid. There were increased numbers of hepatocellular adenomas and carcinomas in males and females at 5000 ppm in the presence of markedly decreased body weights and increased mortality. During the first year, there were no increases in hepatic tumours.

Table 44. Non-neoplastic histopathological lesions in a 2-year study in rats

		Males				Females			
Tissue	**Observation**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
No. of animals examined		70	70	70	70	70	70	70	70
Liver	Necrosis	7	2	4	5	1	8	3	12**
Lung	Alveolar histiocytosis	5	5	6	6	0	1	1	9**
	Interstitial pneumonia	6	5	5	7	6	4	9	10
Tongue	Inflammation	3	1	3	1	1	0	3	8**
Kidneys	Cyst	0	0	1	1	0	2	2	5
Testes	Polyarteritis	7	11	12	17	–	–	–	–
Heart	Thrombosis	2	3	2	1	0	0	0	4
Arteries	Polyarteritis	3	3	3	10	0	0	0	0
Peripheral nerve	Neuropathy	1	0	1	1	0	0	0	4
Stomach	Fibrosis	13	10	13	14	4	5	7	12
	Gastritis	8	3	2	2	0	1	1	3

ppm: parts per million; **: $P \leq 0.01$ (Chi-squared test)
Source: Ahmed (1983a)

Table 45. Neoplastic histopathological lesions in a 2-year study in rats

		Males				Females			
Tissue	**Observation**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**5 000 ppm**
Liver[a]	*No. of tissues examined*	60	60	60	60	60	60	60	60
	Hepatocellular adenoma	2	1	1	6	0	1	1	3
	Hepatocellular carcinoma	1	3	3	6	0	0	0	2
	Hepatocellular adenoma/carcinoma	3	4	4	11**	0	1	1	5*
Nasal turbinates[b]	*No. of tissues examined*[c]	69	70	69	69	69	68	70	69
	Papillary adenoma	0††	1	6*	18**	0	0	2	1
	Papillary adenocarcinoma	0†	0	0	2	0	0	0	0
Thyroid	*No. of tissues examined*	59	60	60	60	60	60	60	60
	Follicular adenoma	0†	0	3	5*	2	0	0	3
	Follicular carcinoma	0	1	0	2	0	0	0	0

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test); †: $P \leq 0.05$; ††: $P \leq 0.01$ (Peto trend test)
[a] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997a).
[b] Data derived from histopathology re-evaluation (Ribelin, 1987).
[c] Includes interim kill animals, as nasal tumours were noted in some animals at 12 months.
Source: Ahmed (1983a)

Interstitial cell tumours of the testes exhibited a dose-related increase in the male test groups compared with the male control group. The overall composite percentage of interstitial cell tumours was as follows: control, 2.8%; low dose, 5.7%; middle dose, 5.7%; and high dose, 10%. Most of these tumours appeared in the terminal sacrifice group of animals. The historical control data show a 2/291 (0.7%) incidence of interstitial tumours. The Peto trend test for interstitial tumours was not statistically significant.

In the thyroid gland, follicular cell adenomas exhibited a dose-related increase in the male mid-dose and high-dose test groups compared with the male control group. The overall incidence of follicular cell adenomas in the males was as follows: control, 0%; low dose, 0%; middle dose, 4.3%; and high dose, 7.1%. These tumours were evenly distributed between the terminal sacrifice animals and the dead and moribund animals from 12 months to termination. There were no follicular adenomas in the historical control data, and two follicular carcinomas were observed in a high-dose male; therefore, thyroid tumours in males were considered to be a compound-related effect.

An increased incidence of nasal tumours (primarily papillary adenomas) was noted at 1500 and 5000 ppm, predominantly in males, and is considered to be treatment related. A slight and not statistically significantly increased incidence of mucosal inflammation was also noted in the nasal turbinates of high-dose males. The inflammation was of mild severity and occurred in a different region of the turbinates than that of the tumours and thus should not be considered related to the development of papillary adenomas.

Several other tumours from various organs appear to have a dose-related increase; however, these were not considered to be toxicologically significant either because of the small number present or because they did not exhibit consistent changes over time or between sexes or a dose–response relationship.

In conclusion, the LOAEL for systemic toxicity in this carcinogenicity study in rats was 500 ppm (equal to 22 mg/kg bw per day), based on decreased body weight in males (14% compared with controls). A NOAEL was not identified in this study. The NOAEL for carcinogenicity was 500 ppm (equal to 22 mg/kg bw per day), based on an increased incidence of nasal tumours at 1500 ppm (equal to 69 mg/kg bw per day) and above (Ahmed, 1983a).

In a second oral carcinogenicity study, acetochlor (purity 96.1%) was administered to 70 Sprague-Dawley rats of each sex per dose in the diet for 24 months at a dose level of 0, 40, 200 or 1000 ppm (equal to 0, 1.9, 9.4 and 47.5 mg/kg bw per day for males and 0, 2.4, 11.8 and 60 mg/kg bw per day for females, respectively). Ten rats of each sex per dose were killed after 12 months for a 1-year interim evaluation.

Survival was not affected by treatment. Reduced body weights were observed in animals receiving 1000 ppm. A statistically significant decrease in body weight of males (5–15%) was observed at 1000 ppm, whereas the difference was not statistically significant for females in the same dose group. The weight differences in females in the 1000 ppm dose group were less than 7%. No treatment-related effects on clinical signs, urinary parameters, haematology or ophthalmoscopic examination were observed in the study. Statistically significant differences in clinical chemistry parameters (Table 46) that were considered to be relevant and related to treatment were increased GGT activity (in high-dose males at months 18 and 24), cholesterol level (high-dose males at month 24) and total bilirubin level (in high-dose females at month 24). There were no gross findings in animals dying spontaneously or killed at sacrifice that were considered to be related to treatment.

Selected treatment-related non-neoplastic histopathological findings are presented in Table 47. Fatty infiltration between the muscles of the tongue was significantly increased in high-dose females. It is a common spontaneous occurrence in aged, obese animals. The fat occurs in a patchy distribution, and the amount encountered depends upon the point of sectioning. There was no dose–response relationship, and the finding was considered to be without meaningful significance.

Table 46. Survival, body weight, blood biochemistry and organ weight data in rats

	Males				Females			
Significant findings	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**
Survival, termination (%)	47	40	37	38	40	43	43	48
Body weight, 23 months (g)	820.2	780.9	787.5	700.6**	505.8	522.1	527.5	469.6
GGT, 18 months (IU/L)	0.94	1.6	0.49	3.8*	4.1	3.7	3.9	4.0
GGT, 24 months (IU/L)	1.4	1.6	5.3	8.4**	0.92	1.2	1.8	1.4
Cholesterol, 24 months (mg/dL)	91.8	109	80.3	153*	50.2	56.3	58.6	73.4
Total bilirubin, 24 months (mg/dL)	0.08	0.09	0.09	0.13	0.11	0.13	0.10	0.51**
Relative liver weight, 24 months (%)	2.941	3.117	2.908	3.479†	3.260	2.903	2.975	3.096

GGT: gamma-glutamyltranspeptidase; IU: International Units; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test); †: $P \leq 0.05$ (Mann-Whitney statistic and Bonferroni inequality)
Source: Naylor & Ribelin (1986)

Table 47. Non-neoplastic histopathological lesions in a 24-month study in rats

		Males				Females			
Tissue	**Observation**	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**
Liver	*No. of tissues examined*	70	69	70	70	70	70	70	70
	Foci of cellular alteration	17†	14	13	25	19	18	14	22
Kidneys	*No. of tissues examined*	70	69	70	70	70	70	70	70
	Tubular casts/cysts/dilatation	50	59	59	64**	38	41	37	44
Tongue	*No. of tissues examined*	67	67	68	68	69	68	63	69
	Fatty infiltration	3	10	11	5	11	17	6	28**

ppm: parts per million; **: $P \leq 0.01$ (Fisher's exact test with Bonferroni inequality); †: $P \leq 0.05$ (Peto trend test)
Source: Naylor & Ribelin (1986)

Also increased were hepatic foci of altered cellular architecture in high-dose males and females (Peto trend test) and hepatic neoplastic nodules and thyroid follicular adenomas in high-dose females. None of these changes was statistically significantly increased over control incidence.

Some of the neoplastic findings were subjected to comprehensive re-evaluation and/or peer review (Hardisty, 1997b). The discussion here reflects the results of the most recent evaluation and may differ from the original study report. Thyroid C-cell hyperplasia was significantly increased in high-dose males that were killed at the end of the study. This lesion was also considered unrelated to treatment, as it was increased only in terminally killed animals, it was not increased in females, the degree was not severe, and there was no clear dose–response relationship.

The most important microscopic finding that was considered to be related to treatment was the occurrence of papillary adenomas of the nasal mucosa (Table 48). The liver samples from this study were re-evaluated by the PWG (Hardisty, 1997a), as an increased incidence of liver tumours was noted at 5000 ppm (Ahmed, 1983a). There was a slight increase in the incidence of hepatocellular adenomas in high-dose females, but this was not statistically significant. These liver tumours were not considered to be treatment related, because they lacked statistical significance and no evidence of

Table 48. Neoplastic histopathological lesions in a 24-month study in rats

		Males				Females			
Tissue	**Observations**	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**40 ppm**	**200 ppm**	**1 000 ppm**
Liver[a]	*No. of tissues examined*	60	60	60	60	60	60	60	60
	Hepatocellular adenoma	0	3	1	2	0	1	1	5
	Hepatocellular carcinoma	1	1	1	1	1	1	0	1
	Hepatocellular adenoma/ carcinoma	1	4	2	3	1	2	1	6
Nasal turbinates	*No. of tissues examined*[b]	70	69	70	70	70	70	70	70
	Papillary adenoma	1	0	0	12**	0	0	0	19**

ppm: parts per million; **: $P \leq 0.01$ (Fisher's exact test)
[a] Data derived from Pathology Working Group re-evaluation (Hardisty, 1997a).
[b] Includes interim kill animals, as nasal tumours were noted in some animals at 12 months.
Source: Naylor & Ribelin (1986)

increased liver tumours was observed at 1500 ppm (Ahmed, 1983a) or at 1750 ppm (Broadmeadow, 1989a).

In conclusion, the NOAEL for systemic toxicity in this 24-month dietary study in rats was 200 ppm (equal to 9.4 mg/kg bw per day), based on decreases in body weight in males, elevated total bilirubin level in females and elevations of GGT activity and cholesterol level in males observed at 1000 ppm (equal to 47.5 mg/kg bw per day). The NOAEL for carcinogenicity was 200 ppm (equal to 9.4 mg/kg bw per day), based on an increased incidence of papillary adenomas of the nasal mucosa at 1000 ppm (equal to 47.5 mg/kg bw per day) (Naylor & Ribelin, 1986).

In a third oral carcinogenicity study, acetochlor (purity 90.5%) was administered to 50 Sprague-Dawley rats of each sex per dose in the diet for 24 months at a dose level of 0, 18, 175 or 1750 ppm (equal to 0, 0.67, 6.4 and 66.9 mg/kg bw per day for males and 0, 0.88, 8.5 and 92.1 mg/kg bw per day for females, respectively). An interim sacrifice was conducted on 10 animals of each sex per group at the low and intermediate doses and on 20 animals of each sex per group for the control and high-dose groups after 12 months.

There were no treatment-related effects on survival, clinical signs of toxicity, clinical chemistry parameters, urine analysis, macroscopic findings or palpable swellings. The overall feed intake of males and females receiving 1750 ppm was, respectively, 13% and 8% lower than that of controls. These differences were statistically significant overall ($P < 0.001$). The overall body weights of males and females receiving 1750 ppm were, respectively, 12% and 33% lower than those of controls. These intergroup differences were statistically significant ($P < 0.05$). The feed conversion ratios (amount of feed consumed per unit of body weight gain) of rats receiving 1750 ppm were slightly higher than those of controls, indicating a reduced efficiency of feed utilization. In the latter phase of the study, the ophthalmoscopic examination after 76 and 101 weeks of treatment indicated that a high proportion of females receiving 1750 ppm had hyperreflection of the ocular fundus (Table 49). In the majority of affected animals, this lesion was bilateral. Examination after 76 weeks, and subsequently, indicated a slightly higher incidence of foci or plaques in the vitreous body or on the posterior capsule of the lens in males receiving 1750 ppm when compared with control males. In the majority of affected animals, this lesion was bilateral.

Low mean platelet counts after 24 weeks of treatment in males receiving 175 or 1750 ppm were considered to be a consequence of a raised mean count in controls caused by two particularly high values. These intergroup differences were not attributed to treatment. After 102 weeks of treatment, the mean cell volume in males and females fed 1750 ppm acetochlor was reduced

Table 49. Selected ophthalmological findings (week 76) in a 24-month study in rats

Finding	Males				Females			
	0 ppm	18 ppm	175 ppm	1 750 ppm	0 ppm	18 ppm	175 ppm	1 750 ppm
Hyperreflection of ocular fundus	0	2	4	1	5	4	6	24**
Vitreous body, foci/opaque spots	3	1	1	11*	3	2	3	0
Lenticular plaques/foci	4	1	4	12*	3	2	4	0

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Student's *t*-test, two-sided)
Source: Broadmeadow (1989a)

(decreases of 7% and 10%, respectively) compared with controls. Other parameters that showed a statistically significant difference from controls were minor and were considered not to be adverse.

After 52 weeks of treatment, there were increased incidences of adenomas of the nasal epithelium in both male ($P < 0.05$) and female ($P < 0.01$) rats that had received 1750 ppm; these tumours were accompanied by statistically significantly increased incidences of hyperplasia of the nasal epithelium in animals treated with 1750 ppm. There were no other changes associated with treatment at 52 weeks (Tables 50 and 51).

Table 50. Non-neoplastic lesions in a 24-month study in rats

Tissue	Observation	Males				Females			
		0 ppm	18 ppm	175 ppm	1 750 ppm	0 ppm	18 ppm	175 ppm	1 750 ppm
Week 52									
Nose	*No. of tissues examined*	20	10	10	20	18	9	10	16
	Purulent rhinitis	0	0	0	2	0	0	0	0
	Epithelial hyperplasia	0	0	0	11**	0	0	0	13**
Week 104									
Nose	*No. of tissues examined*	50	49	50	50	50	50	49	49
	Purulent rhinitis	0	1	3	4	1	1	2	1
	Epithelial hyperplasia	0	0	0	25**	0	0	0	28**
	Squamous metaplasia of olfactory epithelium	0	0	0	4	0	0	1	0
Eye	*No. of tissues examined*	48	43	46	49	49	48	48	45
	Degeneration of outer retinal layer	2	1	2	7	13	7	14	24*
Kidney	*No. of tissues examined*	50	50	50	50	50	50	50	49
	Pelvic epithelial hyperplasia	6	7	10	22**	4	7	9	14**

ppm: parts per million; **: $P \leq 0.01$ (Fisher's exact test)
Source: Broadmeadow (1989a)

There was an increased incidence of hyperplasia, progressing to adenoma, of the nasal epithelium observed in the majority of animals at 1750 ppm. After 104 weeks of treatment, two males and one female had carcinoma of the nasal epithelium. Nasal olfactory hyperplasia and polypoid adenoma were also observed in the interim kill animals at 52 weeks. No nasal hyperplasia or adenoma was observed in the other treatment groups or in the controls. At 1750 ppm, treatment-related

Table 51. Neoplastic lesions in a 24-month study in rats

		Males				Females			
Tissue	**Observation**	**0 ppm**	**18 ppm**	**175 ppm**	**1 750 ppm**	**0 ppm**	**18 ppm**	**175 ppm**	**1 750 ppm**
Week 52									
Nose	*No. of tissues examined*	20	10	10	20	18	9	10	16
	Adenoma of nasal epithelium	0	0	0	5*	0	0	0	8**
Week 104									
Nose	*No. of tissues examined*	50	49	50	50	50	50	49	49
	Adenoma of nasal epithelium	0	0	0	30**	0	0	0	28**
	Carcinoma of nasal epithelium	0	0	0	2	0	0	0	1
Thyroid	*No. of tissues examined*	50	50	48	50	50	50	50	49
	Follicular cell adenoma	2	1	2	5	1	1	3	5

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test)
Source: Broadmeadow (1989a)

increases in the incidence of squamous metaplasia of the olfactory epithelium and purulent rhinitis were also observed in male rats. There was a slightly increased incidence of thyroid follicular cell adenoma in females at 1750 ppm. In females fed 1750 ppm acetochlor, there was an increased incidence of degeneration of the retinal outer layer, which was consistent with the ophthalmological findings. The foci or plaques in the vitreous body or on the posterior capsule of the lens in males at this dose level were not associated with any other histopathological findings.

Two rare tumours were also originally reported in animals of the high-dose group. A benign chondroma of the femur was reported in one male that died during the study and in one female that survived to week 104, and a basal cell tumour was reported in the forestomach of one male and one female that died during the study. However, a subsequent PWG re-evaluation of these lesions indicated that the original diagnoses were erroneous (Hardisty, 2001a). The femur lesions were actually cartilaginous hyperplasia, which was also observed in one control animal. The forestomach lesions were not basal cell tumours, but the more common squamous cell carcinomas, and were not considered to be treatment related.

In conclusion, the NOAEL for systemic toxicity was 175 ppm (equal to 6.4 mg/kg bw per day), based on reduced body weight and feed consumption, changes in the eyes, reduced blood cell parameters and increased incidence of focal hyperplasia in the nasal epithelium observed at 1750 ppm (equal to 66.9 mg/kg bw per day). The NOAEL for carcinogenicity was 175 ppm (equal to 6.4 mg/kg bw per day), based on adenomas and carcinomas of nasal epithelium and thyroid follicular cell adenoma at 1750 ppm (equal to 66.9 mg/kg bw per day) (Broadmeadow, 1989a).

Nasal tumours were observed in the three carcinogenicity studies in rats and also in the two-generation reproductive toxicity study in rats (see section 2.5 below; Milburn, 2001). The incidences of the tumours are summarized in Table 52.

A PWG review was performed on the livers from all animals from the five long-term rat and mouse feeding studies with acetochlor in order to determine the incidence of hepatic neoplasms using currently accepted nomenclature and diagnostic criteria and to discuss the relevance, if any, of these tumours for human risk assessment.

Table 52. Comparison of acetochlor dose with nasal tumour incidence in reproductive toxicity and carcinogenicity studies with acetochlor

			Males		Females		
Study type	**Dietary concentration (ppm)**	**Generation**	**Dose (mg/kg bw per day)**	**Tumour incidence (%)**	**Dose (mg/kg bw per day)**	**Tumour incidence (%)**	**Reference**
Reproduction	200	F_0	18.6	0	21.5	0	Milburn (2001)
		F_1	18.6	0	21.5	0	
	600	F_0	57.0	0	64.6	0	
		F_1	57.0	12	64.6	4	
	1 750	F_0	166	15	199	23	
		F_1	166	31	199	65	
Oncogenicity	500	–	22	1	30	0	Ahmed (1983a)
	1 000	–	47.5	17	60	27	Naylor & Ribelin (1986)
	1 750	–	66.9	53	92.1	57	Broadmeadow (1989a)

ppm: parts per million

The results of the PWG review of hepatocellular neoplasms indicate that there is an increased incidence of combined hepatocellular carcinoma and hepatocellular adenoma in male and female rats and male and female mice only at 5000 ppm acetochlor in the diet. This dose level greatly exceeds the MTD, as it produces hepatic and renal toxicity and is associated with a marked decrease in body weight and an increase in mortality. As an increased incidence of hepatocellular neoplasms occurred only at 5000 ppm, a dose that greatly exceeded the MTD in rats and mice, the use of these neoplasms for risk assessment is considered inappropriate (Hardisty, 1997a).

2.4 Genotoxicity

Results for gene mutation assays are conflicting and provide no clear evidence of a positive effect in either bacterial or mammalian cell test systems. Similarly, the evidence from in vitro and in vivo unscheduled DNA synthesis assays, in vitro sister chromatid exchange studies and an in vivo comet test provides no convincing pattern of genotoxic activity. By contrast, results from chromosomal aberration assays indicate that acetochlor is a confirmed clastogen in cultured human lymphocytes. There is also the possibility that the increased mutant colony counts observed in the positive mouse lymphoma assay resulted from a clastogenic rather than a mutagenic response, as this test system can detect chromosome breakage. Nevertheless, clastogenicity is confined to in vitro mammalian cell test systems, and the types of induced aberrations suggest cytotoxicity. Based on data from three bone marrow assays and three dominant lethal mutation studies in mice or rats, acetochlor-induced clastogenicity is not expressed in either somatic or germinal cells of whole animals. The results of studies of genotoxicity with acetochlor are summarized in Table 53.

Table 53. Results of studies of genotoxicity with acetochlor

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro studies					
Bacterial gene mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	0.001–1 µL/plate in ethanol (±S9)	92.5	Negative	Kulik & Ross (1978)
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	1.6–5 000 µg/plate in DMSO (±S9)	89.9	Weakly positive[a]	Callander & Priestly (1989)
Bacterial gene mutation (Ames)	*S. typhimurium* TA1538	500–5 000 µg/plate in DMSO (±S9)	89.9 (technical standard) 99.6 (analytical standard)	Analytical-grade test material was negative Technical-grade test material was weakly positive	Callander (1992)
Bacterial gene mutation (Ames)	*S. typhimurium* TA1538	100–5 000 µg/plate in DMSO (±S9)	Technical acetochlor (89.9%, batch A) Technical acetochlor (94.4%, batch C) Analytical acetochlor (99.6%, batch B)	Negative[b]	Callander (1998)
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537	33.3–5 000 µg/plate in DMSO (±S9)	95.5	Negative	Xu (2006a)
Bacterial gene mutation (Ames)	*S. typhimurium* TA98	33.3–5 000 µg/plate in DMSO (±S9)	95.5	Negative	Xu (2006b)
Gene mutation	CHO/HGPRT	25–150 µg/plate in ethanol (±S9)	96.3	Weakly positive	Li (1983)
Gene mutation	CHO/HGPRT	50–200 µg/mL in ethanol (±S9)	91.4	Negative	Li & Myers (1989)
Gene mutation	Mouse lymphoma	20–400 µL/L in DMSO (±S9)	Not available	Weakly positive	Mitchell, Rudd & Colman (1982)
Chromosomal aberration	Human lymphocytes	10, 50 and 100 µg/mL in DMSO (±S9)	89.4	Positive[c]	Howard (1989)
Chromosomal aberration	Human lymphocytes	10–150 µg/mL in DMSO (±S9)	99.6	Positive[d]	Fox (1998)

End-point	Test object	Concentration	Purity (%)	Results	Reference
Unscheduled DNA synthesis	Rat hepatocytes	0.016–160 µg/mL in DMSO (±S9)	99.7	Negative	Naismith (1983)
Sister chromatid exchange	Human lymphocytes	10 µmol/L in DMSO (±S9)	Not reported	Equivocal[e]	Hill et al. (1997)
In vivo studies: somatic cells					
Chromosomal aberration	Rat (bone marrow)	40–500 mg/kg bw in corn oil	96.3	Negative	Farrow & Cortina (1983)
Micronucleus	Mouse (bone marrow)	200–2 000 mg/kg bw in corn oil	96.7	Negative	Cavagnaro & Cortina (1985)
Micronucleus	Mouse (bone marrow)	898–1 719 mg/kg bw in corn oil	89.4	Negative	Randall (1989)
DNA damage: comet assay	Rat (nasal)	1750 ppm (87.5 mg/kg bw) in the diet	96.6	Negative[f]	Ashby et al. (1996)
Unscheduled DNA synthesis	Rat (hepatocyte)	500–2 000 mg/kg bw in corn oil	89.4	Weakly positive[g]	Trueman (1989)
Unscheduled DNA synthesis	Rat (hepatocyte)	500–2 000 mg/kg bw in corn oil	89.9	Weakly positive[h]	Ashby & Lefevre (1993, 1994)
In vivo studies: germ cells					
Dominant lethal	Rat	100–2 000 ppm in diet	94.3	Negative	Naylor (1987)
Dominant lethal	Rat	200–2 000 mg/kg bw in corn oil	90.4	Negative	Hodge (1991)
Dominant lethal	Rat	200–1 500 ppm in the diet	94.4	Negative	Milburn (1996a)
Dominant lethal	Mouse	200–3 500 ppm in the diet	94.4	Negative	Milburn (1996b)

bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; GSH: glutathione; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; ppm: parts per million; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Weakly positive (< 2-fold increase) at higher concentrations in TA1538 (+S9). Negative in TA98, TA100, TA1535 and TA1537.

[b] Three different batches of technical-grade acetochlor were tested.

[c] Positive only at a concentration that induced substantial (36–69%) reduction in mitotic index.

[d] Positive response seen with acetochlor, but not with its *tert*-norchloroacetochlor (*t*-NCA; EU reference no. 6) analogue, indicating that the chloro substituent in acetochlor is responsible for its in vitro clastogenicity. Less toxicity observed in studies with whole blood than with isolated lymphocytes (probably due to protective effect of GSH).

[e] Equivocal (1.5-fold) increase in sister chromatid exchange at 10 µmol/L, maybe due to dialkylbenzoquinone metabolite in low-GSH environment.

[f] Negative at 1750 mg/kg bw per day, a dose level that produced rat nasal tumours.

[g] Weakly positive at 2000 mg/kg bw.

[h] Weakly positive at 2000 mg/kg bw, a dose level that also depleted hepatic GSH and produced severe liver toxicity.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a two-generation reproductive toxicity study, acetochlor (purity 94.7%) was administered continuously in the diet to CD (SD) IGS BR (Sprague-Dawley) rats (26 of each sex per dose) at a nominal dose level of 0, 200, 600 or 1750 ppm (equal to 0, 18.6, 57.0 and 166 mg/kg bw per day for F_0 males and 0, 21.5, 64.6 and 199 mg/kg bw per day for F_0 females, respectively). F_0 animals were given test article diet formulations for 10 weeks prior to mating to produce the F_1 litters. On PND 29, F_1 animals (26 of each sex per dose) were selected to become the parents of the F_2 generation and were given the same concentration of test formulation as their dams. F_1 animals were given test formulations for 10 weeks prior to mating to produce the F_2 litters.

The analytical data indicated that the mixing procedure was adequate and that variability was within acceptable ranges. No treatment-related clinical observations were observed in this study. Survival of parental animals was unaffected by treatment at any dose level. Mean premating body weight, body weight gain and feed consumption were consistently and statistically significantly reduced in both F_0 and F_1 animals at the high dose level (1750 ppm; Table 54). When compared with concurrent controls, decreases in body weight occurred in the high-dose F_0 males (reaching a maximum of approximately 8%) and females (reaching a maximum of 10%) from week 2 and throughout the premating period. The maximum decrease in F_1 animals was 16% for males and 14% for females. Body weights of F_1 females in the 600 ppm group were also decreased; the maximum difference from controls was approximately 9% in week 3. Body weights of F_0 and F_1 animals receiving 200 ppm acetochlor in the diet were similar to those of controls.

Table 54. Mean body weights of F_0 and F_1 parents

			Mean body weight (g)							
			Males				Females			
Generation	**Period**	**Weight**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**
F_0	Premating	Initial	153.5	155.8	150.9	150.9	126.9	122.7	122.5	123.3
		Final	463.6	463.2	465.5	433.5**	276.0	275.0	275.0	248.2**
	Gestation	Initial	–	–	–	–	284.3	276.0	273.4*	250.3*
		Final	–	–	–	–	380.5	385.4	378.5	365.4*
	Lactation	Initial	–	–	–	–	285.1	275.1*	271.5	248.4*
		Final	–	–	–	–	317.2	310.0	319.6	303.2*
F_1	Premating	Initial	76.3	80.2	74.8	67.5**	73.6	76.8	67.5**	64.9**
		Final	416.4	402.0	412.6	383.7**	251.5	251.1	246.9	229.8**
	Gestation	Initial	–	–	–	–	259.1	265.4	247.8	231.2**
		Final	–	–	–	–	353.6	354.9	351.6	345.1
	Lactation	Initial	–	–	–	–	257.4	271.9*	248.0	226.1**
		Final	–	–	–	–	298.9	301.7	297.5	294.9

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Milburn (2001)

There was no effect of acetochlor on estrous cyclicity, precoital interval, the duration of gestation or number of successful matings. A decrease in mean number of implantations occurred in a

dose-related manner in both the F_0 and F_1 generations, reaching statistical significance at the high dose level in both generations. The mean number of live pups on PND 1 decreased in a dose-related manner in both the F_1 and F_2 litters. Mean live F_1 pups per litter was significantly decreased at 1750 ppm, and the mean number of live-plus-dead pups per litter was statistically significantly lower in F_1 and F_2 litters at 1750 ppm and also in F_2 litters at 600 ppm (Table 55).

Table 55. Effect of acetochlor on reproductive and offspring toxicity in F_0 and F_1 generations

	F_0				F_1			
Parameters	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**
Estrous cycle (days)	3.89	3.86	3.80	3.70*	4.12	3.95	4.17	3.92
Precoital length (days)	2.27	2.60	2.65	2.58	2.80	3.33	2.88	3.48
Gestation length (days)	22.1	22.0	22.0	21.9**	22.0	21.9	21.9	21.8
Successful matings (%)	96.2	96.2	92.3	96.2	88.5	96.2	92.3	92.3
Whole litter loss (%)	16.0	8.0	12.5	12.0	17.4	8.0	8.3	8.3
No. of implantations	14.7	14.2	13.8	13.1**	14.6	13.6	13.5	12.7**
Postimplantation loss (%)	6.4	8.1	8.0	5.9	4.4	7.4	9.8*	7.7
No. of pups (live + dead)	13.7	13.0	12.6	12.3*	14.0	12.6	12.1*	11.7**
No. of live pups, day 1	13.1	12.3	12.1	12.0	13.6	12.3	11.9	11.4*

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Milburn (2001)

There were no treatment-related gross pathological findings in the F_0 or F_1 adults. At 1750 ppm, relative kidney, liver and thyroid weights were significantly increased for F_0 and F_1 parental males and females, and relative ovarian weights were decreased for F_0 and F_1 females, compared with controls. Increased relative liver, kidney and thyroid weights were also observed at 600 ppm in F_0 males and females. However, there were no gross or histopathological changes in these tissues that appeared to be associated with treatment. Histopathological evaluation revealed treatment-related incidences of benign proliferative lesions (focal epithelial hyperplasia and polypoid adenomata [papillary adenoma]) in the epithelial lining of the ethmoid region of the nasal cavity in F_0 and F_1 adult animals receiving 1750 ppm acetochlor and in F_1 animals at the 600 ppm level (Table 56). Minimally increased brown pigment (lipofuscin) was observed in the olfactory mucosa, mainly in the lamina propria and occasionally in the basal epithelium, in most animals receiving 600 and 1750 ppm in both the F_0 and F_1 generations and also in F_1 females at the 200 ppm dose level; however, this finding was not considered to be of toxicological significance.

Table 56. Incidence of nasal proliferative lesions in F_0 and F_1 adults

		Males				Females			
Generation	**Findings**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**
F_0	*No. of tissues examined*	26	26	26	26	26	26	26	26
	Hyperplasia	0	0	0	3	0	0	0	7
	Papillary adenoma	0	0	0	4	0	0	0	6
F_1	*No. of tissues examined*	26	26	26	26	26	26	26	26
	Hyperplasia	0	0	0	7	0	0	4	14
	Papillary adenoma	0	0	3	8	0	0	1	17

ppm: parts per million
Source: Milburn (2001)

Postnatal survival was not affected by treatment. The adjusted body weights of the pups at preweaning were statistically significantly reduced in the 1750 ppm groups for the F_1 and F_2 generations and in the 600 ppm group for the F_2 generation. There was no effect on body weights of F_1 or F_2 pups in the 200 ppm group. There was no effect of acetochlor on preputial separation in F_1 males. A decrease in anogenital distance in F_2 males on PND 1 and a 3-day treatment-related delay in the day of vaginal opening in F_1 females at the high dose level appeared to be associated with delayed growth. The delay in vaginal opening was attributed to the delay in reaching critical body weight and was not considered to be a specific developmental effect, as the body weight at the time of vaginal opening was similar to that of controls. There was no effect of treatment on vaginal opening in F_1 females in the 200 and 600 ppm groups (Table 57). Sperm measures (epididymal sperm count, motility and morphology; and testicular spermatid count) were not affected by treatment for F_0 or F_1 males. There were no treatment-related histopathological lesions of the reproductive organs, and primordial follicle count data were similar between control and high-dose F_1 females.

Table 57. Effect of acetochlor on developmental parameters of F_1 and F_2 pups

	F_1				**F_2**			
Developmental parameters	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**	**0 ppm**	**200 ppm**	**600 ppm**	**1 750 ppm**
Pup survival (%), day 29	74.7	87.0	86.5	91.0**	90.5	88.4	92.3	92.9
Sex distribution (% male)	49.6	49.7	51.6	47.5	50.2	51.4	54.3	54.7
Pup weight (g), day 29, M	6.0	6.0	5.7*	5.8*	5.7	5.9	5.9	5.5
Pup weight (g), day 29, F	5.7	5.7	5.3	5.3*	5.4	5.6	5.5	5.2
Pup weight (g), day 29, M[a]	76.8	80.9	75.8	69.2*	75.8	73.7	68.3**	65.0**
Pup weight (g), day 29, F[a]	72.6	74.5	70.4	65.1*	71.9	70.3	64.4**	61.0**
Preputial separation (days)	46.9	46.8	47.0	48.0	–	–	–	–
Vaginal opening (days)	36.2	35.8	37.8	39.1**	–	–	–	–
Body weight at vaginal opening (g)	112.9	113.5	110.4	109.4	–	–	–	–
Anogenital distance (mm), M	–	–	–	–	4.49	4.50	4.45	4.32*
Anogenital distance (mm), F	–	–	–	–	2.66	2.72	2.70	2.64

F: female; M: male; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$

[a] Adjusted by covariance on day 1 mean pup weight.

Source: Milburn (2001)

There were no treatment-related macroscopic findings in either sex of the pups at any dose level. Decreases in absolute mean brain weights of F_1 males and F_2 males and females were noted at 1750 ppm. On a relative to body weight basis, ratios were statistically increased for the brain in F_2 pups at the 600 ppm dose as well. Mean absolute spleen weights were also decreased in F_1 males and in both sexes of the F_2 pups at both the middle and high dose levels. Spleen weights were also reduced on a relative basis in 600 ppm F_2 females. Mean absolute thymus weights were also decreased in F_2 pups at the high dose level, but not relative (to body weight) thymus weights.

The NOAEL for parental toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on focal hyperplasia and polypoid adenomata in the nasal epithelium of adult F_1 offspring at study termination and decreased body weight in F_1 females observed at 600 ppm (equal to 57.0 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on decreased F_2 litter size at birth, decreased F_1 and F_2 pup body weights during lactation, and decreased absolute and relative spleen weights in F_2 weanlings seen at 600 ppm (equal to 57.0 mg/kg bw per day). The NOAEL for reproductive toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on

a decreased number of implantations observed at 600 ppm (equal to 57.0 mg/kg bw per day) (Milburn, 2001).

In a second two-generation reproductive toxicity study, acetochlor (purity 91.0%) was administered continuously in the diet to CD (Sprague-Dawley) rats (25 of each sex per dose) at a nominal dose level of 0, 18, 175 or 1750 ppm (equal to 0, 1.3, 12.6 and 124 mg/kg bw per day for F_0 males and 0, 1.6, 15.5 and 157 mg/kg bw per day for F_0 females, respectively). F_0 animals were given test article diet formulations for 8 weeks prior to mating to produce the F_1 litters. On postnatal day (PND) 29, F_1 animals (26 of each sex per dose) were selected to become the F_1 parents of the F_2 generation and were given the same concentration of test formulation as their dams. F_1 animals were given test formulations for 10 weeks prior to mating to produce the F_2 litters. Animals were dosed 40 days prior to mating. Quantitative evaluation of primordial follicles, sperm parameters, vaginal opening, balano-preputial separation and anogenital distance was not conducted.

The analytical data indicated that the mixing procedure was adequate and that variability was within acceptable ranges. No compound-related effects on clinical signs of toxicity or mortality were observed. The body weight gain of animals receiving 1750 ppm acetochlor was reduced compared with that of controls during maturation in both generations, although there was little effect on females during the gestation or lactation phases. Body weight gain of animals receiving 18 or 175 ppm acetochlor was similar to that of controls. The comparison of the mean parental body weights is shown in Table 58.

Feed consumption by males in both generations at 1750 ppm was slightly reduced compared with controls, but there was no effect in females at this dose or in either sex at 18 or 175 ppm acetochlor. The mating performance, conception and fertility indices were similar in all groups at both pairings. The precoital interval was very similar between groups (1–4 days) for the majority of animals in the F_0 (92% and 100% in control and 1750 ppm groups, respectively) and F_1 generations (92% and 84% in control and high-dose groups, respectively). There were no treatment-related macroscopic or histopathological findings in either generation. Organ weights (brain, liver and kidney in both sexes, heart and reproductive organs in F_1 males, and spleen in females) relative to body weight were increased for animals in the 1750 ppm group as a result of the considerably lower final body weights of these animals compared with controls. Thymus weight was reduced in the F_1 males. In addition, there was an increase in the relative weights of epididymides, testes, seminal vesicles and thymus in F_1 males.

There was no evidence of an adverse effect of acetochlor on pup birth weight, pup sex ratio, pup viability or pup development. At the end of the lactation period, the body weight of the pups of both generations at the 1750 ppm dose level was reduced compared with controls (14–20% lower than controls). Macroscopic examination of F_1 and F_2 pups did not reveal any treatment-related abnormalities.

The NOAEL for parental toxicity was 175 ppm (equal to 12.6 mg/kg bw per day), based on decreases in body weight, body weight gain and feed consumption in the premating period as well as body weight reduction at the beginning of the gestation and lactation periods and, at sacrifice, increases in relative weights of brain, kidney, liver and spleen in F_1 females and of testes, seminal vesicles and thymus in F_1 males observed at 1750 ppm (equal to 124 mg/kg bw per day). The NOAEL for offspring toxicity was 175 ppm (equal to 12.6 mg/kg bw per day), based on decreased pup body weights observed at 1750 ppm (equal to 124 mg/kg bw per day). The NOAEL for reproductive toxicity was 1750 ppm (equal to 124 mg/kg bw per day), the highest dose tested (Willoughby, 1989).

In a third two-generation reproductive toxicity study, acetochlor (purity 94.5%) was administered continuously in the diet to COBS CD rats (12 males and 24 females per dose) at a nominal dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 30.8, 90.6 and 316 mg/kg bw per day for F_0 males and 0, 46.2, 130 and 442 mg/kg bw per day for F_0 females, respectively). F_0 animals were given test article diet formulations for 100 days and F_1 parents for 120 days prior to mating. Data were

Table 58. Mean body weights of F_0 and F_1 parents in a reproductive toxicity study in rats

			Mean body weight (g)							
			Males				Females			
F	**Period**	**Weight**	**0 ppm**	**18 ppm**	**175 ppm**	**1 750 ppm**	**0 ppm**	**18 ppm**	**175 ppm**	**1 750 ppm**
F_0	Premating	Initial	252	252	248	251	170	169	170	173
		Final	494	493	488	469**	283	272	279	265**
	Gestation, F_{1A}	Initial	–	–	–	–	284	274	276	261**
		Final	–	–	–	–	396	392	395	374
	Lactation, F_{1A}	Initial	–	–	–	–	306	304	307	275**
		Final	–	–	–	–	327	327	322	304
	Gestation, F_{1B}	Initial	–	–	–	–	318	309	309	284**
		Final	–	–	–	–	439	434	434	397
	Lactation, F_{1B}	Initial	–	–	–	–	340	329	328	291**
		Final	–	–	–	–	356	357	354	323
F_1	Premating	Initial	79	81	77	75	70	74	70	67
		Final	532	522	530	472**	279	276	285	246**
	Gestation, F_{2A}	Initial	–	–	–	–	283	275	285	247**
		Final	–	–	–	–	387	391*	401*	348
	Lactation, F_{2A}	Initial	–	–	–	–	316	301	314	262**
		Final	–	–	–	–	324	323	322	281
	Gestation, F_{2B}	Initial	–	–	–	–	317	312	321	263**
		Final	–	–	–	–	430	432	441	363*
	Lactation, F_{2B}	Initial	–	–	–	–	353	346	357	286**
		Final	–	–	–	–	340	352	357	296

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (analysis of variance and Student's *t*-test)
Source: Willoughby (1989)

lacking on the evaluation of precoital interval, primordial and small growing follicles, mean number of cycles and cycle length, number of implantations, whole litter losses, developmental landmarks and sex ratios. Postmortem examinations and histopathology were limited to those animals that died on test and five F_{1b} and F_{2b} pups and 10 F_1 parents killed at scheduled sacrifice.

There were no treatment-related effects on survival, clinical observations, fertility indices, sperm parameters, gestation length, gross pathology or organ weights. At termination, the mean body weights of F_0 animals at 5000 ppm were statistically significantly decreased by 10.4% (male) and 25.1% (female) compared with controls. Similarly, the mean body weights of F_1 animals at 5000 ppm were statistically significantly decreased by 19.1% (male) and 33.4% (female) compared with controls. At 1500 ppm, body weights of females were statistically significantly decreased (11.4%) compared with controls. At the 5000 ppm level, feed consumption was reduced by 24% and 27% in females of the F_0 and F_1 generations, respectively. At 5000 ppm, the absolute weights of the heart, brain and spleen were decreased in both sexes. As well, the relative weights of kidney and liver were increased at 5000 ppm; relative liver weight was also increased in both sexes in the F_1 generation at 1500 ppm. In females, the relative weight of adrenals decreased and the absolute and relative weights of the thyroid increased at 5000 ppm. In males, the absolute weight of the pituitary decreased at 500 and 5000 ppm, and the relative weight of the pituitary decreased at 500 and 1500 ppm. A slight

increase was observed in the relative weight of adrenals at 5000 ppm. The only histopathological finding in the parent animals was nephritis in 8/10 females of the F_1 generation at 5000 ppm.

There were no effects of acetochlor on the clinical condition of the F_1 or F_2 pups. Statistically significant differences were observed in the number of live pups and the mean body weight of pups (37–45% compared with controls) from the F_1 and F_2 generations at 5000 ppm. At 1500 ppm, there was a slight, but statistically significant, decrease in the body weight of F_{2b} pups at lactation days 14 and 21. There were no test article–related changes in either mean absolute or mean relative (to body weight) organ weights observed among animals killed at the scheduled sacrifice in F_1 and F_2 animals, except for spleen weights in F_{2b} pups at 5000 ppm.

In conclusion, the NOAEL for parental systemic toxicity was 500 ppm (equal to 30.8 mg/kg bw per day), based on decreased body weights and decreased relative weights of liver and kidney observed at 1500 ppm (equal to 90.6 mg/kg bw per day). The NOAEL for offspring toxicity was 1500 ppm (equal to 90.6 mg/kg bw per day), based on the number of liveborn pups and decreased body weights of F_{2b} pups observed at 5000 ppm (equal to 316 mg/kg bw per day). The NOAEL for reproductive toxicity was 5000 ppm (equal to 316 mg/kg bw per day) (Schardein, 1982).

(b) Developmental toxicity

Rats

In a prenatal developmental toxicity study, acetochlor (purity 90.5%) was administered by gavage in corn oil to 25 pregnant Sprague-Dawley CD (SD) BR VAF/Plus rats per dose from gestation day (GD) 6 to GD 15 inclusive at a dose level of 0, 40, 150 or 600 mg/kg bw per day. Animals were killed on GD 20, and uteri were examined for live fetuses and intrauterine deaths. Fetuses were weighed and examined for external, visceral and skeletal alterations.

Four animals were killed moribund. Two females dosed with 600 mg/kg bw per day were killed (one on GD 13 and the other one on GD 15) with signs including piloerection, hunched posture, weight loss and general poor condition. Two further females, one control and one dosed with 40 mg/kg bw per day, revealed perforated oesophagus, indicating an intubation error. There was a dose-related response in the extent of salivation observed post-dosing (15, 25 and 23 of the rats dosed at 40, 150 and 600 mg/kg bw per day, respectively). Signs of toxicity were generally confined to the 600 mg/kg bw per day group and included ungroomed coat, piloerection and hunched posture in two females and stained urogenital region in four females. Rats dosed with 600 mg/kg bw per day reduced their feed consumption slightly, but statistically significantly, during days 6–9 (about 20%); thereafter, feed intake was slightly lower than, but comparable with, that of controls. A statistically significant increase in water consumption (28.5–52.4% increase compared with controls) was observed during GDs 6–15 at 600 mg/kg bw per day. At 600 mg/kg bw per day, the rate of body weight gain when compared with the control group was statistically significantly reduced ($P < 0.01$) from the first dose (GD 6), which was followed by a statistically significant mean weight loss from GD 7 to GD 9 (about 10.6% compared with controls). The mean rate of weight gain thereafter was still slightly lower than that of controls through to GD 12, although the difference from control weight gain was not statistically significant. From day 13, body weight gains were comparable with those of the control group, although parity was not regained by GD 20. Macroscopic findings at postmortem examination did not reveal any treatment-related effects.

Only at the 600 mg/kg bw per day dose level was there a statistically significant increase in early embryonic deaths, total embryonic deaths and postimplantation loss. However, the increases observed were predominantly due to a higher number of litters with one embryonic death at this dose and do not appear to be directly attributable to treatment; the number of litters with more than one embryonic death were one, four, two and two at 0, 40, 150 and 600 mg/kg bw per day, respectively. A statistically significant decrease in mean fetal weight was also observed at the high dose (Table 59).

Table 59. Selected gestational and fetal parameters in a developmental toxicity study in rats

Parameter	0 mg/kg bw per day	40 mg/kg bw per day	150 mg/kg bw per day	600 mg/kg bw per day
Early embryonic deaths	0.2	0.5	0.5	0.8**
Late embryonic deaths	0.1	0	0	0.2
Total embryonic deaths	0.3	0.5	0.5	1.0**
Postimplantation loss (%)	3.4	4.4	4.3	7.6**
Mean fetal weight (g)	3.37	3.32	3.36	3.13**

bw: body weight; **: $P < 0.01$
Source: Brooker, Stubbs & John (1989b)

Some reduction in ossification was also observed at the highest dose level. There was no obvious relationship between treatment and the incidence or distribution of malformations (three, three, six and four malformed fetuses at 0, 40, 150 and 600 mg/kg bw per day, respectively) or visceral anomalies.

The NOAEL for maternal toxicity in rats was 150 mg/kg bw per day, based on mortality, clinical signs of toxicity, decreases in body weight gain and feed consumption and a marked increase in water consumption observed at 600 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day, based on the reduction in mean fetal weight and reduced ossification at 600 mg/kg bw per day. There was no evidence of teratogenicity (Brooker, Stubbs & John, 1989b).

In a second prenatal developmental toxicity study, acetochlor (purity 91.3%) was administered by gavage in corn oil to 25 pregnant Sprague-Dawley COBS CD rats per dose from GD 6 to GD 19 inclusive at a dose level of 0, 50, 200 or 400 mg/kg bw per day. The corn oil gavage dosing volume was 10 mL/kg bw. Caesarean sections were performed on all dams on GD 20, and uteri were examined for live fetuses and intrauterine deaths. Fetuses were weighed and examined for external, visceral and skeletal alterations.

Mortality was not observed in the study. Matting and/or staining of the anogenital region were noted for 13/25 rats in the 400 mg/kg bw per day group, and excessive salivation was observed in three rats as a post-dosing response on one occasion. A slight but not dose-related increase in matting and/or staining of the anogenital region was noted in the 50 and 200 mg/kg bw per day dose groups. At 400 mg/kg bw per day, decreases in body weight gain (30.4% of the control value) and adjusted body weight gain (53.5% of the control value) were noted for the GDs 6–20 and GDs 0–20 periods, respectively. No significant differences in mean maternal body weight gain during the treatment period or adjusted body weights on GD 20 were observed in the 50 or 200 mg/kg bw per day dose groups when compared with the control group.

There were no biologically meaningful or statistically significant differences in the mean number of corpora lutea, total implantations, postimplantation loss, viable fetuses or the fetal sex distribution in the 50, 200 or 400 mg/kg bw per day dose groups when compared with the control group. A slight to moderate decrease in mean fetal weight, although not statistically significant, was noted in the 400 mg/kg bw per day dose group. There were no statistically significant differences in the number of litters with malformations in the 50, 200 or 400 mg/kg bw per day dose groups when compared with the control group. The only malformation noted in the 400 mg/kg bw per day dose group was dwarfism, noted in five fetuses in one litter (1.9% incidence). The incidence of dwarfism fell into the range of the historical control data presented (0–1.9% incidence in fetuses and 0–4.3% incidence in litters).

Based on the results of this developmental toxicity study in rats, the NOAEL for maternal toxicity was 200 mg/kg bw per day, based on the decreased body weight gain and clinical signs of

toxicity seen at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested. There was no evidence of teratogenicity in rats (Rodwell, 1980).

Rabbits

In a developmental toxicity study, acetochlor (purity 90.5%) was administered by gavage in corn oil to 16 pregnant New Zealand White rabbits per dose from GD 6 to GD 18 inclusive at a dose level of 0, 30, 100 or 300 mg/kg bw per day. Caesarean sections were performed on all dams on GD 29, and uteri were examined for live fetuses and intrauterine deaths. Fetuses were weighed and examined for external, visceral and skeletal alterations.

At 300 mg/kg bw per day, one female was killed following an intubation error; a second female was unable to move easily, with restricted movement of the hindlimbs (the toxicological significance of this is unclear), and was killed on GD 11; and a third female was found dead on GD 19 after aborting on GD 18. No clinical signs of toxicity were seen that were considered to be due to treatment in any group. There was a statistically significant decrease in body weight gain (Table 60) during GDs 6–8 at 300 mg/kg bw per day (weight change of −30, −21, −20 and −83 g at 0, 30, 100 and 300 mg/kg bw per day, respectively). Body weight gain adjusted for gravid uterine weight did not show any intergroup differences. At 300 mg/kg bw per day, there was a statistically significant reduction in feed consumption during GDs 6–7 (mean feed consumption of 125, 126, 120 and 86 g per rabbit per day at 0, 30, 100 and 300 mg/kg bw per day, respectively). Macroscopic findings at postmortem examination did not reveal any treatment-related effects.

Table 60. Body weight change in dams in a developmental toxicity study in rabbits

	Body weight change (g)			
Days of gestation	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day[a]**
1–6	59	24	6	47
6–8	−30	−21	−20	−83*
8–11	−2	4	33	39
11–15	102	136	107	145
15–18	15	8	16	−13
18–25	166	126	100	137
25–29	144	75	158	110

bw: body weight; *: $P < 0.05$

[a] Ten animals.

Source: Brooker, Stubbs & John (1989a)

There were no treatment-related effects of acetochlor on litter size, sex ratio, preimplantation and postimplantation losses, litter weight, mean fetal weight or incidence of malformations, visceral and skeletal anomalies or skeletal variants at any dose level.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on decreased feed consumption and body weight (GDs 6–8) and the treatment-related death of two dams observed at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, the highest dose tested (Brooker, Stubbs & John, 1989a).

In a second developmental toxicity study, acetochlor (purity 94.2%) was administered by gavage in corn oil to 20 inseminated New Zealand White rabbits per dose from GD 7 to GD 19 inclusive at a dose level of 0, 15, 50 or 190 mg/kg bw per day. Animals were killed on GD 29, and

uteri were examined for live fetuses and intrauterine deaths. Fetuses were weighed and examined for external, visceral and skeletal alterations. Feed consumption was not recorded, gravid uterine weights were not determined, and dosing was only on GDs 7–19.

There were no treatment-related effects on survival, clinical observations or gestational parameters. Statistically significant decreases in mean body weight on GD 19 (3748 g treated mean versus 4038 g in controls) and mean body weight loss on GDs 7–19 (−146 g in treated versus +81 g in controls) were observed at 190 mg/kg bw per day. Macroscopic findings at postmortem examination did not reveal any treatment-related effects.

Intrauterine survival, fetal sex ratios and fetal weights were not affected by administration of the test article at any of the dose levels. No remarkable differences in the number or type of malformations were apparent in any of the treated groups when compared with the vehicle control group. Fetal developmental variations occurred with similar frequency in the control and treated groups.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on decreased body weight at GD 19 and decreased body weight gain during GDs 7–19 observed at 190 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 190 mg/kg bw per day, the highest dose tested (Adam, 1986).

2.6 Special studies

(a) Neurotoxicity

Rats

In an acute neurotoxicity study, groups of fasted Alpk:ApfSD (Wistar-derived) rats (10 of each sex per dose) were given a single oral dose of acetochlor (purity 94.7%) in corn oil at a dose of 0, 150, 500 or 1500 mg/kg bw and observed for 14 days. Neurobehavioural assessment (functional observational battery and motor activity testing) was performed in 10 animals of each sex per group pretest and at study days 1 (time of peak effect), 8 and 15. At study termination, five animals of each sex per group were euthanized and perfused in situ for neuropathological examination. Of the perfused animals, five rats of each sex from the control and high-dose groups were subjected to histopathological evaluation of brain and peripheral nervous system tissues.

One male given 1500 mg/kg bw was found dead on day 2. This death is attributed to the acute toxicity of acetochlor. At 1500 mg/kg bw, body weights adjusted for initial weight were significantly lower than in the control group on day 8 for males and on days 1, 8 and 15 for females. Feed consumption by the high-dose males and females was significantly reduced during the first week of the study compared with the controls. During the functional observational battery, findings were limited to the time of peak effect at the high dose level. At 1500 mg/kg bw, the following clinical signs were attributable to treatment: decreased activity, chromodacryorrhoea, hunched posture, hypothermia, laboured breathing, piloerection, sides pinched in, signs of salivation, stains around the mouth and nose and upward curvature of the spine. Almost all animals recovered by day 8, but a few presented with slightly reduced splay reflex on day 15. In male and female rats given 500 mg/kg bw, the clinical signs were piloerection and stains around the nose on day 1 only. No effects of treatment on landing foot splay measurement, time to tail flick or grip strength were noted.

No effects of treatment on motor activity were noted in males (Table 61). Total activity counts for high-dose females on day 1 were significantly decreased at 1500 mg/kg bw compared with controls. In females given 500 mg/kg bw, motor activity on day 1 was statistically significantly decreased relative to controls. However, the motor activity level in the 500 mg/kg bw dose group on day 1 was slightly greater than the pretest value. Furthermore, the pattern of habituation was unchanged compared with pretest values. Consequently, this decrease is not considered to be related to treatment with acetochlor. A statistically significant increase in motor activity of 28% was also observed in high-dose females on day 8. There were no treatment-related effects on brain weights or gross and histological pathology or neuropathology. Effects on the motor activity and functional

observational battery parameters at the high dose of 1500 mg/kg bw are considered to be due to the overt toxicity of the compound rather than neurotoxicity.

Table 61. Intergroup comparison of motor activity (total activity and percentage of pretest values)

	Males				Females			
Period (days)	**0 mg/kg bw**	**150 mg/kg bw**	**500 mg/kg bw**	**1 500 mg/kg bw**	**0 mg/kg bw**	**150 mg/kg bw**	**500 mg/kg bw**	**1 500 mg/kg bw**
−7	398.5	390.3	436.9	437.6	423.6	392.7	345.5	448.1
1	361.2	600.5	388.8	269.2	571.4	501.4	401.7**	251.7**
% of pretest value	90.6	77.0	88.6	61.5	134.9	127.7	116.3	56.2
8	566.6	581.6	487.0	575.6	525.8	570.9	573.0	673.2*
% of pretest value	142.2	149.0	111.0	131.5	124.1	145.4	165.8	150.2
15	516.3	538.1	609.4	504.2	622.6	587.3	632.3	642.2
% of pretest value	129.6	137.9	138.8	115.2	147.0	149.6	183.0	143.3

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance)
Source: Kilgour (2001a)

The NOAEL for systemic toxicity was 500 mg/kg bw, based on decreased body weight and body weight gain and reduced feed consumption in both sexes observed at 1500 mg/kg bw. The NOAEL for neurotoxicity was 1500 mg/kg bw, the highest dose tested (Kilgour, 2001a).

In a subchronic oral neurotoxicity study, acetochlor (purity 94.7%) was administered in the diet to 12 Alpk:APfSD rats of each sex per group at a dose level of 0, 200, 600 or 1750 ppm (equal to 0, 15.4, 47.6 and 139 mg/kg bw per day for males and 0, 18.3, 55.9 and 166.5 mg/kg bw per day for females, respectively) for 90 days. A neurobehavioural assessment (functional observational battery and motor activity testing) was performed in all animals of each sex per group at −1 week pretest and at weeks 2, 5, 9 and 14. At study termination, five animals of each sex per group were euthanized and perfused in situ for neuropathological examination (brain, spinal cord and peripheral nervous system of the control and high-dose animals were examined microscopically; brain weights were also measured).

At 1750 ppm, slight but statistically significant decreases in mean body weight (−2.6% to −4.1% less than controls) and weight gain (−14% to −20% for males and −25% to −30% for females) were reported in both sexes in the early weeks of the study. Decreases thereafter were not statistically significant, but continued throughout the study. Body weight gain was also lower in animals given 1750 ppm acetochlor in the diet. The maximum effect was seen in week 2. During the functional observational battery evaluations at week 2, but not at later times, a statistically significant decrease in hindlimb grip strength (−44%) in males was observed, which was considered to be a transient effect, as it was not observed at any other time points. There were no treatment-related increases in clinical signs of toxicity or effects on other neurobehavioural parameters in the functional observational battery, motor activity, brain weight or gross/microscopic neuropathology.

The NOAEL for systemic toxicity was 600 ppm (equal to 47.6 mg/kg bw per day), based on marginal decreases in mean body weight and body weight gain in males and females observed at 1750 ppm (equal to 139 mg/kg bw per day) (Kilgour, 2001b).

(b) Immunotoxicity

Mice

In an immunotoxicity study, acetochlor (purity 95.4%) was administered to 10 female Crl:CD1(ICR) mice per dose in the diet at a dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 119, 334 and 1536 mg/kg bw per day, respectively) for 28 days. A concurrent positive control group received cyclophosphamide monohydrate at 50 mg/kg bw per day once daily during study days 24–27 via intraperitoneal injection. Only female animals were used for the immunotoxicity evaluation because females are considered to be more consistent responders than males for the evaluation of the humoral immunoresponse using the antibody-forming cell (AFC) assay. All animals were immunized with an intravenous injection of sheep red blood cell antigen in the tail vein on study day 24.

Over the entire 4-week study period, there were no test substance–related effects on survival, body weight, feed consumption or parameters evaluated during histopathological examinations. There were no test substance–related effects on absolute or relative (to final body weight) spleen or thymus weights, spleen cellularity or the T cell–dependent antibody response, as measured by the AFC immunoglobulin M specific activity (AFC/10^6 spleen cells) or total spleen activity (AFC/spleen), at any dose level tested.

The NOAEL for immunotoxicity was 5000 ppm (equal to 1536 mg/kg bw per day), the highest dose tested (Crittenden, 2011).

(c) Mechanistic studies on nasal tumours

In vivo studies

A study was designed to determine the overall tissue distribution and localization, especially in the nasal turbinate tissue, of ^{14}C-labelled acetochlor residues following oral administration to rats and mice. The nasal turbinate is a target tissue in rats.

The study consisted of two groups of animals. In the first group, three male and three female Sprague-Dawley rats were administered a single oral gavage dose of ^{14}C-labelled acetochlor (purity 99.5%) at target dose levels of 290 and 375 mg/kg bw for males and females, respectively. The second group consisted of three male and three female B6C3F1 mice administered a single oral gavage dose of ^{14}C-labelled acetochlor at target dose levels of 885 and 1060 mg/kg bw for males and females, respectively. These dose levels correspond to the high dose levels used in the rat and mouse carcinogenicity studies described above in section 2.3 (Ahmed, 1983a,b; Naylor & Ribelin, 1986; Amyes, 1989; Broadmeadow, 1989a). For both mice and rats, one animal of each sex was killed at 6 and 24 hours and 7 days after dosing. All animals were processed by whole-body autoradiography.

In the male rat killed at 6 hours after dosing, intense, nonspecific localization was seen in the frontal region of the nose. Specific localization was also observed in the ethmoid turbinate region. Because of the unusual nature of the nonspecific localization, the 6-hour time point was repeated for both male and female rats. The nonspecific nasal localization was not repeated; however, localization was observed in the ethmoid turbinates of both male and female rats. Few differences were observed in the sites of localization between males and females. Overall, the sites of distribution of radioactivity in the body were similar in the male and female rats. At 7 days after treatment, there was still slight radioactivity present in nasal tissue, blood and highly perfused tissues. At both 24 hours and 7 days, females generally displayed a slightly higher accumulation of radioactivity compared with males.

No evidence of nasal localization of radioactivity was observed in either the male or female mice at any time point. At 7 days, most of the radioactivity appeared to have been cleared from the tissues in male mice, but not in female mice.

Acetochlor and/or its metabolites were found to accumulate specifically in the olfactory region of the nasal turbinates in the rat and remained detectable for at least 7 days. No specific accumulation of acetochlor and/or its metabolites was observed in the olfactory region of the nose in mice (Kraus & Wilson, 1996).

A study was conducted to evaluate the nasal localization of ^{14}C-labelled acetochlor following dietary administration to rats. A mixture of radiolabelled (^{14}C) and unlabelled (^{12}C) acetochlor was administered in the diet at target levels of 1750 and 5000 ppm for 14 days to Sprague-Dawley rats. Three males per group were used in groups M1 and M2, and four males per group were used in groups M3 and M4. Groups M1 and M3 received 1750 ppm acetochlor, and groups M2 and M4 received 5000 ppm acetochlor. Animals in groups M1 and M2 were used in the characterization of nasal protein adducts, whereas those in groups M3 and M4 were used for whole-body autoradiography and nasal turbinate microautoradiography studies.

Results from whole-body autoradiography showed significant localization of radioactivity in nasal turbinates. Localization of radioactivity was apparent at both 1750 and 5000 ppm. Microautoradiography studies showed intense localization of radioactivity in the Bowman's glands of the group M4 animals. A lower degree of localization of radioactivity in the olfactory surface epithelium was observed, whereas no evidence of localization was evident in the respiratory epithelium. For group M3 animals, only slight to moderate localization of radioactivity was observed in the Bowman's glands of the animals. This study demonstrated the formation of an acetochlor protein adduct in rat nasal tissue that is derived from the quinoneimine, 3-ethyl,5-methylbenzoquinone-4-imine (EMIQ).

This result supports the hypothesis that rat nasal tissue is capable of metabolizing acetochlor to EMIQ. The formation of EMIQ in rat nasal tissue could be a critical step for the induction of nasal tumours in rats by acetochlor (Lau et al., 1998a).

A study was conducted to determine and characterize the nasal localization of radiolabel in Sprague-Dawley rats following oral administration of ^{14}C-labelled acetochlor secondary sulfide. A mixture containing radiolabelled (^{14}C) acetochlor sulfide and unlabelled (^{12}C) acetochlor was administered by oral gavage to four groups of male Sprague-Dawley rats at approximately 7 mg/kg bw. Groups M1, M2 and M3 were dosed for 5 consecutive days, and group M4 received a single dose. Two out of four rats from group M1 and one out of two rats from group M2 were killed 5 days after the final dose; all other rats were killed 1 day after their final/single dose. Groups M1, M2 and M4 were used for whole-body autoradiography or microautoradiography, and group M3 was used for characterization of binding to nasal tissue.

HPLC analysis of the acid hydrolysate of rat nasal proteins from rats treated with ^{14}C-labelled acetochlor secondary sulfide showed a major radioactive component in the acid hydrolysate that was structurally related to 3-ethyl,5-methylbenzoquinoneimine-cysteine (EMIQ-cysteine). It was tentatively assigned as the L-cysteine conjugate of EMIQ-(4-amino-2-(cysteine-*S*-yl)-2-ethyl,5-methylphenol. Unlike the results obtained from rats administered ^{14}C-labelled acetochlor, significant amounts of EMA were not observed in the hydrolysate of nasal proteins. The lack of formation of EMA in the acid hydrolysate of acetochlor secondary sulfide–treated animals provides evidence that the chlorine displacement of acetochlor by the protein sulfhydryl was a potential pathway in the binding of acetochlor to rat nasal proteins. Results from whole-body autoradiography showed significant localization and persistence of radioactivity in nasal turbinates. Microautoradiography studies showed intense localization of radioactivity in the Bowman's glands of treated animals.

In conclusion, the formation of an EMIQ-derived protein adduct in rat nasal turbinate after oral treatment with acetochlor secondary sulfide has been reported. As acetochlor secondary sulfide lacks the chlorine atom in its amide side-chain, the binding of sulfhydryl in protein via the chlorine displacement route is not feasible. This is supported by the lack of formation of EMA in the acid hydrolysate of rat nasal turbinate. Autoradiography showed the localization of radioactivity in rat nasal turbinates after oral administration of acetochlor secondary sulfide. This finding is consistent with the hypothesis that acetochlor secondary sulfide is metabolized to the precursor for EMIQ, which binds to protein covalently in the rat nasal turbinate (Lau et al., 1998b).

In a distribution study, groups of Crl:CD(SD)BR rats were given a single oral gavage dose (200 mg/kg bw) of ^{14}C-ring-labelled *sec*-amide methyl sulfoxide (EU reference no. 46; also known as acetochlor sulfoxide; purity > 99%), ^{14}C-carbonyl-labelled acetochlor sulfoxide (purity > 99%) or ^{14}C-phenyl ring–labelled EMA (EU reference no. 34; purity > 97.0%) in polyethylene glycol 600. Nasal tumours were seen in rats, but not in mice. The target specificity in the rat is believed to be due to the metabolism of acetochlor to products such as quinoneimines, which bind covalently to nasal proteins. Acetochlor sulfoxide (EU reference no. 46), which is the major plasma metabolite in rat metabolism studies, has been found to specifically accumulate in the olfactory region of rat nasal tissue. The acetochlor sulfoxide (EU reference no. 46) was metabolized to EMA (EU reference no. 34), which is an intermediate precursor to EMIQ. Two rats from each dose group were killed at 8 and 24 hours and 5 days after dosing. A 2% (w/v) solution of EMA in carboxymethyl cellulose was injected into the external nasal passages, and the carcasses were immediately frozen in hexane/dry ice. The rats were then processed for autoradiography.

Most of the radioactivity of ^{14}C-ring-labelled acetochlor sulfoxide (EU reference no. 46) showed selective labelling in the olfactory region of nasal passages at all of the time points, including 5 days after dosing. At 8 and 24 hours, the radioactivity was concentrated in the olfactory region of the nasal passages and the gastrointestinal tract, and at 5 days, the only radioactivity present in the animal was in the nasal turbinates. ^{14}C-carbonyl-labelled acetochlor sulfoxide (EU reference no. 46) also showed a similar pattern of labelling, with localization in the olfactory region of the nasal passages. ^{14}C-phenyl ring–labelled EMA (EU reference no. 34) also localized in the nasal passages, although this metabolite did not show the olfactory-specific localization seen with acetochlor sulfoxide (EU reference no. 46). Radiolabel was present throughout the nasal passages, in the lining of the buccal cavity and in the nares.

In conclusion, the persistence of acetochlor sulfoxide (EU reference no. 46), the major metabolite circulating in plasma, in the olfactory region of the nasal passage 5 days after dosing strongly suggests that the radioactivity was covalently bound to protein. EMA (EU reference no. 34) also localized in the nasal passages but did not appear to show the olfactory specificity of the acetochlor sulfoxide (Green, 1997a).

A comparative dietary feeding study was conducted to assess the carcinogenic potential of *sec*-amide methyl sulfoxide (also known as acetochlor sulfoxide; EU reference no. 46), a metabolite of acetochlor, to the nasal passages of male CD (Sprague-Dawley) rats. Three groups of 96 rats were fed ad libitum on control diet, diet containing 1750 ppm acetochlor (purity 94.7%) or diet containing acetochlor sulfoxide (purity 96%). The initial dietary concentration of acetochlor sulfoxide (100 ppm) was based on preliminary studies that showed that 100 ppm acetochlor sulfoxide gave plasma sulfoxide levels comparable to those resulting from the 1750 ppm diet selected for acetochlor. Chemical consumption was on average 14.6 mg/kg bw per day for acetochlor sulfoxide and 99.6 mg/kg bw per day for acetochlor, calculated over the entire duration of the study. The plasma sulfoxide concentrations in rats fed on the acetochlor diet increased 2- to 3-fold over the first 13 weeks of the study and remained relatively constant for the remainder of the study. Consequently, the concentration of acetochlor sulfoxide in the diet had to be increased after 2 weeks (to 150 ppm) and then again after 4 weeks (to 300 ppm) in order to try to maintain comparable plasma concentrations. Even with these increases, the concentration of sulfoxide in the plasma of acetochlor sulfoxide–dosed animals was significantly lower than that in acetochlor-treated rats for a large part of the study (about 0.5 and 0.7 µg/mL plasma in acetochlor sulfoxide– and acetochlor-treated rats, respectively).

Groups of rats were killed after 1, 2, 4, 7, 13 and 39 weeks of feeding. Blood was collected by cardiac puncture (n = 8 per group) at each time point. Nasal cell replication rates were measured in rats killed after 1, 2, 4 and 13 weeks. Each animal was given a single intraperitoneal injection of ^{3}H-labelled thymidine (74 kBq/g bw) in water on each of the 3 days prior to sacrifice. The final injection was given 2 hours before the animals were killed by exsanguination under terminal anaesthesia with halothane. The remaining animals were killed after either 26 weeks (n = 32) or 52 weeks (n = 31).

No abnormalities were seen in any of the haematoxylin and eosin–stained sections from the animals examined at 1, 2 and 13 weeks. At 26 weeks, multifocal polypoid adenomas were seen in the olfactory epithelium, with an incidence of 65.5% in the acetochlor group and 21.9% in the acetochlor sulfoxide group. Hyperplasia of the nasal epithelium was seen in both treatment groups at the same sites as the adenomas. At 52 weeks, the incidence of polypoid adenomas was 65.4% and 29.6% in the acetochlor- and acetochlor sulfoxide–treated groups, respectively. The incidence of hyperplasia was 88% and 41% in acetochlor- and acetochlor sulfoxide–treated rats, respectively (Table 62).

Table 62. Histopathological findings in nasal cavities of rats treated with acetochlor and sec-amide methyl sulfoxide

Week	Finding	Control 0 ppm	Acetochlor sulfoxide[a] 300 ppm	Acetochlor[a] 1 750 ppm
26	*No. of tissues examined*	32	32	32
	Polypoid adenoma	0	7** (21.9%)	21** (65.5%)
	Hyperplasia, total	0	2 (6.25%)	6* (18.8%)
52	*No. of tissues examined*	31	27	26
	Rhinitis, total	14	12 (44.5%)	10 (38.5%)
	Polypoid adenoma	0	8** (29.6%)	17** (65.4%)
	Hyperplasia, total	0	11** (40.7%)	23** (88.5%)

ppm: parts per million
[a] Per cent incidence values shown in parentheses.
Source: Mainwaring (2004)

There were no increases in cell proliferation in nasal tissue levels 1–5 after 1 week of treatment. However, in nasal tissue level 6, the mean labelling index in the acetochlor group was 134% of that seen in controls. No increase in cell proliferation was seen in the animals treated with acetochlor sulfoxide.

The sulfoxide metabolite of acetochlor has been shown to be a nasal carcinogen in the rat. The development, morphology and location of the tumours were identical to those seen with acetochlor. Its tumorigenic potency is also comparable to that of acetochlor. These findings conclusively demonstrate that acetochlor sulfoxide, which is the major circulating metabolite in the rat, can be directly associated with the mechanism of tumour formation (Mainwaring, 2004).

A study was conducted to determine the effects of subchronic dietary administration of acetochlor on rat nasal cell proliferation. Groups of 10 male Sprague-Dawley rats were administered acetochlor (purity 95.2%) in the diet at a dose level of 0, 1750 or 5000 ppm (equal to 0, 91.9 and 270 mg/kg bw per day, respectively) for 60, 90 and 160 days. Cell proliferation was determined at 60, 90 and 160 days in both the respiratory and olfactory epithelia of the nasal turbinates by measuring the incorporation of [^{3}H]thymidine into replicating DNA. [^{3}H]Thymidine was administered daily (intraperitoneally) for 3 consecutive days prior to the rats being killed on the fourth day. Additional groups of 10 male rats were administered acetochlor in the diet at 0, 200, 1750 or 5000 ppm (equal to 0, 10.4, 91.9 and 270 mg/kg bw per day, respectively) for 163 days. Three days prior to sacrifice, these rats were implanted with osmotic pumps containing 5-bromo-2′-deoxyuridine (BrdU). After sacrifice, the nasal turbinates were removed from all animals and processed for the determination of cell proliferation.

Terminal body weights of animals administered 5000 ppm for 60, 90 and 160 days were significantly decreased compared with those of control animals (11%, 17% and 19% decrease in body weights at 60, 90 and 160 days, respectively). Cell proliferation was significantly increased (~1.6-fold) in the olfactory epithelium of rats from the 5000 ppm group at 60 days when measured using [^{3}H]thymidine (Table 63). After 90 and 160 days of acetochlor treatment, a significant increase (~1.3- to 2.0-fold) in cell proliferation was observed in rats treated with 1750 and 5000 ppm (measured using either [^{3}H]thymidine or BrdU). Cell proliferation in the olfactory epithelium was significantly increased at 5000 ppm compared with 1750 ppm (measured using [^{3}H]thymidine). There were no significant differences in cell proliferation in the respiratory epithelium at any of the time points or dose levels tested.

Table 63. Intergroup comparison of nasal cell proliferation

Group	Dietary concentration (ppm)	No. of labelled cells/field	
		Olfactory epithelium	Respiratory epithelium
[^{3}H]Thymidine	0	4.23	2.80
60 days	1 750	3.81	1.37
	5 000	6.66*	2.07
[^{3}H]Thymidine	0	4.75	1.37
90 days	1 750	6.37**	1.28
	5 000	7.90**	1.14
[^{3}H]Thymidine	0	3.48	0.52
160 days	1 750	5.24**	0.79
	5 000	7.06**	0.86
BrdU	0	7.17	2.43
160 days	200	6.25	2.23
	1 750	9.78**	2.53
	5 000	11.46**	2.61

BrdU: 5-bromo-2′-deoxyuridine; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test, two-sided)
Source: Hotz & Wilson (1996a)

In conclusion, acetochlor produced a significant increase in cell proliferation in the olfactory region, but not the respiratory region, of the nasal turbinates of the rat in a dose-dependent manner (Hotz & Wilson, 1996a).

A study was conducted to determine and characterize nasal cell proliferation in mice following dietary administration of acetochlor. In this study, acetochlor (purity 95.2%) was administered to groups of 26 male CD-1 mice in the diet at a dose level of 0, 1000 or 5000 ppm (equal to 0, 167 and 888 mg/kg bw per day, respectively). Cell proliferation was determined at 60 and 90 days in both the respiratory and olfactory epithelia of the nasal turbinates by measuring the incorporation of BrdU into replicating DNA. BrdU was administered using micro-osmotic pumps, which were implanted 3 days prior to sacrifice.

There was a significant reduction in body weight gain throughout the study in animals that received 5000 ppm acetochlor. The mean terminal body weight of the mice treated with acetochlor at 5000 ppm was significantly less than that of controls at 90 days (12% reduced compared with controls). Liver weight was significantly increased at 90 days in mice administered 5000 ppm acetochlor. Liver weight relative to body weight was significantly increased in the mice treated with

5000 ppm acetochlor at both 60 and 90 days. These results could be related to the hepatocarcinoma found at the same dose level in chronic mouse feeding studies. Liver cell proliferation was not measured. Cell proliferation was not significantly increased in either the olfactory or the respiratory epithelium of the mouse nasal turbinates at any dose level after 60 or 90 days of dietary treatment.

In conclusion, there was no effect of treatment on cell proliferation in the olfactory or respiratory epithelium of mice administered 1000 or 5000 ppm acetochlor for 60 and 90 days via dietary administration. This finding is consistent with the lack of nasal tumours in the mouse after chronic acetochlor administration (Hotz & Wilson, 1996b).

In vitro and in vivo studies

The binding of acetochlor sulfoxide (EU reference no. 46) to rat nasal proteins was investigated in one in vitro and two in vivo experiments. In the in vitro experiment, two radiolabelled forms of acetochlor sulfoxide were incubated with rat olfactory microsomal proteins, and the binding of radioactivity to protein was assessed by gel electrophoresis. In the first in vivo experiment, rats were given a single oral dose of ^{14}C-labelled acetochlor sulfoxide at 10 mg/kg bw, and the nasal tissues were collected 8 and 24 hours after dosing. The tissues were hydrolysed, and the hydrolysate was examined by HPLC. In the second in vivo experiment, the distribution of radioactivity in the nasal passages of rats dosed orally with ^{14}C-labelled acetochlor sulfoxide at 10 mg/kg bw was studied using histoautoradiography techniques.

HPLC of hydrolysates of nasal tissue taken from the olfactory region following a single dose of ^{14}C-phenyl-labelled acetochlor sulfoxide showed the presence of at least four protein adducts, one of which eluted as a doublet and was significantly greater than the rest. The structures of adducts were not identified. There was no evidence of significant adduct formation in the respiratory region. When rat olfactory microsomal proteins were incubated in vitro with the two radiolabelled forms of acetochlor sulfoxide, the pattern of protein adducts was identical for both forms, clearly indicating that adducts carry the sulfoxide side-chain.

In conclusion, there is strong evidence that acetochlor sulfoxide binds to rat nasal olfactory proteins, probably as a result of metabolism to a quinoneimine that still carries the sulfoxide side-chain. Within the nasal tissues, the principal site of covalent binding was the Bowman's glands. None of the studies provided any evidence of significant binding to proteins in the respiratory region (Green, 2001).

A series of in vitro and in vivo experiments was conducted to assess the binding and association of acetochlor metabolites with rat, mouse and human blood. These experiments included the analysis of haemoglobin adducts, assessment of acetochlor metabolism in cultured hepatocytes, binding to erythrocytes from control rats, binding to erythrocytes from rats dosed with acetochlor and binding to erythrocytes from control mice and human volunteers.

In rats given a single oral dose of [^{14}C]acetochlor at 200 mg/kg bw, most of the radioactivity associated with blood was shown to be bound to the protein moiety of haemoglobin. Acetochlor was metabolized extensively by primary cultures of rat hepatocytes, and the binding of several [^{14}C]acetochlor metabolites to erythrocytes from control rat blood was demonstrated. This binding occurred readily, both during the incubation of hepatocytes with [^{14}C]acetochlor and after incubating freshly collected control rat erythrocytes with blood-free hepatocyte culture supernatant containing acetochlor metabolites. Rat hepatocyte cultures therefore provided a source of acetochlor metabolites with binding potential and that were also stable to storage. The interaction of these [^{14}C]acetochlor metabolites with erythrocytes from control mice and human volunteers was investigated, and no binding of radioactivity was observed. This clearly demonstrated that the rat metabolites of acetochlor that bound readily to control rat erythrocytes had no affinity for mouse or human erythrocytes. Further in vitro experiments using erythrocytes from rats given repeated oral doses of unlabelled acetochlor at 500 or 1000 mg/kg bw per day for 31 days showed little additional binding of radiolabelled metabolites to these erythrocytes, indicating that the binding sites were saturable.

It was concluded that as the rat metabolites of acetochlor, which bound readily to rat erythrocytes, had no affinity for erythrocytes from mouse or human blood, the blood binding observed among these species is a rat-specific phenomenon (Macpherson & Jones, 1991).

In vitro studies

The in vitro metabolism of [^{14}C]acetochlor (phenyl labelled; purity 99%) was investigated in both cytosol and microsomal fractions prepared from male CD rat, male CD-1 mouse and male and female squirrel monkey liver and nasal tissue. Separate nasal olfactory and respiratory tissues were obtained from the rodents.

There were no significant differences in liver fractions from rats and mice for the rates of conjugation of acetochlor with GSH or for the hydrolysis of the secondary sulfide to EMA. The hydroxylation of EMA to *p*-OH-EMA did occur at a faster rate, 5-fold, in mouse hepatic microsomal fractions than in rat fractions.

In nasal tissue fractions from rats and mice, the rates for the conjugation of acetochlor with GSH and for the oxidation of EMA to *p*-OH-EMA were comparable. There was a marked difference between rats and mice in the rate of hydrolysis of the secondary sulfide to EMA, the rate in rat nasal tissues being significantly higher, 9.8- and 24.5-fold for olfactory and respiratory nasal tissue fractions, respectively.

Marked differences were seen between rats and primates for all three metabolic reactions, particularly those in nasal tissue fractions. Compared with the highest rate in the rat, which occurred in olfactory fractions, the rate in fractions prepared from total primate nasal tissue for the conjugation of acetochlor with GSH was 26-fold lower, that for the hydrolysis of the secondary sulfide, 86-fold lower, and that for the *p*-hydroxylation of EMA, 24-fold lower. Based on these comparisons, the overall flux through the metabolic pathway from acetochlor to *p*-OH-EMA is several orders of magnitude lower in primates than in rats. In mice, the flux was up to 18-fold lower than in rats. However, as GSH conjugation of acetochlor is minimal in the mouse, the potential conversion of acetochlor to *p*-OH-EMA would be even lower than the 18-fold measured in vitro.

In conclusion, the study results show that the rate of metabolism of acetochlor to a quinoneimine precursor in animals was markedly higher in the rat than in other species, particularly primates. The study author suggested that the results provide a clear explanation for the unique sensitivity of the rat to acetochlor-induced tumours (Green, 1997b).

Acetochlor sulfoxide (*sec*-amide methyl sulfoxide; EU reference no. 46), the major metabolite circulating in the plasma of rats dosed with acetochlor, was investigated in liver and nasal tissues from CD rats, CD-1 mice and a single human nasal tissue sample. Acetochlor sulfoxide was rapidly metabolized in olfactory microsomal fractions from both rats and mice to two major products, including the *para*-hydroxy metabolite of acetochlor sulfoxide, a precursor in the formation of a quinoneimine, the proposed toxic species in rat nasal tissue. The rates for the hydroxylation reaction were comparable in rat and mouse olfactory microsomes and 6-fold higher than the rate in rat respiratory microsomes. Metabolism could not be detected in mouse liver microsomes, and that in rat liver was extremely low, 260-fold lower than in olfactory microsomes. The hydroxylation of acetochlor sulfoxide could not be detected in the single human nasal tissue sample (Green, 1998).

In vitro metabolism of U-^{14}C-phenyl-labelled acetochlor sulfoxide (*sec*-amide methyl sulfoxide; EU reference no. 46) was studied in the nasal tissues of male CD (Crl:CD(SD)BR) rats, male CD-1 mice, male and female squirrel monkeys and human nasal explants.

The rates for the hydroxylation reaction were comparable in rat and mouse olfactory microsomes and 6-fold higher than the rate in rat respiratory microsomes. Acetochlor sulfoxide did not inhibit coumarin metabolism. The hydroxylation of acetochlor sulfoxide could not be detected in 33 human nasal tissue samples, even though these were fresh surgical samples. Similarly, the primate

nasal samples did not metabolize acetochlor sulfoxide. Purified human CYP2A6 did not metabolize acetochlor sulfoxide, although it did hydroxylate coumarin at significant rates.

In conclusion, the localization of acetochlor sulfoxide, the major circulating metabolite of acetochlor, in rat nasal tissues has been attributed to metabolism by an isoform of cytochrome P450 that is unique to the nasal tissues in the rat and is concentrated mainly in the olfactory region. The product formed, *p*-hydroxyacetochlor sulfoxide, is an immediate precursor of a quinoneimine, the protein-reactive metabolite believed to be responsible for the nasal tumours seen in rats given acetochlor. A complete lack of detectable metabolism of acetochlor sulfoxide by nasal tissues from primates and humans suggests that the particular form of cytochrome P450 responsible in the rat could not be detected in primates and humans. Consequently, acetochlor-induced rat nasal tumours are not indicative of a hazard to humans (Green et al., 2000).

The in vitro metabolism of acetochlor *sec*-amide methyl sulfide (abbreviated by study authors as ASMS, EU reference no. 36), the precursor of the major circulating rat metabolite acetochlor *sec*-amide methyl sulfoxide (abbreviated by study authors as ASMSO, EU reference no. 46), was investigated for its possible conversion to reactive dialkylbenzoquinoneimine (DABQI) metabolites. The purpose of the study was to determine the relative importance in Sprague-Dawley rats of the formation of DABQI metabolites through the EMA metabolic pathway compared with other pathways, specifically the ASMSO pathway. Incubations of ASMS were conducted with rat liver and rat olfactory nasal turbinate microsomes in the presence of NADPH and GSH. GSH served to trap reactive DABQI metabolites formed in the microsomal incubations producing GSH conjugates. For comparison purposes, rat liver and nasal microsomal incubations were also conducted using ASMSO as substrate.

Four major non-conjugated metabolites, ASMSO, *p*-hydroxy acetochlor *sec*-amide methyl sulfoxide (*p*-OH-ASMSO), EMA and *p*-OH-EMA, were formed and identified from both rat liver and nasal microsomal incubations of ASMS in the presence of NADPH and GSH. ASMSO, the result of the oxidation of sulfur, was the major metabolite and was formed at similar levels in liver and nasal incubations. EMA, derived by amidase hydrolysis of the amide, was also formed at comparable levels in liver and nasal incubations, but at levels at least 10 times lower than ASMSO levels. The *para*-hydroxylated metabolites *p*-OH-EMA and *p*-OH-ASMSO were formed at levels similar to those of EMA in nasal microsomes. These two metabolites were formed at much higher levels (at least 30 times higher) in nasal microsomes than in liver microsomes.

In all, 12 GSH conjugates resulting from trapping of reactive quinoneimine (DABQI) metabolites of *para*-hydroxylated ASMSO, ASMS or EMA were formed in rat nasal microsomal incubations of ASMS in the presence of NADPH and GSH. In contrast, only three detectable quinoneimine GSH conjugates were formed in rat liver microsomal incubations in the presence of NADPH and GSH, and these were at levels much lower than those in nasal olfactory incubations. The *p*-OH-EMA GSH conjugate was the major GSH adduct metabolite formed in both liver and nasal turbinate incubations. However, the *p*-OH-EMA GSH levels in nasal microsomal incubations were more than 25-fold higher than those in rat liver microsomal incubations.

Similar non-conjugated and GSH-conjugated metabolites were formed in limited incubations of ASMSO with rat liver or nasal olfactory turbinate microsomes in the presence of NADPH and GSH. At similar substrate concentrations, the incubation of ASMSO with rat nasal microsomes formed almost 5 times more total GSH conjugates than the incubation of ASMS. The vast majority of GSH conjugates (trapped quinoneimine metabolites) formed in nasal microsomal incubations of ASMSO are formed from *p*-OH-ASMSO (> 98%), unlike the incubations of ASMS, which formed reactive quinoneimine metabolites primarily via *p*-OH-EMA.

The major toxicity of acetochlor in nasal tissues is believed to result from site-specific formation of reactive DABQI metabolites (e.g. *p*-OH-EMA quinoneimine and *p*-OH-ASMSO quinoneimine) within the olfactory epithelium. These metabolites can bind to cysteine residues in nasal proteins and produce oxidative stress. The relative levels of GSH conjugate formation (a

quantitative estimate of the formation of those reactive metabolites) in nasal olfactory turbinate microsomes provide a measure of the relative contributions of the various quinoneimine metabolites to the observed toxicity. ASMSO is the predominant metabolite circulating in the blood plasma of rats treated with acetochlor. Therefore, the results of this study, which show that more than 98% of reactive quinoneimine metabolites formed from ASMSO in nasal microsomes are derived from *p*-OH-ASMSO (not *p*-OH-EMA), indicate that the *p*-OH-ASMSO pathway is the predominant pathway contributing to acetochlor-induced nasal toxicity (Zhang, Hansen & Saghir, 2010).

A study was conducted to determine and characterize the binding of radioactivity to CD-1 mouse nasal tissue protein following dietary administration of ^{14}C-labelled acetochlor. In this study, CD-1 mice were administered ^{14}C-labelled acetochlor at target dietary concentrations of 1750 and 5000 ppm for 14 days. After treatment, the animals were killed, the nasal turbinate protein was isolated and the binding of acetochlor to the nasal turbinate protein was determined.

No evidence of EMIQ-cysteine or related benzoquinoneimine protein adduct was detected in the nasal turbinates of either dose group (Lau & Wilson, 1998a).

A study was conducted to characterize the binding of radioactivity to rhesus monkey nasal tissue protein following oral administration of ^{14}C-labelled acetochlor. In this study, three male rhesus monkeys were orally administered ^{14}C-labelled acetochlor at a dose level of 126 mg/kg bw for 14 days. After treatment, the animals were killed, and the nasal turbinates were isolated.

No evidence of an EMIQ-derived or related benzoquinoneimine protein adduct was detected in the nasal turbinates of any of the monkeys (Lau & Wilson, 1998b).

The mode of action for nasal tumours observed in rats following long-term exposure to acetochlor is analysed using the International Programme on Chemical Safety (IPCS) conceptual framework for cancer risk assessment (Sonich-Mullin et al., 2001; Boobis et al., 2006) in Appendix 1.

(d) In vitro elucidation of metabolic pathway in liver

In an in vitro study, the metabolism of acetochlor by rat liver and kidney cell–free preparations was investigated to elucidate the initial metabolic pathways of acetochlor in the rat and to complement the results obtained from the in vivo rat metabolism studies. Liver and kidney homogenates were fractionated by centrifugation to separate membrane-bound and soluble enzymes, allowing the separate study of the metabolic pathways involving cytochrome P450 enzymes and glutathione transferases, respectively.

The in vitro studies led to the identification of two pathways of acetochlor metabolism in the rat. One pathway involved glutathione transferases and the enzymes of the mercapturic acid pathway, and the other pathway involved the cytochrome P450 and glucuronyl transferase enzymes. The initial detoxification metabolites of these two pathways were identified as GSH and glucuronic acid conjugates, respectively. These two types of metabolites were not observed in the urine and faeces of the rat from the in vivo studies, indicating extensive further metabolism prior to their elimination from the rat. Metabolites resulting from oxidative or reductive dehalogenation and aryl hydroxylation were not observed (Feng, 1984).

A study was conducted to compare the rat and human liver metabolism of the chloroacetamide herbicides acetochlor, alachlor, butachlor and metolachlor in vitro. Only the test conditions and results of acetochlor are presented and discussed in this summary.

Acetochlor was incubated with rat and human liver microsomes and human cytochrome P450 isoforms 1A1, 1A2, 2B6, 2D6, 2E1 and 3A4 at a substrate concentration of 50 µmol/L for up to 1 hour in the presence of an NADPH regenerating system, and the metabolites formed were analysed by HPLC at several time points. 2-Chloro-*N*-(2-methyl-6-ethylphenyl) acetamide (CMEPA; *sec*-amide chloride; EU reference no. 8), a metabolite formed after *N*-dealkylation of acetochlor, was incubated with rat and human liver microsomes (but without an NADPH regenerating system) at a substrate concentration of 50 µmol/L for up to 1 hour, and the metabolites formed were analysed by HPLC at several time points.

Both rat and human hepatocytes metabolized CMEPA to EMA. The human cytochrome P450 isoforms involved in the metabolic pathway studied (acetochlor → CMEPA → EMA → *p*-OH-EMA) were CYP3A4 and CYP2B6. The rates of metabolic conversion at the different steps were roughly comparable between rat and human liver microsomes, with only a few slight differences (Coleman et al., 2000).

(e) Mode of action of related to thyroid tumour formation

The objective of this study was to determine the effect of acetochlor on end-points relevant to thyroid homeostasis in order to evaluate the mechanism of thyroid neoplasia. In one study, male Sprague-Dawley rats (20 per group) were fed diets containing acetochlor (purity 95.2%) at 0, 1750 or 5000 ppm (equal to 0, 92 and 270 mg/kg bw per day, respectively) for 14, 28 and 56 days. The end-points measured included terminal body weights, liver weights, thyroid weights, serum concentrations of thyroid stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3) and hepatic T_4 uridine diphosphate-glucuronosyltransferase (UGT) activity. In the other study, four groups of male Sprague-Dawley rats were fed diets containing acetochlor at 0, 200, 1750 or 5000 ppm (equal to 0, 10.4, 92 and 270 mg/kg bw per day, respectively) for 90 and 160 days. The end-points as listed above were measured at 90 days; terminal body weights, liver weights and thyroid weights were evaluated only at 160 days.

Significant decreases in final body weight were observed for the animals treated with 5000 ppm acetochlor when compared with their respective controls at 14, 28, 56, 90 and 160 days. The decreases in body weight at 5000 ppm were 9%, 8%, 7%, 11% and 17%, compared with respective controls, at 14, 28, 56, 90 and 160 days, respectively. Significant reductions in body weight gain were observed during the first 3 weeks of the study for animals that received 5000 ppm acetochlor. Acetochlor caused significant increases in liver weights in animals treated with 1750 ppm for 28, 56 and 90 days. Liver weights were increased at 14, 26, 56 and 90 days in animals treated with 5000 ppm acetochlor. Thyroid weights were increased in animals treated with 1750 ppm acetochlor for 28 days. Thyroid weights were increased at 14 and 28 days in animals treated with 5000 ppm acetochlor. Concentrations of serum TSH were significantly increased at 14 and 28 days in the animals treated with 5000 ppm acetochlor. TSH was significantly increased at 56 days only in animals receiving 1750 ppm.

Serum T_4 concentrations were significantly increased only at 14 days in animals administered 1750 and 5000 ppm acetochlor. Serum T_3 concentrations were significantly decreased at 14 days in animals administered 5000 ppm acetochlor. Dose-related increases in hepatic T_4 UGT activity were observed at 14, 28 and 56 days in both the 1750 and 5000 ppm acetochlor groups and at 90 days only in the 5000 ppm group. Increased UGT was also generally observed when expressed on either a milligram protein or gram liver basis (Table 64).

The results of these studies indicate that the effect of acetochlor on circulating levels of TSH is an important event in thyroid tumour formation. It appears that increased hepatic enzymatic conjugation causes a compensatory increase in TSH levels. Taken together, the results support the view that acetochlor indirectly produces thyroid hyperplasia and ultimately neoplasia in the male rat by alterations in thyroid hormone homeostasis. This process is considered to be threshold sensitive and not relevant to humans (Hotz & Wilson, 1996c).

Table 64. Intergroup comparison of liver and thyroid gland parameters

Exposure time (days)	Dietary level (ppm)	Mean TSH level (ng/mL)	Mean T_3 level (ng/dL)	Mean T_4 level (ng/dL)	Mean hepatic T_4 UGT activity (pmol/min)			Relative liver weight (%)	Relative thyroid weight (%)
					per mg protein	per g liver	per total liver		
14	0	4.18	60.81	2.98	0.49	5.81	117.4	4.2	0.004 2
	1 750	4.42	53.69	4.18**	0.57	7.81	167.2*	4.6**	0.004 8*
	5 000	5.41**	52.3*	4.22**	0.78**	10.81**	256.1**	5.4**	0.005 4**
28	0	2.50	56.33	3.16	0.46	5.04	118.4	3.9	0.004 3
	1 750	3.32	52.04	3.30	0.63**	6.65*	149.9**	4.5**	0.005 1**
	5 000	4.56**	53.21	3.11	0.82**	9.55**	237.1**	5.1**	0.005 3**
56	0	3.22	55.94	2.13	0.51	6.68	125.7	3.7	0.004 1
	1 750	4.52*	57.27	2.60	0.69*	8.58	226.0**	4.2**	0.004 3
	5 000	4.35	60.36	2.57	0.78**	8.98	237.2**	5.0**	0.004 9**
90	0	1.45	60.99	3.60	0.38	7.59	189.0	3.8	0.003 9
	200	1.03	63.36	3.58	0.46	8.56	181.2	3.7	0.004 3
	1 750	1.70	72.14	3.85	0.45	7.77	210.6	4.2**	0.004 3
	5 000	1.42	62.46	3.44	0.63**	13.72**	361.1**	4.9**	0.005 0**
160	0	–	–	–	–	–	–	3.4	0.004 4
	200	–	–	–	–	–	–	3.5	0.004 4
	1 750	–	–	–	–	–	–	3.9**	0.005 2
	5 000	–	–	–	–	–	–	4.4**	0.005 2

ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UGT: uridine diphosphate-glucuronosyltransferase; –: no values; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test, two-sided)
Source: Hotz & Wilson (1996c)

2.7 Studies on metabolites

Acetochlor is extensively metabolized in plants. Although several metabolites were found to represent more than 10% of the total residue in forage or fodder, none of these metabolites was found at significant levels in grain. Some plant metabolites were not detected in rat metabolism studies. EFSA (2008) decided to include one of the metabolites, *N*-(6-ethyl-3-hydroxy-2-methylphenyl) oxamic acid (EU reference no. 68), in the residue definition for human dietary risk assessment (but not for enforcement). This metabolite was found at only very low levels in maize grain and has not been detected in other crops. The oxamic acid metabolite was not formed in the rat metabolism study. Therefore, although human exposure is negligible, several studies were conducted with the oxamic acid metabolite to evaluate its toxicological potential.

Acetochlor is rapidly degraded by soil microbes, with a mean half-life of approximately 12–13 days under aerobic conditions. The four most prominent environmental degradates or soil metabolites are acetochlor *t*-ethanesulfonic acid (*t*-ESA; EU reference no. 7), acetochlor *t*-oxanilic acid (*t*-OXA; EU reference no. 2) and acetochlor *t*-sulfinylacetic acid (*t*-SAA; EU reference no. 3), each accounting for more than 10% of the applied product, and acetochlor *s*-ethanesulfonic acid (*s*-ESA; EU reference no. 13), which was found at levels above 5% in one soil type. All four of these acid metabolites are much more polar than acetochlor and have the potential to leach to groundwater. However, none of these degradates has been identified as a metabolite of acetochlor in rats or mice.

Therefore, several studies have been conducted with each of these degradates to evaluate their potential toxicity.

Another environmental metabolite, *t*-norchloroacetochlor (*t*-NCA; EU reference no. 6) has also been detected in some studies, particularly under anaerobic conditions. However, *t*-NCA represents less than 5% of the total soil residue under the aerobic conditions typical of acetochlor use, is not polar and has no tendency to accumulate. A few toxicology studies were also conducted with *t*-hydroxyacetochlor (EU reference no. 17), which was also detected at low levels (< 5%) in some soil degradation studies, but is considered a transient metabolite and unlikely to persist.

The studies conducted on selected metabolites include absorption, distribution, metabolism and excretion (ADME) studies, acute oral toxicity studies, 28- and 90-day dietary studies in rats and mice, developmental toxicity studies and genotoxicity studies. Some of the study types and key findings are summarized in Tables 65–69.

Table 65. ADME studies on selected metabolites of acetochlor

Metabolite	Study	Key findings	Reference
t-ESA (EU reference no. 7)	ADME (mouse)	Relatively poorly absorbed, 24–33% (urine) and 60–70% (faeces) in 7 days, and rapidly excreted after oral administration, with minimal metabolism (majority excreted as unchanged parent).	Hansen et al. (2009b)
t-ESA (EU reference no. 7)	ADME (rat)	Poorly absorbed, 10–13% (urine) and 77–80% (faeces) in 5 days, and rapidly excreted after oral administration, with minimal metabolism (76–79% unchanged parent).	Albin & Kraus (2000a)
t-OXA (EU reference no. 2)	ADME (mouse)	Incompletely absorbed, 54–61% (urine) and 34–39% (faeces) in 7 days, and rapidly excreted after oral administration, with minimal metabolism (predominantly unchanged parent in excreta).	Hansen et al. (2009a)
t-OXA (EU reference no. 2)	ADME (rat)	Incompletely absorbed, 34–39% (urine) and about 56% (faeces) in 7 days, and rapidly excreted after oral administration, with minimal metabolism (predominantly unchanged parent in excreta).	Albin & Kraus (2000b)
t-SAA (EU reference no. 3)	WBA (rat)	No evidence of localization. No radioactivity detected in nasal turbinates. Undetectable level of residues in organs in 5 days.	Meeker (2006a)
s-ESA (EU reference no. 13)	WBA (rat)	No evidence of localization. No radioactivity detected in nasal turbinates. Undetectable level of residues in organs in 5 days.	Meeker (2006b)

ADME: absorption, distribution, metabolism and excretion; EU: European Union; WBA: whole-body autoradiography

3. Observations in humans

No local or systemic signs of toxicity were observed in employees who handled acetochlor in laboratories or during manufacturing process development and operations. No adverse effects were reported in pesticide applicators as a result of mixing and loading and field application of acetochlor (Monsanto, 2014).

Lerro et al. (2015) examined the relationship between occupational use of acetochlor and cancer outcomes in pesticide applicators enrolled as part of the Agricultural Health Study in the USA. Phone interviews were conducted from 1999 to 2005 to obtain information on use of pesticides, including acetochlor. In total, 4026 male pesticide applicators from an available cohort of 33 484 had reported using acetochlor. Poisson regression was used to estimate relative risk (RR) and the 95%

Table 66. Acute toxicity studies on selected metabolites of acetochlor

Metabolite	Study	Strain	Purity (%)	Results	Reference
Oxamic acid (EU reference no. 68)	Acute oral (rat)	Wistar-derived	96	LD_{50}: > 2 000 mg/kg bw[a]	Duerden (1992)
t-ESA (EU reference no. 7)	Acute oral (rat)	Wistar-derived	97	LD_{50}: > 2 000 mg/kg bw[a]	Lees (1997b)
t-OXA (EU reference no. 2)	Acute oral (rat)	Wistar-derived	97	LD_{50}: > 2 000 mg/kg bw[a]	Lees (1997a)
t-SAA (EU reference no. 3)	Acute oral (rat)	Wistar-derived	99	LD_{50}: > 2 000 mg/kg bw[a]	Lees. (1997c)
s-ESA (EU reference no. 13)	Acute oral (rat)	Sprague-Dawley	86.8	LD_{50}: > 2 000 mg/kg bw[a]	Smedley (2006)
t-NCA (EU reference no. 6)	Acute oral (rat)	Sprague-Dawley	99.5	LD_{50}: 2 499 mg/kg bw (males)[b] LD_{50}: 1 002 mg/kg bw (females)[b]	Bonnette (2000)
t-Hydroxyacetochlor (EU reference no. 17)	Acute oral (rat)	Sprague-Dawley	99	LD_{50}: 1 098 mg/kg bw (female)[a]	Durando (2013)

bw: body weight; EU: European Union; LD_{50}: median lethal dose
[a] No significant clinical signs of toxicity or macroscopic findings.
[b] Decreased activity, wobbly gait, convulsions, tremors, prostration, apparent hypothermia, hunched posture.

confidence interval (CI) for cancers that occurred from the time of the interview to 2010 or 2011. As acetochlor has been registered for use in the USA only since 1994, such a time frame is unlikely to be sufficient for solid tumours to arise. Although a number of statistically significant increases in cancer incidences in applicators were calculated based on ever-use of acetochlor versus never-use of acetochlor (lung: RR = 1.74, CI = 1.07–2.84; melanoma: RR = 1.61, CI = 0.98–2.66; pancreas = 2.36, CI = 0.98–5.65), the low number of exposed applicators limits the strength of the calculated statistical associations (23, 23 and 7, respectively). No statistical associations were calculated for all cancer sites, bladder, lymphohaematopoietic, colon, colorectal, oesophagus, kidney, leukaemia, non-Hodgkin lymphoma, prostate and rectal cancer.

Further analysis by days and intensity-weighted days found statistical associations with cancer at all sites in the high-use cohort (RR = 1.19, CI = 1.00–1.43); increased colorectal cancer in the high-use cohort (RR = 1.75, CI = 1.08–2.83), but decreased colorectal cancer in the low-use cohort (RR = 0.31, CI = 0.11–0.83); increased lung cancer in the low-use but not high-use cohort (RR = 2.64, CI = 1.47–4.74); and increased melanoma in the high-use cohort (RR = 1.78, CI = 0.90–3.52). The authors also found statistical associations when acetochlor and atrazine had been used in relation to lung cancer (RR = 2.01, CI = 1.17–3.46) and melanoma (RR = 1.75, CI = 0.97–3.14).

The authors considered that the analysis was inconclusive because of minimal evidence for exposure–response relationships, the small numbers of exposed applicators and the short latency period between the use of acetochlor and the analysis of cancer outcomes (Lerro et al., 2015).

Table 67. Short-term toxicity studies on selected metabolites of acetochlor

Metabolite	Study	Doses[a]	NOAEL (mg/kg bw per day)	Key findings	Reference
Oxamic acid (EU reference no. 68)	28-day (Wistar rat)	0, 1 400, 4 000 or 14 000 ppm (equal to 0, 109, 319 and 1 142 mg/kg bw per day for males)	1 142 (HDT)	No treatment-related adverse findings at any dose level	Walraven (2009)
t-ESA (EU reference no. 7)	28-day (Sprague-Dawley rat)	0, 3 000, 6 000 or 12 000 ppm (equal to 0, 370, 767 and 1 579 mg/kg bw per day for males)	1 579 (HDT)	No treatment-related adverse effects, including on thyroid hormones, at any dose level	Lees (2000a)
	90-day (Sprague-Dawley rat)	0, 1 000, 3 000 or 12 000 ppm (equal to 0, 75.0, 225 and 919 mg/kg bw per day for males)	225	Decreased feed consumption and weight gain, slight changes in haematology and clinical chemistry in both sexes; no evidence of nasal cell proliferation	Lees (2000b)
t-OXA (EU reference no. 2)	28-day (Sprague-Dawley rat)	0, 3 000, 6 000 or 12 000 ppm (equal to 0, 373, 769 and 1 468 mg/kg bw per day for males)	769	Decreased feed consumption and weight gain; no effect on thyroid hormones	Williams (2000a)
	90-day (Sprague-Dawley rat)	0, 1 000, 3 000 or 12 000 ppm (equal to 0, 77.2, 230 and 955 mg/kg bw per day for males)	230	Decreased body weight and increased motor activity in males; no evidence of nasal cell proliferation	Williams (2000b)
t-SAA (EU reference no. 3)	28-day (Sprague-Dawley rat)	0, 1 000, 4 000 or 12 000 ppm (equal to 0, 68, 267 and 820 mg/kg bw per day for males)	820 (HDT)	No treatment-related adverse effects, including on thyroid hormones	O'Neill (2001)
	90-day (Sprague-Dawley rat)	0, 1 000, 4 000 or 14 000 ppm (equal to 0, 67, 265 and 962 mg/kg bw per day for males)	265	Decreased feed consumption and body weight	O'Neill (2002)

bw: body weight; HDT: highest dose tested; NOAEL: no-observed-adverse-effect level; ppm: parts per million

[a] Doses converted to mg/kg bw per day values for males only (female values are not shown in the table because they are usually higher numbers).

Comments

Biochemical aspects

Following gavage dosing of rats, acetochlor was rapidly and almost completely absorbed and rapidly excreted. Male bile duct–cannulated rats given 10 mg/kg bw excreted on average 85.1% of the dose in bile, 8.0% in urine and 4.2% in faeces over 48 hours. Similar results were obtained with the high dose and following repeated oral dosing (Jones, 1990). No pronounced sex differences were observed following the administration of single low or high doses or repeated dosing (Hawkins, Kirkpatrick & Dean, 1989b). Radiolabel was widely distributed throughout the body (< 4% of the

Table 68. Genotoxicity studies on selected metabolites of acetochlor

Study	Test system	Purity (%)	Results	Reference
Oxamic acid (EU reference no. 68)				
Bacterial gene mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538; *Escherichia coli* WP2P and WP2P uvrA	99	Negative	Callander (1991)
In vitro chromosomal aberration	Human lymphocytes	99	Negative	Fox (1995)
In vivo unscheduled DNA synthesis	Rat hepatocytes	99	Negative	Barber & Mackay (1995)
t-ESA (EU reference no. 7)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 and WP2P uvrA	99	Negative	Callander (1997b)
In vitro mammalian gene mutation	Mouse lymphoma	97.6	Negative	Clay (2000b)
In vitro chromosomal aberration	Human lymphocytes	97.6	Negative	Fox (2000c)
In vivo micronucleus	Mouse (bone marrow)	97.6	Negative	Fox (2000d)
t-OXA (EU reference no. 2)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 and WP2P uvrA	97	Negative	Callander (1997a)
In vitro mammalian gene mutation	Mouse lymphoma	93.2	Positive (+S9) at concentrations ≥ 7.5 mmol/L	Clay (2000a)
In vitro chromosomal aberration	Human lymphocytes	93.2	Negative	Fox (2000a)
In vivo micronucleus	Mouse (bone marrow)	93.2	Negative	Fox (2000b)
t-SAA (EU reference no. 3)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 and WP2P uvrA	99	Negative	Callander (1997c)
In vitro mammalian gene mutation	Mouse lymphoma	100.3	Negative	Clay (2001)
In vitro	Human lymphocytes	100.3	Negative	Fox (2001)

Study	Test system	Purity (%)	Results	Reference
chromosomal aberration				
s-ESA (EU reference no. 13)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2P uvrA	86.8	Negative	Xu (2006c)
In vitro mammalian gene mutation	CHO/HGPRT	86.8	Negative	Cifone (2006)
In vitro chromosomal aberration	Human lymphocytes	86.8	Negative	Murli (2006)
t-NCA (EU reference no. 6)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 and WP2P uvrA	99.5	Negative	Callander (2002)
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 and WP2P uvrA	99	Negative	Wagner (2013a)
In vitro mammalian gene mutation	Mouse lymphoma	99.5	Weakly positive (up to 2.6 times control)	Clay (2002)
In vitro chromosomal aberration	Human lymphocytes	99.5	Negative	Fox (2002a)
In vivo micronucleus	Mouse (bone marrow)	99.5	Negative	Fox (2002b)
In vivo transgenic gene mutation	Mouse	> 99	Negative	Beevers (2014)
t-Hydroxyacetochlor (EU reference no. 17)				
Bacterial gene mutation (Ames)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2P uvrA	99	Negative	Wagner (2013b)

CHO: Chinese hamster ovary; EU: European Union; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

administered dose in tissues and carcass); highest concentrations were observed in blood (but not in plasma), liver, heart, lung and spleen (Hawkins, Kirkpatrick & Dean, 1989b). There was some accumulation in nasal turbinates in rats, but not in mice (Kraus & Wilson, 1996). The combined urinary and faecal excretion showed biphasic elimination, with a half-life of 5.4–10.4 hours for the α phase and 129–286 hours for the β phase (Carr et al., 1983).

Acetochlor was extensively metabolized. No parent compound was detected in the urine, and less than 1% was found in faeces. Species differences were observed, particularly with respect to the formation of sulfur-containing precursors to the DABQI metabolites that are believed to be responsible for the induction of nasal tumours in rats. The primary metabolic pathway in the rat

Table 69. Developmental toxicity studies on selected metabolites of acetochlor

Metabolite	Study	NOAEL (mg/kg bw per day)	Key findings	Reference
Acetochlor *t*-OXA (EU reference no. 2)	Developmental (Sprague-Dawley rat) Doses: 0, 250, 500 or 1 000 mg/kg bw per day	Maternal: 500 Developmental: 1 000 (HDT)	Mortality and clinical toxicity in the dams at 1 000 mg/kg bw per day, and no developmental toxicity at any dose level	Holson (2000)
Alachlor *t*-ESA (closely related compound)	Developmental (Sprague-Dawley rat) Doses: 0, 150, 400 or 1 000 mg/kg bw per day	Maternal: 1 000 (HDT) Developmental: 1 000 (HDT)	No test article–related maternal or developmental toxicity at any dose level	Holson (1995)

bw: body weight; EU: European Union; HDT: highest dose tested; NOAEL: no-observed-adverse-effect level

involves *O*-dealkylation and subsequent glucuronidation or GSH conjugation, enterohepatic circulation and excretion. The predominant metabolite in rat plasma following oral administration of acetochlor was a secondary amide, *sec*-amide methyl sulfoxide (or acetochlor sulfoxide). In contrast, in mice, acetochlor is metabolized primarily to a number of glucuronides, which are excreted in the urine (Green et al., 1998). In rhesus monkeys, GSH conjugation and subsequent metabolism via the mercapturic acid pathway occur preferentially. However, as a result of the higher molecular weight threshold for biliary excretion in primates compared with rats, the metabolites appear to be excreted primarily via the urine and not the bile and thus would not be subjected to the formation of *sec*-amide methyl sulfoxide, as in rats (Livingston, 1983; Kurtzweil, 2014).

Toxicological data

In rats, the acute oral LD_{50} was 1929 mg/kg bw (Branch, 1982b), the acute dermal LD_{50} was 4166 mg/kg bw (Branch, 1982a) and the acute inhalation LC_{50} was greater than 2.07 mg/L (Duerden & Lewis, 1990). Acetochlor was severely irritating to the skin of rabbits (Barlow & Ishmael, 1989) and mildly irritating to the eyes of rabbits (Branch, 1982c). It was a skin sensitizer in guinea-pigs, as determined by the Buehler test (Auletta, 1983) and Magnusson and Kligman maximization test (Botham & Ishmael, 1989). It gave a positive response for phototoxicity in an in vitro mouse fibroblast assay (Bowen, 2014).

The finding observed most consistently in short- and long-term toxicity studies in mice, rats and dogs is decreased body weight, with changes in haematology and clinical chemistry in some studies.

In a 91-day toxicity study in mice using dietary acetochlor concentrations of 0, 800, 2000 and 6000 ppm (equivalent to 0, 120, 300 and 900 mg/kg bw per day, respectively), the NOAEL was 2000 ppm (equivalent to 300 mg/kg bw per day), based on decreased body weight observed at 6000 ppm (equivalent to 900 mg/kg bw per day) (Ahmed, 1981a).

In a 29-day toxicity study in rats using dietary acetochlor concentrations of 0, 300, 600, 1200, 2400, 4800 and 9600 ppm (equal to 0, 33.3, 67.7, 132, 267, 519 and 1012 mg/kg bw per day for males and 0, 35.2, 69.3, 139, 279, 539 and 1081 mg/kg bw per day for females, respectively), the NOAEL was 600 ppm (equal to 67.7 mg/kg bw per day), based on slight decreases in body weight gain and prothrombin time observed at 1200 ppm (equal to 132 mg/kg bw per day) (Broadmeadow, 1985).

Two 3-month dietary toxicity studies were conducted in rats. In the first study, using dietary acetochlor concentrations of 0, 800, 2000 and 6000 ppm (equal to 0, 53.2, 134 and 460 mg/kg bw per

day for males and 0, 69.3, 173 and 530 mg/kg bw per day for females, respectively), marginal decreases in feed consumption and body weight gain were observed at 800 ppm (equal to 53.2 mg/kg bw per day), the lowest dose tested (Ahmed, 1980a).

In the second study, using dietary acetochlor concentrations of 0, 20, 200 and 2000 ppm (equal to 0, 1.60, 16.1 and 161 mg/kg bw per day for males and 0, 1.92, 18.8 and 191 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 16.1 mg/kg bw per day), based on significantly decreased feed consumption and body weight gain seen at 2000 ppm (equal to 161 mg/kg bw per day) (Broadmeadow, 1986a).

The overall NOAEL for the two 3-month toxicity studies in rats was 200 ppm (equal to 16.1 mg/kg bw per day). The overall LOAEL was 800 ppm (equal to 53.2 mg/kg bw per day).

In a 91-day toxicity study in dogs administered acetochlor by capsule at a dose of 0, 2, 10 or 60 mg/kg bw per day, the NOAEL was 10 mg/kg bw per day, based on decreased body weight gain, clinical signs, increased relative liver weights, increased serum ALT activity and decreased blood glucose levels observed in both sexes and reduced haemoglobin, haematocrit and erythrocyte counts observed in females at 60 mg/kg bw per day (Broadmeadow, 1986b).

In a 119-day toxicity study in dogs administered acetochlor by capsule at a dose of 0, 25, 75 or 200 mg/kg bw per day, a NOAEL could not be identified, as effects were observed at all doses. At the LOAEL of 25 mg/kg bw per day, decreased red blood cell counts, decreased haematocrit, increased alkaline phosphatase activity, increased ALT activity and increased relative adrenal and liver weights occurred in females (Ahmed, 1980b).

Two 1-year toxicity studies in dogs were conducted. In the first study, in which dogs were administered acetochlor by capsule at a dose of 0, 4, 12 or 40 mg/kg bw per day, the NOAEL was 12 mg/kg bw per day, based on decreased body weights and feed consumption, testicular atrophy, increased absolute liver and kidney weights and decreased relative testis weight observed at 40 mg/kg bw per day (Ahmed, 1981b).

In the second study, in which dogs were administered acetochlor by capsule at a dose of 0, 2, 10 or 50 mg/kg bw per day, the NOAEL was 2 mg/kg bw per day, based on decreased feed consumption and body weight gain in females and changes in kidneys (interstitial nephritis) and tubular degeneration in testes in males observed at 10 mg/kg bw per day (Broadmeadow, 1989b).

The overall NOAEL for the two 1-year toxicity studies in dogs was 2 mg/kg bw per day. The overall LOAEL was 10 mg/kg bw per day.

Two long-term toxicity and carcinogenicity studies were conducted in mice. In the first study, in which mice were given acetochlor in the diet at a concentration of 0, 500, 1500 or 5000 ppm (equal to 0, 75, 227 and 862 mg/kg bw per day for males and 0, 95, 280 and 1084 mg/kg bw per day for females, respectively) for 23 months, increases in absolute and relative kidney weights (both sexes) and liver weights (males only), a dose-related increase in interstitial nephritis in both sexes and retinal degeneration in females (positive trend) were observed at all dose levels. The high dose of 5000 ppm was considered to be excessive and above the MTD (Ahmed, 1983b). Statistically significant increases in the incidence of lung tumours in females of all dose groups were considered not to be related to treatment based on the consideration of historical control data (Hardisty, 1997b). The Meeting noted that the incidence of histiocytic sarcomas in females at 1500 ppm was marginally outside the historical control range for the performing laboratory, that this tumour occurs commonly in aged mice and that this tumour is of unknown relevance to humans (Hardisty, 1997c).

In the second study, in which mice were administered acetochlor in the diet at a concentration of 0, 10, 100 or 1000 ppm (equal to 0, 1.1, 11 and 116 mg/kg bw per day for males and 0, 1.4, 13 and 135 mg/kg bw per day for females, respectively) for 78 weeks, the NOAEL was 10 ppm (equal to 1.1 mg/kg bw per day), based on slight anaemia and increased incidences of bronchiolar hyperplasia and interstitial fibrosis in the kidney in males observed at 100 ppm (equal to 11 mg/kg bw per day) (Amyes, 1989). A significantly increased incidence of renal tubular basophilia was also noted in males at 10 and 100 ppm; however, these increased incidences were not considered to be adverse

because they were of minimal severity and because of the lack of a clear dose–response relationship, the lack of associated histopathological findings, the lack of similar effects in females or in either sex at higher dose levels in a study of longer duration and the lack of corroborative renal findings. There was a slight increase in the incidence of adenoma in the lungs of males and females at 1000 ppm; however, the increase was not considered to be treatment related based on the lack of a dose–response relationship, the low incidence in concurrent controls, the lack of an increase in tumour multiplicity with increasing dose and the comparable incidence of lung tumours in historical controls (Hardisty, 1997b).

Three long-term toxicity and carcinogenicity studies have been conducted in rats. In a 27-month study using dietary acetochlor concentrations of 0, 500, 1500 and 5000 ppm (equal to 0, 22, 69 and 250 mg/kg bw per day for males and 0, 30, 93 and 343 mg/kg bw per day for females, respectively), decreased body weight in males was observed at 500 ppm (equal to 22 mg/kg bw per day), the lowest dose tested. Treatment-related neoplastic findings (liver, thyroid and nasal tumours) occurred at 5000 ppm, but this dose was well above the MTD. An increased incidence of nasal tumours was observed at 1500 ppm (equal to 69 mg/kg bw per day) (Ahmed, 1983a).

In the second study, in which rats were administered acetochlor in the diet at a concentration of 0, 40, 200 or 1000 ppm (equal to 0, 1.9, 9.4 and 47.5 mg/kg bw per day for males and 0, 2.4, 11.8 and 60 mg/kg bw per day for females, respectively) for 24 months, the NOAEL was 200 ppm (equal to 9.4 mg/kg bw per day), based on decreased body weight in males, elevated total bilirubin level in females and elevations of GGT activity and cholesterol level in males at 1000 ppm (equal to 47.5 mg/kg bw per day). An increased incidence of papillary adenomas of the nasal mucosa was observed at 1000 ppm (equal to 47.5 mg/kg bw per day) (Naylor & Ribelin, 1986).

In the third study, in which rats were administered dietary acetochlor at a concentration of 0, 18, 175 or 1750 ppm (equal to 0, 0.67, 6.4 and 66.9 mg/kg bw per day for males and 0, 0.88, 8.5 and 92.1 mg/kg bw per day for females, respectively) for 24 months, the NOAEL was 175 ppm (equal to 6.4 mg/kg bw per day), based on reduced body weight and feed consumption, changes in the eyes (degeneration of outer retinal layer), reduced blood cell parameters and increased incidence of focal hyperplasia in the nasal epithelium at 1750 ppm (equal to 66.9 mg/kg bw per day). Adenomas and carcinomas of nasal epithelium and thyroid adenoma were observed at 1750 ppm (equal to 66.9 mg/kg bw per day) (Broadmeadow, 1989a).

The overall NOAEL for systemic toxicity in the three long-term toxicity studies in rats was 200 ppm (equal to 9.4 mg/kg bw per day), and the LOAEL was 500 ppm (equal to 22 mg/kg bw per day).

A comparative 52-week toxicity study was conducted in rats using a dietary acetochlor concentration of 1750 ppm (equal to 99.6 mg/kg bw per day) and a dietary *sec*-amide methyl sulfoxide (EU reference no. 46; also known as acetochlor sulfoxide) concentration of 100/150/300 ppm (equal to 14.6 mg/kg bw per day). The results of this study clearly demonstrate that the sulfoxide metabolite of acetochlor is a nasal carcinogen in the rat. The development, morphology and location of the tumours were identical to those seen with acetochlor. The tumorigenic potency of the sulfoxide metabolite is also comparable with that of acetochlor (Mainwaring, 2004).

A number of other mode of action studies (e.g. Green, 1997a,b; Lau et al., 1998a,b) are also available for acetochlor, indicating that the nasal tumours observed in rats likely result from the site- and species-specific formation of reactive quinoneimine metabolites within the nasal epithelium and the associated formation of adducts with nasal proteins. It is postulated that this results in cytotoxicity, based on the observation of inflammation and metaplasia in vivo, leading to prolonged nasal cell proliferation and eventual development of nasal olfactory tumours. Because of large differences in the in vitro rate of formation of quinoneimines in human and rat nasal tissues and in the rate of formation of quinoneimine adducts in vivo in rats, mice and squirrel monkeys, this mode of action is unlikely to lead to nasal tumours in humans at levels of exposure arising from pesticide residues in food.

A mechanistic study (Hotz & Wilson, 1996c) has shown that the thyroid tumours observed in rats are caused by induction of hepatic UGT. This, in turn, leads to decreased levels of thyroid

hormone and a compensatory increase in TSH, which acts upon the rat thyroid to induce hyperplasia and, ultimately, neoplasia. Therefore, thyroid tumours in rats are not considered relevant to humans because of the well-known differences in thyroid hormone homeostasis between rats and humans.

The Meeting concluded that acetochlor induces tumours in mice of unknown relevance for humans and that the modes of action for the nasal epithelial and thyroid tumours in rats have been established.

Acetochlor has been evaluated in an adequate range of in vitro and in vivo genotoxicity studies. Acetochlor exhibited weak positive responses in some in vitro gene mutation assays conducted with less than pure material. It was clastogenic at cytotoxic concentrations in human lymphocytes; this response is attributable to the metabolism of acetochlor to the chloromethyl group and low GSH levels. However, no evidence of clastogenicity or other genotoxic effects was observed in a number of in vivo assays, including a rat bone marrow chromosomal aberration assay, two mouse micronucleus assays and several dominant lethal mutation assays in rats and mice. No evidence of DNA damage was noted in an in vitro unscheduled DNA synthesis assay in rat hepatocytes. A weakly positive response was noted in an in vivo rat hepatocyte unscheduled DNA synthesis assay, but only at a high dose level (2000 mg/kg bw, which is higher than the LD_{50} value) associated with hepatic GSH depletion and severe hepatotoxicity.

The Meeting noted the absence of a specific assay for gene mutations in vivo. However, the Meeting concluded that, on the basis of the weight of evidence, acetochlor was unlikely to be genotoxic in vivo.

As acetochlor is unlikely to be genotoxic in vivo, the histiocytic sarcomas in mice are commonly observed as animals age and occur only at high doses and the proposed modes of action for tumours of the thyroid and nasal epithelium in rats involve a threshold and are highly unlikely to occur in humans, the Meeting concluded that acetochlor is unlikely to pose a carcinogenic risk to humans from the diet.

Three two-generation reproductive toxicity studies in rats are available. In the first study, in which rats were given diets containing acetochlor at a concentration of 0, 200, 600 or 1750 ppm (equal to 0, 18.6, 57.0 and 166 mg/kg bw per day for F_0 males and 0, 21.5, 64.6 and 199 mg/kg bw per day for F_0 females, respectively), the NOAEL for parental toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on focal hyperplasia and polypoid adenomata in the nasal epithelium of adult F_1 offspring at study termination and decreased body weight in F_1 females at 600 ppm (equal to 57.0 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on decreased F_2 litter size at birth, decreased F_1 and F_2 pup body weights during lactation, and decreased absolute and relative spleen weights in F_2 weanlings observed at 600 ppm (equal to 57.0 mg/kg bw per day). The NOAEL for reproductive toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), based on a decreased number of implantations seen at 600 ppm (equal to 57.0 mg/kg bw per day) (Milburn, 2001).

In the second study, in which rats were given diets containing acetochlor at a concentration of 0, 18, 175 or 1750 ppm (equal to 0, 1.3, 12.6 and 124 mg/kg bw per day for F_0 males and 0, 1.6, 15.5 and 157 mg/kg bw per day for F_0 females, respectively), the NOAEL for parental toxicity was 175 ppm (equal to 12.6 mg/kg bw per day), based on decreases in body weight, body weight gain and feed consumption and increases in relative weights of brain, kidney, liver and spleen in F_1 females and of testes, seminal vesicles and thymus in F_1 males at 1750 ppm (equal to 124 mg/kg bw per day). The NOAEL for offspring toxicity was 175 ppm (equal to 12.6 mg/kg bw per day), based on decreased pup body weights observed at 1750 ppm (equal to 124 mg/kg bw per day). The NOAEL for reproductive toxicity was 1750 ppm (equal to 124 mg/kg bw per day), the highest dose tested (Willoughby, 1989).

In the third study, in which rats were given diets containing acetochlor at a concentration of 0, 500, 1500 or 5000 ppm (equal to 0, 30.8, 90.6 and 316 mg/kg bw per day for F_0 males and 0, 46.2, 130 and 442 mg/kg bw per day for F_0 females), the NOAEL for parental toxicity was 500 ppm (equal to 30.8 mg/kg bw per day), based on decreased body weights and decreased relative liver and kidney

weights at 1500 ppm (equal to 90.6 mg/kg bw per day). The NOAEL for offspring toxicity was 1500 ppm (equal to 90.6 mg/kg bw per day), based on the number of liveborn pups and decreased body weights of F_{2b} pups at 5000 ppm (equal to 316 mg/kg bw per day). The NOAEL for reproductive toxicity was 5000 ppm (equal to 316 mg/kg bw per day), the highest dose tested (Schardein, 1982).

The overall NOAEL for parental toxicity was 200 ppm (equal to 18.6 mg/kg bw per day), and the overall LOAEL was 600 ppm (equal to 57.0 mg/kg bw per day). The overall NOAELs for reproductive and offspring toxicity were 200 ppm (equal to 18.6 mg/kg bw per day), and the overall LOAELs for reproductive and offspring toxicity were 600 ppm (equal to 57.0 mg/kg bw per day).

Two developmental toxicity studies in rats are available. In the first study, which used oral gavage acetochlor doses of 0, 40, 150 and 600 mg/kg bw per day, the NOAEL for maternal toxicity was 150 mg/kg bw per day, based on mortality, clinical signs of toxicity, decreased body weight gain and feed consumption and a marked increase in water consumption observed at 600 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day, based on a reduction in mean fetal weight and reduced ossification at 600 mg/kg bw per day (Brooker, Stubbs & John, 1989b).

In the second study, which used oral gavage acetochlor doses of 0, 50, 200 and 400 mg/kg bw per day, the NOAEL for maternal toxicity was 200 mg/kg bw per day, based on decreased body weight gains and clinical signs of toxicity seen at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Rodwell, 1980).

The overall NOAEL and LOAEL for maternal toxicity in rats were 200 and 400 mg/kg bw per day, respectively. The overall NOAEL and LOAEL for embryo and fetal toxicity in rats were 400 and 600 mg/kg bw per day, respectively.

Two developmental toxicity studies in rabbits are available. In the first study, which used oral gavage acetochlor doses of 0, 30, 100 and 300 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on decreased feed consumption and body weight (GDs 6–8) and the treatment-related death of two dams at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, the highest dose tested (Brooker, Stubbs & John, 1989a).

In the second study, which used oral gavage acetochlor doses of 0, 15, 50 and 190 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on decreased body weight at GD 19 and decreased body weight gain during GDs 7–19 at 190 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 190 mg/kg bw per day, the highest dose tested (Adam, 1986).

The overall NOAEL and LOAEL for maternal toxicity in rabbits were 100 and 190 mg/kg bw per day, respectively. The overall NOAEL for embryo and fetal toxicity in rabbits was 300 mg/kg bw per day, the highest dose tested.

The Meeting concluded that acetochlor is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats administered a single oral gavage acetochlor dose of 0, 150, 500 or 1500 mg/kg bw, decreased body weights and body weight gain and reduced feed consumption were observed at 1500 mg/kg bw. No neurotoxicity was observed (Kilgour, 2001a).

In a 93-day study of neurotoxicity in rats given diets containing acetochlor at a concentration of 0, 200, 600 or 1750 ppm (equal to 0, 15.4, 47.6 and 139 mg/kg bw per day for males and 0, 18.3, 55.9 and 166.5 mg/kg bw per day for females, respectively), marginal decreases in mean body weight and body weight gain in males and females were observed at 1750 ppm (equal to 139 mg/kg bw per day). There was no evidence for neurotoxicity or neuropathological effects up to 1750 ppm (equal to 139 mg/kg bw per day), the highest dose tested (Kilgour, 2001b).

The Meeting concluded that acetochlor is not neurotoxic.

No evidence of immunotoxicity was observed in an immunotoxicity study in female mice administered acetochlor in the diet at a dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 119, 334 and 1536 mg/kg bw per day, respectively) for 28 days (Crittenden, 2011).

The Meeting concluded that acetochlor is not immunotoxic by the oral route of exposure.

Biochemical and toxicological data on metabolites and/or degradates

Biochemical and toxicological studies were conducted on plant metabolites, soil degradates and environmental metabolites of acetochlor.[1] The ADME studies in rats and mice indicate that some of these metabolites were absorbed, minimally metabolized and rapidly excreted. There is no significant accumulation in the body. The acute oral LD_{50} values were greater than 2000 mg/kg bw in rats except for two metabolites, for which LD_{50} values were slightly lower than 2000 mg/kg bw in female rats. The 28-day and 90-day dietary toxicity studies in rats indicate NOAELs of 3000 ppm (equal to 225 mg/kg bw per day) and above, primarily based on decreases in body weight. No evidence of thyroid toxicity or effects on the nasal epithelium was observed in these studies. No evidence of embryo and fetal toxicity was observed at 1000 mg/kg bw per day in rats. No evidence of genotoxicity was observed in various in vivo and in vitro assays, except for a mouse lymphoma assay, which gave a weak positive response for two metabolites; however, these two metabolites were negative in a mouse micronucleus assay.

The Meeting concluded that these plant metabolites, soil degradates and environmental metabolites of acetochlor appear to be less toxic than the parent compound.

Human data

There were no reports of adverse health effects in manufacturing plant personnel. Also, there were no reports of poisonings with acetochlor. A recent epidemiological study (Lerro et al., 2015) reported weak associations between exposure to acetochlor and a number of human cancers. However, the authors stated that a lack of exposure–response trends, the small number of exposed cases and the relatively short time between acetochlor use and cancer development prohibit definitive conclusions.

The Meeting concluded that the existing database for acetochlor 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 0–0.01 mg/kg bw on the basis of a NOAEL of 1.1 mg/kg bw per day in the 78-week dietary study in mice, based on slight anaemia and an increased incidence of bronchiolar hyperplasia and interstitial fibrosis in the kidney in males observed at 11 mg/kg bw per day. A safety factor of 100 was applied.

The upper bound of the ADI provides a margin of exposure of at least 28 000 relative to the LOAEL for histiocytic sarcomas in mice (280 mg/kg bw per day) and 4700 relative to the LOAEL for nasal tumours in rats (47.5 mg/kg bw per day).

An acute reference dose (ARfD) of 1 mg/kg bw was established on the basis of a NOAEL of 100 mg/kg bw per day in a study of developmental toxicity in rabbits, based on decreased feed consumption, decreased body weight (GDs 6–8) and the treatment-related death of two dams observed at 300 mg/kg bw per day. A safety factor of 100 was applied.

[1] *N*-(6-ethyl-3-hydroxy-2-methylphenyl) oxamic acid (EU reference no. 68), acetochlor *t*-ethanesulfonic acid (*t*-ESA) (EU reference no. 7), acetochlor *t*-oxanilic acid (*t*-OXA) (EU reference no. 2), acetochlor *t*-sulfinylacetic acid (*t*-SAA) (EU reference no. 3), acetochlor *s*-ethanesulfonic acid (*s*-ESA) (EU reference no. 13), *t*-norchloroacetochlor (*t*-NCA) (EU reference no. 6) and *t*-hydroxyacetochlor (EU reference no. 17).

Levels relevant to risk assessment of acetochlor

Species	Study	Effect	NOAEL	LOAEL
Mice	Twenty-three-month study of toxicity and carcinogenicity[a]	Toxicity	–	500 ppm, equal to 75 mg/kg bw per day[b]
		Carcinogenicity	500 ppm, equal to 95 mg/kg bw per day	1 500 ppm, equal to 280 mg/kg bw per day
	Seventy-eight-week study of toxicity and carcinogenicity	Toxicity	10 ppm, equal to 1.1 mg/kg bw per day	100 ppm, equal to 11 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 116 mg/kg bw per day[c]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,d]	Toxicity	200 ppm, equal to 9.4 mg/kg bw per day	500 ppm, equal to 22 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 22 mg/kg bw per day	1 000 ppm, equal to 47.5 mg/kg bw per day
	Two-generation studies of reproductive toxicity[a,d]	Reproductive toxicity	200 ppm, equal to 18.6 mg/kg bw per day	600 ppm, equal to 57.0 mg/kg bw per day
		Parental toxicity	200 ppm, equal to 21.5 mg/kg bw per day	600 ppm, equal to 64.6 mg/kg bw per day
		Offspring toxicity	200 ppm, equal to 18.6 mg/kg bw per day	600 ppm, equal to 57.0 mg/kg bw per day
	Developmental toxicity studies[d,e]	Maternal toxicity	200 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	400 mg/kg bw per day	600 mg/kg bw per day
	Acute neurotoxicity study[e]	Neurotoxicity	1 500 mg/kg bw[c]	–
	Ninety-day neurotoxicity study[a]	Neurotoxicity	1 750 ppm, equal to 139 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity studies[d,e]	Maternal toxicity	100 mg/kg bw per day	190 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day[c]	–
Dog	Ninety-day and 1-year studies of toxicity[d,e]	Toxicity	2 mg/kg bw per day	10 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.01 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 acetochlor

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly and extensively absorbed from gastrointestinal tract (≥ 93% in 48 h)
Dermal absorption	Low; 9.7% in rhesus monkey
Distribution	Widely distributed; highest concentrations in blood (but not plasma), liver, heart, lung, spleen and nasal turbinates
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid; ~77% excreted within first 24 h following a single low dose
Metabolism in animals	Extensive, species differences in metabolism, *sec*-amide mercapturic acid in rats and glucuronides in mice
Toxicologically significant compounds in animals and plants	Acetochlor
Acute toxicity	
Rat, LD_{50}, oral	1 929 mg/kg bw
Rabbit, LD_{50}, dermal	4 166 mg/kg bw
Rat, LC_{50}, inhalation	> 2.07 mg/L (4 h)
Rabbit, dermal irritation	Severely irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Sensitizing (maximization test and Buehler test)
Short-term studies of toxicity	
Target/critical effect	Kidney and testes
Lowest relevant oral NOAEL	2 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	100 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	Anaemia, kidney and liver (mouse and rat)
Lowest relevant oral NOAEL	1.1 mg/kg bw per day (mouse)
Carcinogenicity	Adenomas in nasal epithelium (rat)[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	Decreased body weights in adults and pups, liver and kidney weights in adults, decreased number of implantations per dam
Lowest relevant parental NOAEL	21.5 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	18.6 mg/kg bw per day (rat)

Lowest relevant reproductive NOAEL	18.6 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Decreased body weights and mortality (rat, rabbit), decreased fetal weight and reduced ossification (rat)
Lowest relevant maternal NOAEL	100 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	300 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	1 500 mg/kg bw per day (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	139 mg/kg bw per day (highest dose tested; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 536 mg/kg bw per day (highest dose tested; mouse)
Mechanistic studies	Nasal tumours: Studies establishing a rat-specific mode of action involving a production of cytotoxic quinoneimine metabolites that result in compensatory hyperplasia leading to tumours Thyroid tumours: Studies establishing rodent-specific thyroid tumour mode of action
Medical data	
	No adverse effects reported in workers at manufacturing plants and agricultural workers

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

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw	Seventy-eight-week toxicity study (mouse)	100
ARfD	1 mg/kg bw	Developmental toxicity study (rabbit)	100

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Appendix 1. Application of the IPCS conceptual framework for cancer risk assessment

(IPCS framework for analysing the relevance of a cancer mode of action for humans)

This framework, developed by an International Programme on Chemical Safety (IPCS) working group, provides a generic approach to the principles commonly used in evaluating a postulated mode of action (MOA) for tumour induction by a chemical (Sonich-Mullin et al., 2001; Boobis et al., 2006). Thus, the framework was used by the 2015 JMPR to provide a structured approach to the assessment of the overall weight of evidence for the postulated MOA for nasal tumours observed in rats after long-term exposure to acetochlor.

A. Nasal tumours in rats

A.1 Introduction

The incidence of nasal olfactory tumours was statistically significantly increased in all three long-term studies in rats (Ahmed, 1983; Naylor & Ribelin, 1986; Broadmeadow, 1989) at dose levels of 1000 ppm and above. Although a single nasal tumour was noted in one animal at 500 ppm in one study (Ahmed, 1983), a nasal tumour was also noted in a control animal from a different study (Naylor & Ribelin, 1986). Nasal tumours were also reported at the high dose of 5000 ppm in a carcinogenicity study; however, the dose was considered excessive based on sharply reduced body weights in females and increased mortality (Ahmed, 1983). Nasal tumours were also observed in a two-generation rat reproductive toxicity study (Milburn, 2001) at 1750 ppm in parental F_0 males and females and at 600 and 1750 ppm in parental F_1 males and females. Olfactory epithelial hyperplasia was also observed in both sexes at 1750 ppm (and in F_1 females at 600 ppm).

A.2 Postulated mode of action (theory of the case)

The postulated non-genotoxic MOA for the induction of nasal tumours by acetochlor in rats involves the biotransformation of acetochlor into reactive metabolites that result in cytotoxicity and subsequent cellular proliferation in nasal tissues. The primary metabolic pathways in the rat involve *O*-dealkylation/glucuronidation, glutathione (GSH) conjugation and excretion into the bile. The predominant metabolite in rat plasma following administration of acetochlor is a *sec*-amide methyl sulfoxide (EU reference no. 46; also known as acetochlor sulfoxide or sulfoxide metabolite), which is subsequently *para*-hydroxylated by the olfactory tissue to the reactive dialkylbenzoquinoneimine (DABQI). This sulfoxide metabolite has been shown to be similar to acetochlor in its ability to induce rat nasal tumours after repeated oral administration (Mainwaring, 2004). The reactive DABQI forms protein adducts in the olfactory epithelium, disrupting cell function and leading to cytotoxicity and regenerative proliferation. Prolonged nasal cell proliferation leads to the eventual development of nasal olfactory tumours (Ashby et al., 1996; Green et al., 2000; USEPA, 2004).

A.3 Key events

Analysis of the available toxicological data for acetochlor, including extensive mechanistic data, suggested a cytotoxicity and regenerative proliferation MOA for nasal tumours in rats that would operate through the following key events:

1. *Conjugation with GSH*. Acetochlor conjugates with GSH and is excreted in the bile.
2. *Biotransformation of GSH conjugate*. The biotransformation of the GSH conjugate results in a series of sulfur-containing products. The predominant metabolite in rat plasma following GSH conjugation of acetochlor is *sec*-amide methyl sulfoxide. Enterohepatic circulation of these products creates a pool of metabolites that are delivered to the nose.
3. *Formation of tissue-reactive metabolites in nasal tissue.* Metabolism by nasal enzymes within the olfactory epithelium results in formation of the reactive DABQI metabolite.
4. *DABQI binding to cellular proteins.* DABQI interacts with nasal tissue proteins to form adducts.
5. *Cytotoxicity.* Protein adducts disrupt cell structure and function in the olfactory epithelium, resulting in cytotoxicity.
6. *Regenerative nasal cell proliferation*. Cell loss due to cytotoxicity induces a regenerative cell proliferative response in nasal tissue.
7. *Increase in nasal olfactory tumours*. Sustained cytotoxicity and cell proliferation results in nasal olfactory tumours.

A.4 Concordance of dose–response relationship

All of the biological processes considered to contribute to the key events occurred with dose–response relationships and occurred at or below the tumorigenic dose. Key events occurring later in the MOA did not occur at lower doses than those key events considered to occur earlier in the MOA. The dose levels for the key events in the MOA for acetochlor-induced nasal tumours are presented in Table A1.

Table A1. Dose levels for key events in the MOA for acetochlor-induced nasal tumours in rats

Key event	Dose level
Conjugation with GSH	GSH conjugate is formed during biotransformation of labelled acetochlor in SD rats (Ashby et al., 1996)
Biotransformation of GSH conjugate	Metabolism studies show formation of sulfur-containing metabolites (Ashby et al., 1996)
Formation of tissue-reactive metabolites in nasal tissue	1750 ppm (14-day in vivo mechanistic study; and in vitro metabolism; Green et al., 2000)
DABQI binding to cellular proteins	1750 ppm (protein adducts; 14-day mechanistic study; Lau & Wilson, 1998)
Cytotoxicity	1750 ppm (metaplasia of olfactory mucosa; 24-month study in SD rats; Broadmeadow, 1989)
Regenerative nasal cell proliferation	1750 ppm ([^{3}H]thymidine and BrdU labelling; 160-day mechanistic study in rats; Hotz & Wilson, 1996)
Increase in nasal olfactory tumours	1750 ppm (24-month study in rats; Broadmeadow, 1989)

BrdU: 5-bromo-2′-deoxyuridine; DABQI: dialkylbenzoquinoneimine; GSH: glutathione; SD: Sprague-Dawley

A.5 Temporal association

The temporal relationships for the events associated with metabolism, tissue distribution and protein adduct formation appear to occur early following exposure to acetochlor. Whole-body autoradiography of acetochlor-dosed rats shows that distribution of the metabolites and binding to nasal turbinates take place after a single dose. Administration of acetochlor at and above 1750 ppm for 14 days resulted in formation of a protein adduct in the nasal tissues that was derived from a quinoneimine reactive species (Lau et al., 1998). There are no data to assess the earliest time of the proliferative response for acetochlor; however, acetochlor produced significantly increased cell proliferation in cells of the nasal olfactory epithelium after 60 days (approximately 8 weeks) of treatment. Polypoid adenomas of the olfactory epithelium were observed in F_0 and F_1 animals in a rat multigeneration reproduction study with acetochlor (Milburn, 2001). Additionally, preliminary data from a 1-year rat feeding study using the sulfoxide metabolite of acetochlor show a statistically significant increase in nasal polypoid adenomas in rats treated for 26 weeks (about 6.5 months), and an increased incidence of nasal tumours was seen in the 24-month rat carcinogenicity studies (Mainwaring, 2004). Overall, there is a logical temporal association of the key events with tumour formation.

A.6 Strength, consistency and specificity of association of tumour response with key events

The biological processes in the formation of reactive metabolites in nasal tissue, tissue binding and tumour formation for acetochlor are consistent with the MOA for nasal tumours for its analogue, alachlor (USEPA, 2004). The interaction of the reactive metabolite DABQI with cellular proteins is limited to the nasal olfactory epithelium, which is the site of tumour formation. Nasal tumours were also induced in rats treated with the sulfoxide metabolite of acetochlor, a proximate precursor of the quinoneimine, indicating that tumour response is associated with that metabolic pathway. The specificity of the response is further supported by mechanistic studies showing that the quinoneimine does not bind to nasal olfactory epithelium in the mouse and the fact that no nasal tumours were observed in the mouse carcinogenicity studies.

A.7 Biological plausibility and coherence

Dietary administration of acetochlor in rats resulted in the early key events (the hepatic biotransformation of acetochlor) that lead to the formation of reactive metabolites in the nasal olfactory epithelium, leading to protein adduct formation and cytotoxicity in the tissue. The MOA for acetochlor is biologically plausible and consistent with the MOA for other chemicals that induce a sustained cytotoxic response leading to regenerative proliferation and tumour formation. The data for acetochlor are reflective of a non-genotoxic, threshold MOA (USEPA, 2004).

A.8 Other modes of action

A number of MOA studies (e.g. Green, 1997a,b; Lau & Wilson, 1998; Lau et al., 1998) are available for acetochlor, indicating that the nasal tumours observed in rats likely result from the site- and species-specific formation of reactive quinoneimine metabolites within the nasal epithelium and the associated formation of adducts with nasal proteins. It is postulated that this results in cytotoxicity, based on the observation of inflammation and metaplasia in vivo, leading to prolonged nasal cell proliferation and eventual development of nasal olfactory tumours.

A.8.1 Mutagenicity

Genetic toxicity, including DNA reactivity, is one possible MOA to consider for the induction of nasal tumours in rats. Acetochlor has been evaluated for its genotoxic potential in a number of in vitro and in vivo studies. The gene mutation tests provide no clear evidence of mutagenicity in either bacterial or mammalian cell test systems. Acetochlor exhibited weak positive responses in some in vitro gene mutation assays conducted with less than pure material. Positive findings for chromosomal aberrations were reported in cultured human lymphocytes and an in vitro mouse lymphoma assay

(Howard, 1989; Fox, 1998). It is possible that the increased mutant colony count observed in the mouse lymphoma assay is the result of a clastogenic event, as that assay also detects chromosome breakage. Acetochlor was clastogenic at cytotoxic concentrations in human lymphocytes; this response is attributable to the metabolism of acetochlor to the chloromethyl group and low GSH levels. However, clastogenicity was confined only to in vitro mammalian systems. Acetochlor was negative for clastogenicity in in vivo studies, including three bone marrow (somatic cell) assays in mice or rats (Farrow & Cortina, 1983; Cavagnaro & Cortina, 1985; Randall, 1989), and was negative in four dominant lethal (germ cell) mutation studies in mice or rats (Naylor, 1987; Hodge, 1991; Milburn, 1996a,b). Additionally, when a single-cell gel electrophoresis assay (comet assay) was performed on nasal olfactory cells isolated from rats exposed to 1750 ppm (tumorigenic dose) in diet for 1 or 18 weeks, no evidence of DNA damage was detected in the target tissue (Ashby et al., 1996). No evidence of DNA damage was noted in an in vitro unscheduled DNA synthesis assay in rat hepatocytes. A weakly positive response was noted in an in vivo rat hepatocyte unscheduled DNA synthesis assay, but only at a high dose level (2000 mg/kg bw, which is higher than the median lethal dose [LD_{50} value]) associated with hepatic GSH depletion and severe hepatotoxicity. Based on a weight of evidence of the available genotoxicity data, acetochlor was unlikely to be genotoxic in vivo, and the findings do not support a mutagenic MOA for nasal tumours in rats.

A.9 Uncertainties, inconsistencies and data gaps

Although there are no data to directly demonstrate cytotoxicity in nasal tissues, evidence of respiratory metaplasia seen in the 24-month rat studies at the tumorigenic dose was used as a surrogate measure of cytotoxicity, as the presence of metaplasia is indicative of the death (and loss) of olfactory epithelial cells.

A.10 Assessment of postulated mode of action

The data provide adequate support for a non-genotoxic, threshold-based MOA for the development of nasal tumours in rats following chronic exposure to acetochlor. The key events for the MOA have been identified and are supported by data that show strong dose–response relationships and temporal concordance. Additionally, the data supporting the MOA for acetochlor are consistent with the data for its analogue, alachlor, which has similar toxicological properties and induces nasal tumours through a cytotoxicity and regenerative proliferation MOA.

Human relevance analysis

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

The toxicological data support a non-genotoxic, threshold-based MOA for the development of nasal cell tumours in rats, and the weight of evidence supports a cytotoxicity and regenerative proliferation MOA.

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 non-genotoxic MOA for the induction of nasal tumours by acetochlor in rats is plausible in humans and cannot be excluded. Because of large differences in the in vitro rate of formation of quinoneimines in human and rat nasal tissues and in the rate of formation of quinoneimine adducts in vivo in rats, mice and squirrel monkeys, this MOA is unlikely to lead to nasal tumours in humans at levels of exposure arising from pesticide residues in food.

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

Human relevance of the MOA can reasonably be excluded in humans based on the quantitative differences in toxicokinetics and toxicodynamics. The large species differences in the overall metabolism of acetochlor indicate that the amount of DABQI produced in rats is significantly

higher than in mice, which do not develop nasal tumours, and at least several orders of magnitude higher than in primates and humans.

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CYAZOFAMID

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Explanation

Cyazofamid is the International Organization for Standardization (ISO)–approved common name for 4-chloro-2-cyano-*N*,*N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service number 120116-88-3. It is a cyanoimidazole class fungicide.

Cyazofamid 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.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with regulated test guidelines. The Meeting considered that the database was adequate for the risk assessment.

Evaluation for acceptable intake

1. Biochemical aspects

Structures of the parent compound and metabolites and degradates of cyazofamid in various crops and animals are shown in Table 1.

1.1 Absorption, distribution and excretion

(a) Absorption

A single dose of radiolabelled cyazofamid – the benzene [^{14}C-Bz] cyazofamid (lot no. CP-1863-2; purity > 99.5%) or the imidazole [^{14}C-Im] cyazofamid (lot no. CP-1947; purity > 98.5%) (see Fig. 1) – suspended in a 0.75% aqueous methyl cellulose solution at 0.5 mg/kg body weight (bw) (low dose) or 1000 mg/kg bw (high dose) was administered by gavage to five male and five female Sprague-Dawley rats. One control animal of each sex received the vehicle. Blood, urine and faeces were collected. Blood samples were taken at 0.25, 0.5, 1, 1.5, 3, 6, 8, 24, 48 and 72 hours after dosing. Data from the radioassay were used to calculate various kinetic parameters.

The kinetics data are shown in Table 2. There were no statistically significant differences in kinetics between animals of the same sex that were treated with [^{14}C-Bz]cyazofamid or [^{14}C-Im]cyazofamid. The concentration of radiolabel in the blood rose rapidly, reaching a maximum (C_{max}) within 15–30 minutes (T_{max}), regardless of dose level, sex or label position. The concentration then decreased rapidly from this time to about 1.5 hours post-dosing. The elimination phase, from which the biological half-life is derived, began about 3 hours post-dosing. The limits of radiocarbon quantification – approximately 2 ng equivalents (eq)/g for the low dose and 1 µg eq/g for the high dose – were reached within 24 hours post-dosing (Huhtanen & Savides, 1998a).

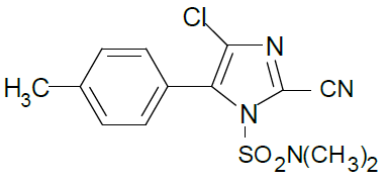
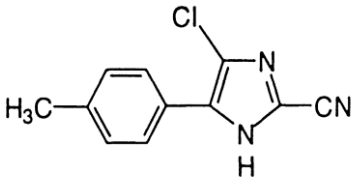
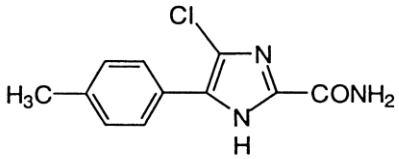
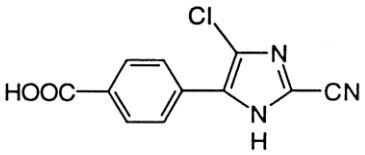
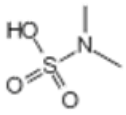
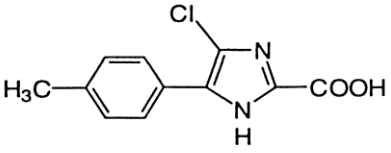
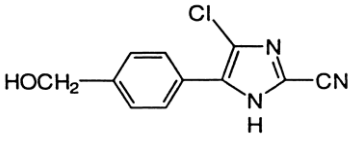
To compare the toxicokinetics of cyazofamid after different routes of administration, eight male Sprague-Dawley rats per group were treated orally or intravenously with [^{14}C]cyazofamid at a dose of 30 or 3 mg/kg bw, respectively. One animal in the intravenous treatment group was excluded from the analysis because the dose volume given and the resulting dose level were different from those for the other animals. Blood samples were collected at eight time points from 0.25 to 24 hours after each dosing.

After oral administration, absorption of total radioactivity was rapid, with maximum concentrations of radioactivity in blood attained at 0.5 hour after dosing. Absolute oral bioavailability (calculated as the area under the concentration–time curve [AUC] after a single dose from time of dosing until infinity normalized to 1 mg/kg bw in the intravenous group / the AUC after a single dose from time of dosing until infinity normalized to 1 mg/kg bw in the oral group) was 14%. The apparent terminal half-life of radioactivity in blood was slightly longer in the intravenous treatment group (5.73 hours) than in the oral dose group (4.90 hours) (Wenker, 2014).

(b) Distribution

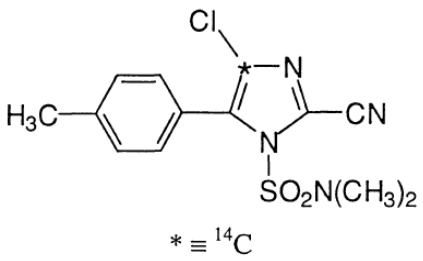
Sprague-Dawley rats (3–5 rats of each sex per group) were treated with a single dose of 0.5 mg/kg bw (low dose) or 1000 mg/kg bw (high dose) radiolabelled cyazofamid – [^{14}C-Bz]cyazofamid (lot no. CP-1863, purity ≥ 99.5%; lot no. CP-1863-2, purity ≥ 99.5%; lot no. 2074, purity ≥ 99.6%) or [^{14}C-Im]cyazofamid (lot no. CP-1864, purity ≥ 98.5%; lot no. CP-1947, purity ≥ 98.3%; lot no. CP-2073, purity ≥ 99.5%) – suspended in a 0.75% aqueous methyl cellulose solution by gavage. Two control animals of each sex received the vehicle. Experimental groups and their controls were terminated at times corresponding to 1) T_{max}, 2) the biological half-life, 3) 24 hours after dosing or 4) 168 hours after dosing. Blood and tissue samples were collected immediately following termination.

Table 1. Structures of the parent compound and metabolites and degradates of cyazofamid in various crops and animals

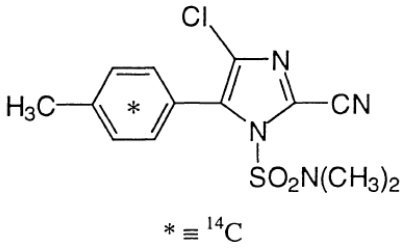
Code/common name (main sources)	Chemical name (IUPAC)	Structural formula
Cyazofamid	4-Chloro-2-cyano-*N*,*N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide	
CCIM (a first metabolite in rats, large animals, plants)	4-Chloro-5-*p*-tolylimidazole-2-carbonitrile	
CCIM-AM (large animals, plants)	4-Chloro-5-*p*-tolylimidazole-2-carboxamide	
CCBA (a major urinary metabolite in rats)	4-(4-Chloro-2-cyanoimidazol-5-yl)benzoic acid	
Dimethylsulfamic acid (proposed metabolite in rats)	Dimethylsulfamic acid	
CTCA (plants)	4-Chloro-5-*p*-tolylimidazole-2-carboxylic acid	
CHCN (rats)	4-Chloro-5-(4-hydroxymethylphenyl) imidazole-2-carbonitrile	

IUPAC: International Union of Pure and Applied Chemistry

Fig. 1. Positions of radiolabelling



Benzene [^{14}C-Bz] cyazofamid



Imidazole [^{14}C-Im] cyazofamid

Table 2. Kinetics data in rats

	[^{14}C-Bz]Cyazofamid				[^{14}C-Im]Cyazofamid			
	0.5 mg/kg bw		1 000 mg/kg bw		0.5 mg/kg bw		1 000 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
C_{max} (µg/g)	0.34	0.24	48.1	75.6	0.35	0.28	54.2	66.6
T_{max} (h)	0.50	0.50	0.25	0.25	0.50	0.50	0.25	0.25
$t_{½}$ (h)	4.37	4.63	7.58	9.72	4.84	5.82	10.40	11.6
AUC (h·µg/g)	0.67	0.48	103	104	0.81	0.63	96.2	102

AUC: area under the concentration–time curve; Bz: benzene; C_{max}: maximum concentration; Im: imidazole; $t_{½}$: biological half-life; T_{max}, time to reach maximum concentration
Source: Huhtanen & Savides (1998a)

In animals treated at the low and high doses and terminated at T_{max}, the mean concentration of radiolabel was highest in the kidneys, followed by the liver and the blood, at the low dose only; at the high dose, the radiolabel was diffusely distributed in various organs (Table 3). At the low dose, the concentration of radiolabel distributed in these tissues and organs was higher for [^{14}C-Bz]cyazofamid than for [^{14}C-Im]cyazofamid. The radioactivity distributed in these tissues at T_{max} was rapidly depleted from all tissues; in low-dose animals terminated at the biological half-life, mean total residues in liver and kidney were less than 0.2 µg/g in both sexes for both radiolabels. Mean total residues also decreased in tissues of high-dose animals terminated at the biological half-life, except in some dose groups in which residues in the carcass increased.

In animals terminated at 24 hours after dosing, levels of mean total radioactivity in blood were below the limit of quantification (LOQ) in three of the four low-dose groups and below 1 µg/g in three of the four high-dose groups. Mean total residues in all other tissues, including carcass, also decreased.

In low-dose animals terminated at 168 hours after dosing, mean total residues in all tissues except liver, kidney and adrenals were less than 0.001 µg/g. The levels in the liver, kidneys and adrenals were less than 0.002 µg/g (McFadden, Yoshida & Huhtanen, 1999).

(c) Excretion

Sprague-Dawley rats (3–5 rats of each sex per group) were treated with a single dose of 0.5 mg/kg bw (low dose) or 1000 mg/kg bw (high dose) radiolabelled cyazofamid – [^{14}C-Bz]cyazofamid (lot no. CP-1863, purity ≥ 99.5%; lot no. CP-1863-2, purity ≥ 99.5%; lot no. 2074, purity ≥ 99.6%) or [^{14}C-Im]cyazofamid (lot no. CP-1864, purity ≥ 98.5%; lot no. CP-1947, purity ≥ 98.3%; lot no. CP-2073, purity ≥ 99.5%) – suspended in a 0.75% aqueous methyl cellulose solution by gavage. Two control animals of each sex received the vehicle. Urine and faeces were collected continuously from

Table 3. Main tissue distribution of cyazofamid in rats

	Concentration of cyazofamid (µg/g)							
	[^{14}C-Bz]Cyazofamid				[^{14}C-Im]Cyazofamid			
	0.5 mg/kg bw		1 000 mg/kg bw		0.5 mg/kg bw		1 000 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
T_{max}								
Kidneys	1.72	1.23	64.9	69.9	0.715	0.535	35.7	57.1
Liver	0.455	0.776	25.1	41.2	0.182	0.310	23.8	31.3
Blood	0.424	0.334	28.9	34.2	0.179	0.152	22.1	29.4
Thyroids	< 0.2[a]	< 0.2[a]	22.4	28.0	< 0.2[a]	< 0.2[a]	< 10.0[a]	< 10.0[a]
Adrenals	< 0.2[a]	< 0.2[a]	13.4	< 10.0[a]	< 0.2[a]	< 0.2[a]	< 10.0[a]	15.3
Fat	< 0.2[a]	< 0.2[a]	11.0	62.4	< 0.2[a]	< 0.2[a]	10.3	10.0
Testes/ovaries	< 0.2[a]	< 0.2[a]	< 10.0[a]	21.7	< 0.2[a]	< 0.2[a]	< 10.0[a]	18.4
Lung	< 0.2[a]	< 0.2[a]	< 10.0[a]	14.6	< 0.2[a]	< 0.2[a]	< 10.0[a]	< 10.0[a]
Uterus	–	< 0.2[a]	–	12.7	–	< 0.2[a]	–	10.7
Heart	< 0.2[a]	< 0.2[a]	< 10.0[a]	10.5	< 0.2[a]	< 0.2[a]	< 10.0[a]	< 10.0[a]
168 h after dosing								
Kidneys	0.001 2	0.001 7	< 0.5[a]	< 0.5[a]	< 0.001[a]	0.001 3	< 0.5[a]	< 0.5[a]
Liver	0.001 4	0.001 7	< 0.5[a]	< 0.5[a]	< 0.001[a]	< 0.001[a]	< 0.5[a]	< 0.5[a]
Adrenals	< 0.001[a]	0.001 1	< 0.5[a]	< 0.5[a]	< 0.001[a]	< 0.001[a]	< 0.5[a]	< 0.5[a]

bw: body weight; Bz: benzene; Im: imidazole; LOQ: limit of quantification; T_{max}: time to reach maximum concentration
[a] Below the LOQ.
Source: McFadden, Yoshida & Huhtanen (1999)

the animals after the administration. As previously, each experimental group was associated with a termination time corresponding to 1) T_{max}, 2) the biological half-life, 3) 24 hours after dosing or 4) 168 hours after dosing. Urine, faeces, cage rinses, blood, tissues and carcasses were analysed for radiolabel content. Samples collected from control animals were used to determine background radioactivity.

The mean recovery of radioactivity was 97.7% for all [^{14}C-Bz]cyazofamid dose groups and 96.8% for all [^{14}C-Im]cyazofamid dose groups. Dosed radioactivity was rapidly excreted from both sexes for both radiolabels, and greater than 90% of the administered dose was excreted in 24 hours. In rats at 168 hours, less than 0.5% of the administered dose remained in the tissues in all dose groups, indicating rapid removal of cyazofamid and its metabolites from rat tissues (Table 4). At the low dose, both radiolabelled cyazofamids were excreted more in urine than in faeces in both sexes, whereas more than 95% of the radiolabelled cyazofamids administered to both sexes were excreted in faeces at the high dose (Table 4) (McFadden, Yoshida & Huhtanen, 1999).

In a repeated-dose study, an oral dose of cyazofamid at 0.5 mg/kg bw was administered once daily for 14 days to Sprague-Dawley rats. On the 15th day, a single dose of [^{14}C-Bz]cyazofamid (purity 98.9%) suspended in 0.75% methyl cellulose at 0.5 mg/kg bw was administered orally, and the distribution and elimination of the dose were determined in two rats of each sex at 0.5, 24 and 168 hours after the last treatment.

Table 4. Excretion of cyazofamid in urine and faeces of rats

	% of administered dose							
	[^{14}C-Bz]Cyazofamid				[^{14}C-Im]Cyazofamid			
	0.5 mg/kg bw		1 000 mg/kg bw		0.5 mg/kg bw		1 000 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
24 h after dosing								
Urine[a]	60.5	50.9	2.1	2.1	60.8	44.7	2.7	1.9
Faeces	39.1	47.4	99.0	89.1	32.0	50.2	92.2	95.0
Tissues	0.70	3.77	2.50	10.17	1.27	2.08	4.09	4.55
168 h after dosing								
Urine[a]	64.8	50.8	2.6	2.6	68.2	49.0	3.6	2.1
Faeces	30.4	44.8	94.2	95.7	29.7	46.7	96.9	97.5
Tissues	0.07	0.04	0.00	0.00	0.27	0.23	0.03	0.02

bw: body weight; Bz: benzene; Im: imidazole
[a] Including the recovery from cage wash.
Source: McFadden, Yoshida & Huhtanen (1999)

Repeated administration of cyazofamid resulted in a somewhat higher excretion in urine than was found after a single dose. At 168 hours after the treatment with radiolabelled cyazofamid, 62.8–72.8% of the administered dose was excreted in urine, and 20.8–31.6% of the administered dose was excreted in faeces (McFadden & Savides, 1999).

The biliary excretion of ^{14}C-labelled cyazofamid was determined following administration of [^{14}C-Bz]cyazofamid or [^{14}C-Im]cyazofamid suspended in a 0.75% aqueous methyl cellulose solution by oral gavage to male and female bile duct–cannulated Sprague-Dawley rats at 0.5 mg/kg bw (low dose) or 1000 mg/kg bw (high dose). Three rats of each sex per dose level and label group were compared, except for [^{14}C-Im]cyazofamid, which had four rats of each sex at the low dose. The animals were terminated 72 hours after dosing.

At the low dose, both radiolabelled cyazofamids were mainly excreted in the urine, with lesser amounts found in faeces and bile (Table 5). At the high dose, the radiolabelled cyazofamids were mainly excreted in the faeces. For each group, the faecal radiocarbon accounted for more than 94.7% of the administered dose. However, the biliary radiocarbon collected over the 72-hour period accounted for approximately 1% of the administered dose for the males and 1.3% for the females. The biliary excretion was rapid, with the maximum levels of radiocarbon collected within the first 2 hours after dosing (Huhtanen & Savides, 1998b).

1.2 Biotransformation

(a) In vivo

[^{14}C-Bz]Cyazofamid (lot no. CP-1863-2; purity 99.5%) at 0.5 mg/kg bw or [^{14}C-Bz]CCIM (lot no. CP-1982; purity 97.6%) at 0.33 mg/kg bw was administered by oral gavage to male Sprague-Dawley rats, and the rats were terminated 0.5 hour later. Each of the two groups consisted of five animals. Blood was collected by exsanguination at termination. Liver and stomach (with contents) were also collected at termination. Blood, liver and stomach contents were analysed for radiolabel content and were used for the characterization of metabolites to identify the pathway of metabolism.

At 0.5 hour after dosing with [^{14}C-Bz]cyazofamid, most (97.2%) of the radiolabel in the stomach contents was the parent, and a small fraction was CCIM. Analysis of the liver from this

Table 5. Excretion of radiolabel in bile, urine and faeces of rats

	% of administered dose							
	[^{14}C-Bz]Cyazofamid				[^{14}C-Im]Cyazofamid			
	0.5 mg/kg bw		1 000 mg/kg bw		0.5 mg/kg bw		1 000 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
Bile	22.1	38.8	0.8	1.4	12.2	28.9	1.1	1.3
Urine[a]	61.6	40.5	5.2	3.6	41.0	43.6	4.1	2.7
Faeces	9.8	18.6	95.0	96.0	42.3	22.4	94.7	94.7

bw: body weight; Bz: benzene; Im: imidazole
[a] Including the recovery from cage wash.
Source: Huhtanen & Savides (1998b)

group showed that only 6.1% of the radiolabel was cyazofamid, whereas 24.2% was CCIM, and 41.9% was CCBA. In the plasma, there was no cyazofamid, and the majority of radiolabel was CCIM. These data demonstrate that a dose of cyazofamid is rapidly metabolized by hydroxylation and that CCIM is the first metabolite of cyazofamid.

At 0.5 hour after a dose of [^{14}C-Bz]CCIM, all of the radiolabel in the stomach contents was CCIM, and most of the radiolabel in liver (76.5%) and plasma (67.9%) was CCIM. CCBA, the main metabolite seen in these tissues 0.5 hour after dosing with CCIM, was also found in the blood and liver from the animals dosed with cyazofamid. Concentrations of CCIM in blood and liver were similar to those found after dosing with cyazofamid, suggesting that CCIM was absorbed similarly to cyazofamid (Table 6).

The metabolism of ^{14}C-labelled cyazofamid was determined following administration of [^{14}C-Bz]cyazofamid or [^{14}C-Im]cyazofamid suspended in a 0.75% aqueous methyl cellulose solution by oral gavage to male and female bile duct–cannulated Sprague-Dawley rats at 0.5 mg/kg bw (low dose) or 1000 mg/kg bw (high dose). Study details are as described in section 1.1 (Huhtanen & Savides, 1998b).

Table 6. Summary of tissue residues 0.5 hour after a single dose treatment with [^{14}C-Bz]cyazofamid and [^{14}C-Bz]CCIM in rats[a]

	% of dose in region of interest					
	[^{14}C-Bz]Cyazofamid			[^{14}C-Bz]CCIM		
	Stomach contents	Liver	Plasma	Stomach contents	Liver	Plasma
Cyazofamid	97.2	6.1	ND	ND	ND	ND
CCIM	2.8	24.2	61.7	100.0	76.5	67.9
CHCN	ND	ND	4.0	ND	3.8	5.6
CCBA	ND	41.9	34.4	ND	18.2	26.6
Unknown	ND	27.2	ND	ND	ND	ND
Total	100.0	99.9	100.1	100.0	98.9	100.1

Bz: benzene; Im: imidazole; ND: not detected
[a] For full names of metabolites, see Table 1.
Source: Murray & Savides (1999)

Table 7. Characterization of metabolites in bile, urine and faeces of rats 72 hours after dosing at 0.5 mg/kg bw[a]

	% of administered dose							
	Females				Males			
	Bile	**Urine**	**Faeces**	**Total**	**Bile**	**Urine**	**Faeces**	**Total**
Cyazofamid	ND	ND	15.0	15.0	ND	ND	21.4	21.4
CCBA	4.5	37.9	ND	42.4	4.1	49.8	ND	53.9
CCIM	ND	ND	< 0.4	< 0.4	ND	ND	< 0.5	< 0.5
Conjugates	29.4	4.2	ND	33.6	13.3	1.5	ND	14.8
Non-extractables	ND	ND	3.2	3.2	ND	ND	2.2	2.2

bw: body weight; ND: not detected

[a] For full names of metabolites, see Table 1.

Source: Huhtanen & Savides (1998b)

As shown in Table 7, the main metabolites were CCBA, at 4.1–4.5% of the administered dose in bile and 37.9–49.8% of the administered dose in urine; and the conjugates of CCBA, CCIM and CHCN, at 13.3–29.4% of the administered dose in bile and 1.5–4.2% of the administered dose in urine. Unchanged cyazofamid was detected in faeces at 15.0–21.4% of the administered dose (Huhtanen & Savides, 1998b).

Sprague-Dawley rats (3–5 rats of each sex per group) were treated by gavage with a single dose of 0.5 mg/kg bw (low dose) or 1000 mg/kg bw (high dose) radiolabelled cyazofamid – [^{14}C-Bz]cyazofamid (lot no. CP-1863, purity ≥ 99.5%; lot no. CP-1863-2, purity ≥ 99.5%; lot no. 2074, purity ≥ 99.6%) or [^{14}C-Im]cyazofamid (lot no. CP-1864, purity ≥ 98.5%; lot no. CP-1947, purity ≥ 98.3%; lot no. CP-2073, purity ≥ 99.5%) – suspended in a 0.75% aqueous methyl cellulose solution. Two control animals of each sex received the vehicle. Urine and faeces were collected continuously from the animals after the administration. Selected urinary and faecal samples were used for the characterization and/or isolation of metabolites by high-performance liquid chromatography. This study was the same as the excretion study described above.

CCBA, CH_3SO-CCIM and CH_3SO_2-CCIM accounted for more than 90% of the urinary radiocarbon in urine from animals at 168 hours. CCBA was the major metabolite in the urine in males at the low and high doses and in females at the high dose. In the urine from females in both low-dose groups, the average CCBA content accounted for over 50% of the urinary radiocarbon. The total percentage of the metabolites CH_3SO-CCIM and CH_3SO_2-CCIM accounted for approximately 30% of the urinary radiocarbon from low-dose female rats (McFadden, Yoshida & Huhtanen, 1999).

(b) In vitro

To examine the metabolism of cyazofamid in vitro, blood from male Sprague-Dawley rats was fortified with 0.40 µg/mL of [^{14}C-Bz]cyazofamid (lot no. CP-1863-2-P; purity 97.5%) or with 0.27 µg/mL of [^{14}C-Bz]CCIM (lot no. CP-1982; purity 97.6%). After the fortification, the samples were incubated in a water bath at 37 °C and analysed at 0, 15, 30 and 60 minutes for cyazofamid and at 0 or 60 minutes for CCIM. Stomach content samples from male Sprague-Dawley rats were fortified with [^{14}C-Bz]cyazofamid (lot no. CP-1863-2-P; purity 97.5%) or with [^{14}C-Bz]CCIM (lot no. CP-1982; purity 97.6%). After the fortification, the samples with cyazofamid or CCIM were analysed 0 and 60 minutes after incubation in a water bath at 37 °C.

The in vitro metabolism of cyazofamid and CCIM in blood and stomach is shown in Table 8.

Table 8. In vitro metabolism of cyazofamid in blood and stomach

Sampling time (min)	Average % of applied dose						
	Cyazofamid				CCIM		
	Cyazofamid	CCIM	Others	Total	CCIM	Others	Total
Blood							
0	94.0	3.0	1.6	98.6	95.2	3.1	98.3
15	79.6	14.1	1.9	95.6	NE	NE	NE
30	74.5	17.6	0.9	93.1	NE	NE	NE
60	62.1	31.3	1.7	95.1	96.1	2.6	98.7
Stomach							
0	101.4	ND	2.2	103.6	103.6	ND	106.1
60	104.7	ND	2.1	106.8	106.8	ND	98.8

ND: not detected; NE: not examined
Source: Sakai, Tada & Kanza (1999)

Cyazofamid was metabolized rapidly in blood. After 60 minutes, approximately 30% of the cyazofamid was metabolized, mainly to CCIM. CCIM was stable in blood. Both cyazofamid and CCIM were stable in the stomach contents. CCIM was a major first metabolite of cyazofamid in rats.

Based on the results of these studies, it can be concluded that the metabolism of cyazofamid proceeds through formation of CCIM as the main pathway. A proposed metabolic scheme in rats is presented in Fig. 2 (Sakai, Tada & Kanza, 1999).

2. Toxicological studies

2.1 Acute toxicity

Studies on the acute toxicity, skin or eye irritation, and skin sensitization potential of cyazofamid are summarized in Table 9.

(a) Lethal doses

Oral administration

Cyazofamid was dispersed in 0.5% aqueous methyl cellulose and administered at 5000 mg/kg bw to five CD-1 mice of each sex per group.

No deaths were observed. No treatment-related clinical findings and no effects on body weight were observed in the mice. The oral median lethal dose (LD_{50}) of cyazofamid in mice was greater than 5000 mg/kg bw (Yoshida & Watson, 1999).

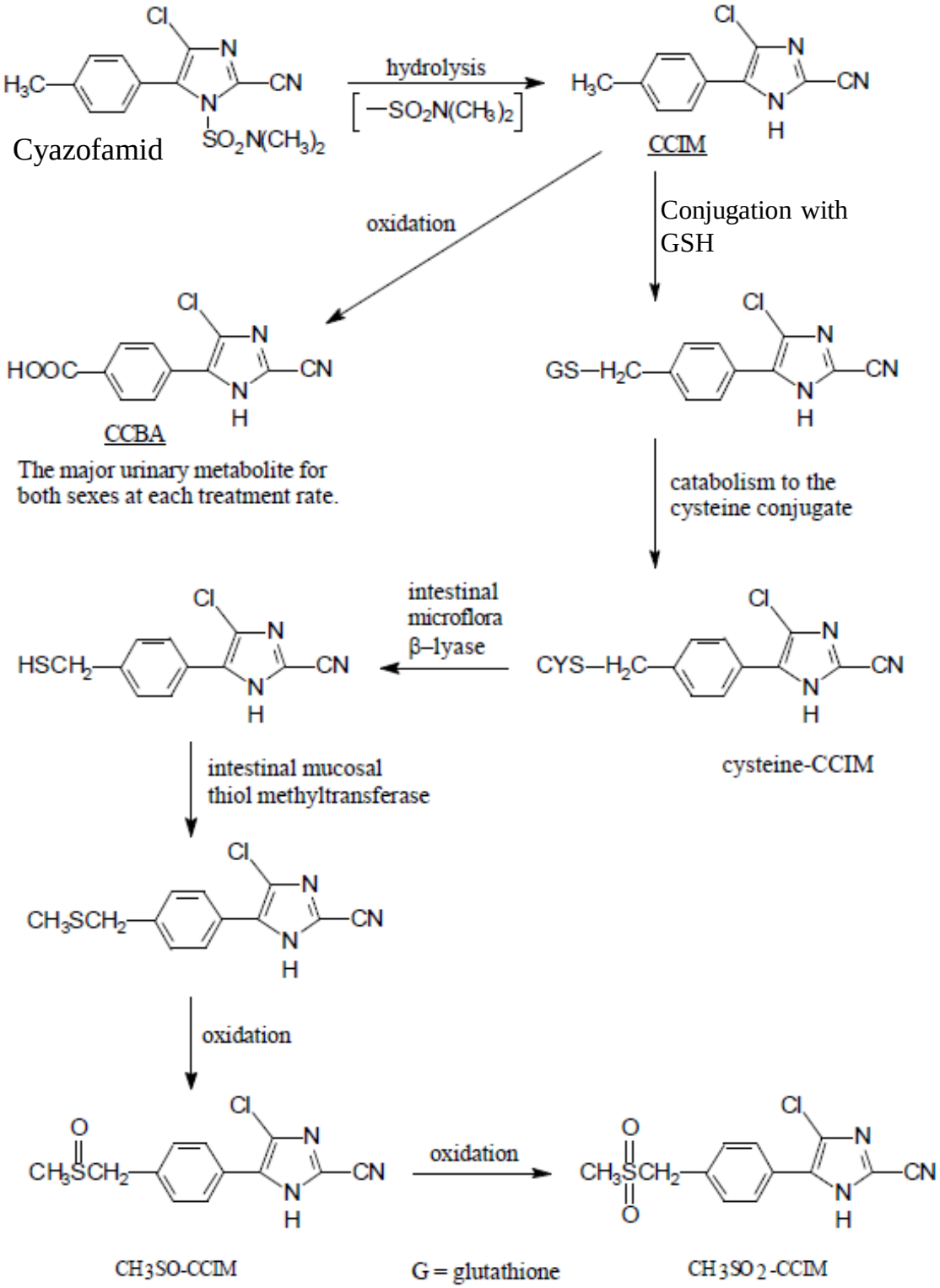
Cyazofamid was dispersed in 0.5% aqueous methyl cellulose and administered at 5000 mg/kg bw to five Sprague-Dawley rats of each sex per group.

No deaths were observed. No treatment-related clinical findings and no effects on body weight were noted in the rats. The oral LD_{50} of cyazofamid in rats was greater than 5000 mg/kg bw (Yoshida, Brock & Watson, 1998b).

Dermal application

Slightly moistened (distilled water) cyazofamid was applied to the shaved skin of five Sprague-Dawley rats of each sex at 2000 mg/kg bw. The test material was covered with a gauze patch

Fig. 2. Proposed metabolic pathway of cyazofamid



GSH: glutathione

Source: Huhtanen & Savides (1998c)

Table 9. The acute toxicity, skin or eye irritation, and skin sensitization potential of cyazofamid

Route (method)	Species/ strain	Lot no.; purity	Result	Reference
Oral (gavage)	CD-1 mice	9506; 95.7%	LD_{50}: > 5 000 mg/kg bw in both sexes	Yoshida & Watson (1999)
Oral (gavage)	Sprague-Dawley rats	9506; 95.5%	LD_{50}: > 5 000 mg/kg bw in both sexes	Yoshida, Brock & Watson (1998b)
Dermal	Sprague-Dawley rats	9506; 95.5%	LD_{50}: >2 000 mg/kg bw in both sexes	Yoshida, Brock & Watson (1998a)
Inhalation (nose only)	Sprague-Dawley rats	9506; 95.5%	LC_{50}: > 5.5 mg/L in both sexes	Ulrich (1998)
Skin irritation	NZW rabbits	9506; 95.5%	Slightly irritating	Yoshida, Brock & Watson (1998c)
Eye irritation	NZW rabbits	9506; 95.5%	Slightly irritating	Yoshida, Brock & Watson (1998d)
Dermal sensitization (maximization method)	Hartley guinea-pig	9506; 95.5%	Not sensitizing	Yoshida, Brock & Watson (1998e)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NZW: New Zealand White

backed with hypoallergenic tape and was secured in place by an elastic wrapping. After a 24-hour exposure period, the cyazofamid was removed, and all rats were euthanized 14 days after the treatment.

No deaths were observed. Very slight erythema at the application site and incidences of dried red material around the eyes and/or nose and of anogenital staining were observed within several days after the treatment. These dermal changes were considered to be indicative of the slight irritant potential of cyazofamid. The dermal LD_{50} of cyazofamid in rats was greater than 2000 mg/kg bw (Yoshida, Brock & Watson, 1998a).

Inhalation

Five Sprague-Dawley rats of each sex per group were given a single inhaled exposure to a dust aerosol of cyazofamid at a concentration of 5.5 mg/L via whole-body exposure for 4 hours. The aerosol was characterized by a mass median aerodynamic diameter of 2.9 µm, with a geometric standard deviation of 2.2. Fourteen days after the inhalation, all rats were euthanized.

No deaths were observed. After the exposure, rales were noted in two animals. There were no other treatment-related clinical signs. Slight body weights were decreased in four female rats from day 0 to day 3; however, mean body weights for the female rats increased thereafter. No other differences were noted in body weights or body weight gains. There were no treated-related changes at necropsy. The inhalation median lethal concentration (LC_{50}) of cyazofamid in rats was greater than 5.5 mg/L in both sexes (Ulrich, 1998).

(b) Dermal irritation

Cyazofamid was administered to a shaved dorsal skin site on each of three male and three female New Zealand White (young adult) rabbits. Cyazofamid was applied at each site over a 4-hour exposure period by the use of a gauze patch and bandage.

Slight erythema (scores 1–2) was observed in all animals 30–60 minutes after exposure. This change was continued in three animals at the same severity as observed at 30–60 minutes up to 72 hours after the exposure, but disappeared in the remaining rabbits. No findings were noted in any of

the rabbits thereafter. Cyazofamid was considered slightly irritating to the skin of rabbits (Yoshida, Brock & Watson, 1998c).

(c) Ocular irritation

A dose of 0.09 g of cyazofamid was administered to the conjunctival sac of the right eye of each of nine New Zealand White rabbits. In the washed group (three animals), both the treated and untreated eyes of each animal were flushed with deionized water for approximately 30 seconds approximately 30 seconds after instillation.

Conjunctival redness, chemosis and/or discharge were observed in all animals of the unwashed group at 1 hour after the treatment. Most of these effects disappeared within 24 hours, although mild redness and/or discharges lasted through 48 hours. Conjunctival redness, chemosis and discharge were also noted in all animals of the washed group at 1 hour after administration. These effects disappeared 24 hours after the administration. Cyazofamid was considered slightly irritating to the eyes of rabbits (Yoshida, Brock & Watson, 1998d).

(d) Dermal sensitization

The method of Magnusson and Kligman (maximization design) was utilized. Cyazofamid was injected intradermally into sites on the scapular region of each of 10 male and 10 female Hartley guinea-pigs at a 1% (weight per volume [w/v]) concentration in sterile distilled water at the study initiation (day 0) and applied topically at a 75% (w/v) concentration in distilled water on day 7. Two weeks after the application (day 21), the test material was topically administered on the left flank of each of the animals at a 25% (w/v) concentration in distilled water. One week after the initial challenge dose (day 28), the animals were rechallenged at the same concentration. Dinitrochlorobenzene was used as a positive control.

The sensitization rates for cyazofamid were less than 25% at 48 hours in both the initial challenge and rechallenge phases. The results indicate that cyazofamid is not sensitizing in the maximization test (Yoshida, Brock & Watson, 1998e).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Cyazofamid (lot no. 9506; purity 95.5%) was administered in the diet to 10 CD-1 mice of each sex per group at 0, 40, 200, 1000, 3500 or 7000 parts per million (ppm) (equal to 0, 8, 38, 193, 653 and 1419 mg/kg bw per day for males and 0, 9, 47, 248, 854 and 1796 mg/kg bw per day for females, respectively) for 6 weeks. Feed and water were available ad libitum. The mice were checked for viability twice a day. Physical examinations, body weights and feed consumption were recorded weekly. After 6 weeks of treatment, surviving animals had blood withdrawn from the orbital sinus under anaesthesia with ether and were necropsied. The adrenals, brain, liver with gallbladder, kidneys and testes were weighed. Haematology and blood chemistry examinations were performed. Histopathological examination was conducted for adrenals, bone marrow (sternum), kidneys, lungs, heart, liver, spleen, and ovaries or testes in the control and 7000 ppm groups and for any gross lesions.

There were no treatment-related deaths or clinical signs in any treated group. Although slightly depressed body weights and lower body weight gains were observed at weeks 5 and 6 in males and at weeks 0–6 in females at 1000 ppm and higher, the decreases were less than 10% and were not statistically significant. Therefore, they were considered not to be toxicologically significant.

There were no treatment-related changes in feed consumption, haematology or blood biochemistry. Liver weights relative to body weight were statistically significantly increased at 7000 ppm in both sexes, but the increases in both sexes were less than 10% and were not accompanied by any histopathological findings indicating hepatotoxicity. The increases were therefore considered not

to be toxicologically significant. Microscopically, no treatment-related changes were observed in any treated groups.

The no-observed-adverse-effect level (NOAEL) for 6-week oral toxicity in mice was 7000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested (O'Meara, Serrone & Lorence, 1996).

Rats

In a 28-day oral toxicity study, cyazofamid (lot no. 9506; purity 97.3%) was administered to six F344/DuCrj rats of each sex per group at a dietary concentration of 0, 50, 500, 5000 or 20 000 ppm (equal to 0, 3.77, 38.5, 370 and 1490 mg/kg bw per day for males and 0, 3.64, 37.1, 389 and 1540 mg/kg bw per day for females, respectively) for 4 weeks. Clinical signs were monitored daily. Body weights and feed consumption were measured weekly. At termination, all animals were necropsied after urine analysis, haematology and blood chemistry examinations. The brain, pituitary, thyroid (including parathyroid), thymus, heart, liver, spleen, kidneys, adrenals, and testes or ovaries were weighed. Histopathological examination was conducted for the livers and kidneys from all animals.

All animals survived the study. There were no treatment-related clinical signs or changes in body weight or feed consumption. In haematological analysis, a very slight increase in mean corpuscular haemoglobin was not considered to be treatment related. In blood biochemistry, glucose level was increased at 5000 ppm and above in males, triglyceride level was decreased at 20 000 ppm in males, and chloride concentration was very slightly increased at 5000 ppm and above in both sexes. Total protein and globulin levels were slightly, but statistically significantly, increased at all treated doses in males. In addition, albumin level was slightly increased at the two highest doses in males. All changes were not considered to be toxicologically significant because they were slight increases (< 10%) compared with the control values. In addition, relative liver weight was slightly (approximately 10%) increased at 5000 ppm and above in males, and absolute liver weight was increased at 20 000 ppm in males. These increases were not considered to be toxicologically significant, as there were no histopathological findings indicating hepatotoxicity. In the kidney, relative weight was increased at 20 000 ppm in males. Microscopically, basophilic tubules in the kidney were increased at incidences of 4/6 at 5000 ppm and 6/6 at 20 000 ppm in males. The severity of the lesion at 5000 ppm was less than that at 20 000 ppm. In females, no histopathological changes were observed.

The NOAEL for the 28-day rat oral toxicity study was 500 ppm (equal to 38.5 mg/kg bw per day), based on histopathological changes in the kidney in male rats at 5000 ppm (equal to 370 mg/kg bw per day) (Nakashima, 1999a).

In a 90-day oral toxicity study, cyazofamid (lot no. 9506; purity 95.5%) was administered in the diet to 12 F344/DuCrj rats of each sex per group for 13 weeks at 0, 10, 50, 500 or 5000 ppm (equal to 0, 0.597, 2.91, 29.5 and 295 mg/kg bw per day, respectively) for males and at 0, 50, 500, 5000 or 20 000 ppm (equal to 0, 3.30, 33.3, 338 and 1360 mg/kg bw per day, respectively) for females. The rats were checked daily for clinical signs. Body weights and feed consumption were measured weekly. Ophthalmological examination was conducted for the males and females in the control and high-dose groups at week 13. All animals were subjected to urine analysis at week 13. After the 13-week treatment period, the animals were subjected to haematology and blood biochemistry examinations, organ weight analysis, necropsy and histopathology. At necropsy, the brain, liver, kidneys, adrenals and testes were weighed.

There were no deaths and no treatment-related clinical signs during the study. There were no treatment-related differences in body weight or feed consumption. There were no ocular abnormalities in either sex at the high dose. In urine analysis, significant increases in protein and urine volume were observed in males at 5000 ppm (Table 10). Urinary pH was also significantly elevated in this group. Other statistically significant differences in urine analysis parameters were not observed in the males

Table 10. Summary of urine analysis, blood biochemistry, organ weight and microscopic findings in a 90-day oral toxicity study in rats

	Males					Females				
	0 ppm	**10 ppm**	**50 ppm**	**500 ppm**	**5 000 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**500 ppm**	**20 000 ppm**
Urine analysis										
Protein grade −/+/++[a]	1/9/2	0/6/6	0/9/3	1/5/6	0/4/8*	1/10/1	3/9/0	2/9/1	5/7/0	4/8/0
Volume (mL)	6.4	6.3	6.2	6.4	7.6**	4.9	5.0	4.7	4.8	6.0
pH										
6.0	–	–	–	–	–	–	–	3	4	–
6.5	1	6	8	7	–	–	3	5	4	–
7.0	–	4	3	4	–	1	7	3	3	–
7.5	7	2*	1**	1	2	2	2	1*	1*	4
8.0	8	–	–	–	10*	7	–	–	–	7
8.5	–	–	–	–	–	2	–	–	–	–
Blood biochemistry										
Chlorine (meq/L)	104.5	105.1	105.0	105.1	106.0**	108.0	107.9	108.6	107.4	108.5
Total cholesterol (mg/dL)	50	49	52	49	44**	56	59	58	57	55
Triglyceride (mg/dL)	130	135	132	132	99**	54	59	56	53	51
Organ weights										
Relative liver weight[b]	2.28	2.31	2.32	2.28	2.36	2.25	2.18	2.16	2.27	2.40*
Relative kidney weight[b]	0.59	0.57	0.58	0.56*	0.60	0.63	0.63	0.62	0.66*	0.66*
Histopathological findings										
Basophilic tubule in the kidney	0	0	0	0	12	0	0	0	0	0

eq: equivalents; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Grade of urine protein: −: negative; +: slight; ++: moderate.

[b] Relative weight: absolute weight (g) / body weight (g) × 100.

Source: Nakashima (1999b)

or in all the females. There were no significant haematology changes in any treated group of either sex. In blood biochemistry, treatment-related changes were increased chlorine and total cholesterol levels and decreased triglyceride levels in males at 5000 ppm. No other treatment-related differences were noted in the analysis (Table 10).

No treatment-related macroscopic findings were observed. There was no difference in organ weights in males. In females, relative kidney weights at 5000 ppm and above and relative liver weights at 20 000 ppm were significantly increased compared with controls. Other changes noted were not related to dose. The very slight increases in relative kidney weights, which were less than 5% and not accompanied by alterations in parameters related to renal toxicity, were not considered to be treatment related. Microscopically, an incidence of basophilic tubules was significantly increased

in the kidneys of all male rats at 5000 ppm. This change was exclusively noted at the portion of the pars recta of the proximal tubules. There were no other microscopic treatment-related changes in any treated groups of females or in males in the 500 ppm group and lower. The kidney is considered a major target organ for cyazofamid in males based on the changes in urine analysis parameters and histopathological analysis.

The NOAEL for the 90-day oral toxicity study in rats was 500 ppm (equal to 29.5 mg/kg bw per day), based on kidney effects in males at 5000 ppm (equal to 295 mg/kg bw per day) (Nakashima, 1999b).

Dogs

In a 28-day oral toxicity study, cyazofamid (lot no. 9506; purity 96.4%) was administered by capsule to two Beagle dogs of each sex per group at 10, 100 or 1000 mg/kg bw per day. A control group received capsules only. Haematology, blood chemistry and urine analysis were performed prior to initiation of test material administration and at termination of the treatment period. All animals were necropsied, and the adrenals, brain, heart, liver, kidneys, ovaries or testes, and thyroid with parathyroids were weighed. All tissues were fixed except ear, cervix, vagina and tongue from the control group animals. All dogs in the treatment groups were examined microscopically.

There were no deaths during the treatment period. No treatment-related changes were observed in clinical signs, body weights, body weight gain, feed consumption, haematology, blood biochemistry, gross necropsy findings, organ weights or histopathology in any treatment group. Both high-dose females exhibited diffuse, unilateral mottling of one lobe of the lung, which may be explained by the histopathological findings suggesting inhalation of regurgitated stomach contents. Therefore, these findings were not considered to be treatment related.

There were no treatment-related effects observed in this study up to 1000 mg/kg bw per day, the highest dose tested (Savides, Yoshida & Watson, 1998).

In a 90-day oral toxicity study, cyazofamid (lot no. 9506; purity 96.2%) was administered by capsule to four Beagle dogs of each sex per group at 40, 200 or 1000 mg/kg bw per day for 13 weeks. Control animals received empty capsules equal to the number of capsules required for the high-dose animals. Animals were observed twice daily for mortality and morbidity, cage-side observations were made for signs of toxic effects approximately 1 hour after test material administration, and a detailed physical examination of each animal was performed weekly. Feed consumption and body weights were measured weekly. An ophthalmological examination was performed on each animal prior to and after 13 weeks of treatment. Haematology, blood biochemistry and urine analysis were performed for all animals prior to treatment and after 45 days and 13 weeks of treatment. At blood sampling, the animals were fasted for 16–24 hours. After necropsy, the adrenal glands, brain, heart, liver, kidneys, ovaries or testes, and thyroid glands with parathyroid glands were weighed. Fixed tissues and organs were examined microscopically.

There were no deaths during the treatment period. No treatment-related clinical signs were observed in either sex. No treatment-related changes were noted in body weight, feed consumption or ophthalmology. There were no treatment-related effects on haematology or blood biochemistry, although no consistent or dose-related changes were observed. At necropsy, a small prostate gland was noted in one male at each of 40, 200 and 1000 mg/kg bw per day. The small prostate gland was observed to be immature microscopically and was considered to be within the normal range for the age of Beagles used in this study. Therefore, the change was not considered to be treatment related. For organ weights, the thyroid weight relative to brain weight was statistically significantly decreased in males at 1000 mg/kg bw per day. However, there was no decrease in thyroid weight relative to body weight, and there were no corresponding histopathological changes in the thyroid, indicating that the decrease was not biologically significant. No treatment-related histopathological changes were noted.

The NOAEL for the 90-day oral toxicity study in dogs was 1000 mg/kg bw per day, the highest dose tested (Savides, Lucas & Watson, 1998).

In a 1-year oral toxicity study, cyazofamid (lot no. 9506; purity 96.4%) was administered by capsule to six Beagle dogs of each sex per group at 40, 200 or 1000 mg/kg bw per day for 52 weeks. Control animals received empty capsules equal to the number of capsules required for the high-dose animals. Animals were observed twice daily for mortality and morbidity, cage-side observations were made for signs of toxic effects after administration, and a detailed physical examination of each animal was performed weekly. Feed consumption and body weights were measured weekly. An ophthalmological examination was performed prior to and after 52 weeks of treatment. Haematology, blood biochemistry and urine analysis were performed prior to treatment and after 14, 27, 40 and 52 weeks of treatment. The animals were fasted for 16–24 hours before blood sampling. At termination, all animals were necropsied, and the adrenal glands, brain, heart, liver, lungs, kidneys, ovaries or testes, spleen, and thyroid glands with parathyroid glands were weighed. After all tissues and organs were fixed, histopathological analysis was performed. A male (animal number: 300131) at 200 mg/kg bw per day was excluded from toxicological evaluation because it was medicated to improve soft stools/diarrhoea for 9 days.

There were no deaths throughout the treatment period. In all groups, sporadic clinical signs were observed, but no treatment-related changes were found. There were no treatment-related differences in body weight, body weight gain or feed consumption between the control and treated groups. There were no treatment-related ophthalmological effects. There were no treatment-related changes observed in haematology, blood biochemistry or urine analysis parameters. The increases in total bilirubin level at 40 and 1000 mg/kg bw per day in males at week 40 were not treatment related owing to a lack of dose dependency. At necropsy, no gross lesions were considered to be treatment related. Absolute spleen weights were dose-dependently lowered in all treated groups in males, and relative spleen weights were statistically significantly decreased at 1000 mg/kg bw per day in males; however, no corresponding histopathological findings were observed in the spleen of any dogs in this study (Table 11). The spleen weights in two males of the control group were approximately 50–100% higher than those of the remaining control group males. Although it was possible that incomplete exsanguination at necropsy resulted in artificial congestion in the spleen in these males, the absolute and relative spleen weights in males at 1000 mg/kg bw per day were still approximately 20% lower than those in control dogs after excluding these two males. The absolute spleen weights were also statistically significantly lower in females at 1000 mg/kg bw per day. Therefore, the decrease in spleen weights in both sexes in the 1000 mg/kg bw per day group was considered to be treatment related. Microscopically, atrophy of the thymus was increased in females at 1000 mg/kg bw per day, although the number of thymuses examined was not sufficient, owing to inadequate tissue sampling of the thymus. This change is not frequent in young adult dogs (Sato et al., 2008). Therefore, it could not be excluded that the thymus atrophy observed in females at 1000 mg/kg bw per day was treatment related.

The NOAEL for the 1-year oral toxicity study in dogs was 200 mg/kg bw per day, based on decreased spleen weight in both sexes and atrophy of the thymus in females at 1000 mg/kg bw per day (Savides & Watson, 1999).

(b) Dermal application

No data on toxicity following dermal application were provided by the company.

A 4-week dermal toxicity study of cyazofamid (lot number and purity were not provided) was conducted in Sprague-Dawley rats at concentrations of 0, 250, 500 and 1000 mg/kg bw per day. The NOAEL for systemic toxicity via dermal exposure was 1000 mg/kg bw per day, the highest dose tested (Food Safety Commission, 2014).

Table 11. Summary of blood biochemistry and pathological findings in 1-year dog toxicity study

	Males				Females			
	0 mg/kg bw per day	**40 mg/kg bw per day**	**200 mg/kg bw per day**	**1 000 mg/kg bw per day**	**0 mg/kg bw per day**	**40 mg/kg bw per day**	**200 mg/kg bw per day**	**1 000 mg/kg bw per day**
No. of dogs examined	6	6	5	6	6	6	6	6
Final body weight (kg)	13.61	12.06	11.51	11.93	10.23	10.17	9.85	10.37
Spleen weight, absolute (g)	86.75[a]	75	66	61*	78	56	66	66*
Spleen weight, relative ($g/g \times 10^3$)	6.478[a]	6.208	5.672	5.195	7.565	5.527	6.743	6.297
No. of thymuses examined	2	3	2	3	1	6	0	5
Atrophy, thymus	1	2	2	3	1	4	0	5
Severity of the atrophy (minimum/mild/moderate)	0/0/1	0/2/0	1/0/1	1/2/0	0/1/0	2/2/0	0/0/0	3/1/1

bw: body weight; *: $P < 0.05$

[a] Two males showing approximately 50–100% heavier spleens than those of the other control males were excluded.

Source: Savides & Watson (1999)

(c) Exposure by inhalation

Inhalation studies were not performed.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Cyazofamid (lot no. 9506; purity 95.5 ± 0.7%) was administered in the diet to 60 CD-1 mice of each sex per group at 0, 70, 700 or 7000 ppm (equal to 0, 9.5, 94.8 and 985 mg/kg bw per day for males and 0, 12.2, 124 and 1203 mg/kg bw per day for females, respectively) for 18 months. The high dose of 7000 ppm (equal to approximately 1000 mg/kg bw per day) was selected because it is a limit dose accepted by many regulatory agencies. The mice in the two control groups received basal diet. The animals were monitored twice daily for viability and clinical signs and checked for tumour formation by palpation. Body weights and feed consumption were periodically recorded. Blood smears were prepared for each surviving animal at 12 and 18 months. The mice were not fasted prior to the blood sample collection from the orbital sinus. Differential leukocytes from animals in the control and high-dose groups were counted at 12 and 18 months. All animals were subjected to necropsy. Adrenals, brain, liver with gallbladder, kidneys and testes were weighed. All organs and tissues were fixed and routinely processed for histopathological examination.

Mortality in the treated groups was comparable with that in the control groups. There were no treatment-related clinical signs or changes in body weight, feed consumption, haematology or blood biochemistry. Cyazofamid did not affect organ weights or cause any macroscopic changes. Microscopically, non-neoplastic and neoplastic lesions were comparable between the treated and control groups.

The NOAEL for the 18-month oral toxicity and carcinogenicity study in mice was 7000 ppm (equal to 985 mg/kg bw per day), the highest dose tested (O'Meara & Watson, 1999).

Rats

Cyazofamid (lot no. 9506; purity 95.5%) was administered in the diet to 85 F344/DuCrj rats of each sex per group for 104 weeks at a concentration of 0, 10, 50, 500 or 5000 ppm (equal to 0, 0.336, 1.68, 17.1 and 171 mg/kg bw per day, respectively) for males and a concentration of 0, 50, 500, 5000 and 20 000 ppm (equal to 0, 2.01, 20.2, 208 and 856 mg/kg bw per day, respectively) for females. Fifty rats of each sex per group were allocated to the carcinogenicity study group at the end of the 104 weeks, and 35 rats of each sex per group were used for the interim kill. All rats were observed daily for clinical signs. Body weights of all rats were periodically recorded, and mean body weights per dose group were obtained from the data on the carcinogenicity study group. Feed consumption was periodically recorded for the carcinogenicity study group only. At 25, 52 and 78 weeks after the treatment, 10 rats of each sex per group in the interim kill group had blood withdrawn for haematology and blood biochemistry and were necropsied. Before the necropsy, these rats were subjected to urine analysis. The remaining rats in the interim kill group were not used for these assays. The surviving rats in the control and high-dose groups were examined after 104 weeks of treatment. Before termination, 10 rats of each sex per group were examined for urine analysis, and these animals were then subjected to haematology, blood biochemistry and organ weight measurement after 104 weeks of treatment. All animals in the main group were subjected to necropsy. Histopathological examination was performed on the animals killed after 52 and 104 weeks of treatment and the animals found dead or killed in extremis during the treatment period.

Cyazofamid did not affect mortality in any treated groups. In clinical observations, the incidences of bradypnoea were increased in males at 500 ppm and above. However, there were no treatment-related abnormalities in the respiratory tracts at these doses, suggesting that the increases were not treatment related. Body weight changes in males and feed consumption in both sexes of the treated groups were comparable with those in control group animals. Body weights in females at 20 000 ppm were slightly (approximately 5%), but continuously, depressed during weeks 3–100, indicating that the slight depression was treatment related. At termination, no treatment-related changes at ophthalmological examination were found in either sex in the highest-dose group. In urine analysis, slight but statistically significant increases in urinary volume were observed at week 77 in males and at weeks 51 and 77 in females at the highest dose (Table 12). Other parameters related to urinary function, such as increased kidney weights, were found in the same dose group. In addition, the kidney was the target site of this compound at the same dose in the short-term rat study. Therefore, the increase in urinary volume were considered to be a treatment-related adverse effect. Increases in blood sodium and chloride levels at week 25 in males were not considered to be treatment related because the changes in electrolytes were very slight (approximately 1%) and transitory. The slight increase in blood urea nitrogen (approximately 15%) observed in males at 5000 ppm at week 51 was considered to be a treatment-related change. Relative kidney weights in males at week 26 at 5000 ppm and in females at weeks 26 and 78 at 5000 ppm and higher were statistically significantly increased (Table 12). The increases in relative kidney weight and blood urea nitrogen in males at 5000 ppm were slight but considered to be treatment related, because the kidney was the target of cyazofamid. Although relative kidney weights were increased in females at the highest dose at weeks 26 and 52, the increases were not considered to be treatment related owing to the lack of corresponding changes indicating renal toxicity at this dose. Increased liver weights occurred at the highest dose tested. As no change indicating hepatotoxicity was observed, the increases in liver weight were not considered to be toxicologically significant. In histopathological examination, the incidences of neoplastic changes in all treated groups were also comparable with those in control group animals of both sexes.

Cyazofamid was not carcinogenic in rats at dietary concentrations up to 5000 ppm (equal to 171 mg/kg bw per day) for males and 20 000 ppm (equal to 856 mg/kg bw per day) for females. The slight but primary toxicity target of cyazofamid was the kidney in rats.

The NOAEL for 2-year oral toxicity in male rats was 500 ppm (equal to 17.1 mg/kg bw per day), based on kidney effects at 5000 ppm (equal to 171 mg/kg bw per day). The NOAEL for carcinogenicity in male rats was 5000 ppm (equal to 171 mg/kg bw per day), the highest dose tested (Nakashima, 1999c). The NOAELs for both of these end-points in females were higher than the NOAELs in males.

Table 12. Summary of treatment-related changes observed in the 2-year oral toxicity study in rats

	Males					Females				
	0 ppm	**10 ppm**	**50 ppm**	**500 ppm**	**5 000 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**5 000 ppm**	**20 000 ppm**
Urinary volume (mL)										
Week 25	6.8	6.7	6.5	6.6	7.3	4.2	3.7	4.8	4.0	5.0
Week 51	6.3	6.7	6.8	6.1	6.9	3.5	3.7	3.7	4.2	5.6**
Week 77	6.9	6.9	6.5	7.1	8.4*	5.3	5.5	5.1	6.8	7.8*
Week 103	7.1	7.1	8.2	7.6	7.9	5.4	5.5	6.2	5.8	6.7
BUN (mg/dL)										
Week 52	13.8	13.4	13.7	14.8	15.6*	16.6	17.8	17.1	17.8	16.5
Kidney weight, absolute (mg)										
Week 26	1 930	1 938	1 910	1 939	2 058*	1 210	1 189	1 142	1 232	1 194
Week 52	2 231	2 177	2 283	2 142	2 158	1 391	1 316	1 389	1 436	1 395
Week 78	2 317	2 260	2 277	2 325	2 382	1 475	1 518	1 526	1 553	1 508
Week 104	2 343	2 376	2 367	2 551	2 536	1 623	1 601	1 652	1 634	1 686
Kidney weight, relative (g/g bw × 100)										
Week 26	0.53	0.52	0.52	0.54	0.55*	0.60	0.60	0.60	0.63**	0.64**
Week 52	0.51	0.50	0.52	0.51	0.53	0.60	0.59	0.62	0.65*	0.64
Week 78	0.53	0.51	0.51	0.54	0.56	0.56	0.62*	0.60	0.62*	0.63**
Week 104	0.54	0.53	0.57	0.58	0.59	0.59	0.57	0.58	0.60	0.62
Liver weight										
Absolute (g), week 26	7.42	7.55	7.53	7.33	8.04*	4.33	4.20	4.07*	4.43	4.37
Relative (g/g bw × 100)	2.02	2.03	2.06	2.05	2.15*	2.14	2.11	2.13	2.26	2.35**

BUN: blood urea nitrogen; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Nakashima (1999c)

2.4 Genotoxicity

The genotoxicity of cyazofamid was investigated in a comprehensive array of studies. One in vivo (mouse micronucleus test in bone marrow) and four in vitro genotoxicity tests (a bacterial assay for reverse mutations, a DNA repair test [rec-assay], a clastogenicity test in human lymphocytes and a gene mutation assay in mouse lymphoma cells) were conducted (Table 13).

Cyazofamid demonstrated no mutagenic properties in *Salmonella typhimurium* (strains TA98, TA100, TA1535 and TA1537) and a tryptophan-dependent mutant of *Escherichia coli* (strain CM891), with or without metabolic activation (S9). Cyazofamid did not damage DNA repair in *Bacillus subtilis* strains H17 and M45. Cyazofamid did not induce clastogenicity in the mammalian chromosomal aberration test using human lymphocytes. In mutation assays in mammalian cells, cyazofamid did not induce mutation in L5178Y mouse lymphoma cells. In in vivo genotoxicity testing, cyazofamid did not induce chromosomal aberrations in mouse bone marrow cells.

Table 13. Summary of genotoxicity studies with cyazofamid

Test	Test object	Concentration	Lot no./purity	Result	Reference
In vitro					
Bacterial reverse mutation	*Salmonella typhimurium* (strains TA98, TA100, TA1535 and TA1537) and a tryptophan-dependent mutant of *Escherichia coli* (strain CM891)	50, 150, 500, 1 500 and 5 000 µg/plate	9506/95.5%	Negative ±S9	Kitching (1998)
DNA repair test (rec-assay)	*Bacillus subtilis* recombination-wild (rec+) strain H17 and recombination-deficient (rec−) strain M45	250, 500, 1 000, 2 000, 4 000 and 8 000 µg/disc	LAP-0298/ 90.4%	Negative	Akanuma (1998)
Clastogenicity in mammalian cells	Mammalian chromosomal aberration test in human lymphocytes	1.563, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL	9506/95.5%	Negative	Akhurst (1998)
Mutation assay in mammalian cells	L5178Y mouse lymphoma cells	0.5, 1, 5, 10, 25, 50, 75 and 100 µg/mL	9506/95.5%	Negative ±S9	Kirkpatrick (1998)
In vivo					
Micronucleus test	Mouse bone marrow polychromatic erythrocytes	500, 1 000 and 2 000 mg/kg bw by gavage to ICR mice of both sexes	9506/95.5%	Negative	Proudlock & Dawe (1998)

bw: body weight; S9: 9000 × *g* supernatant fraction from rat liver homogenate

In conclusion, cyazofamid was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

Two reproductive toxicity studies were conducted using rats. In a single-generation reproductive toxicity study, cyazofamid (lot no. 9506; purity 95.5 ± 0.7%) was administered via the diet to 10 Sprague-Dawley rats of each sex per group at 0, 1000, 3000, 7000 or 20 000 ppm (equal to 0, 66.5, 200, 450 and 1327 mg/kg bw per day for males and 0, 77.5, 252, 562 and 1613 mg/kg bw per day for females, respectively) during the 8-week growth phase. The parental animals were treated continuously throughout the entire study, and the offspring were exposed to the test diets throughout the lactation period. Body weights and feed consumption were recorded weekly during the 8-week growth period. Each female was randomly assigned for mating with a male in the same group for a 2-week period. After a check for mating, females were weighed on days 0, 7, 14 and 20 of gestation. Following delivery, dams were weighed on days 0, 7 and 14. Litters were culled to eight pups, if possible, on day 4 of lactation. The resulting offspring were held for a 14-day lactation period.

Necropsies were not conducted on any animals except to verify pregnancy of any females dying during the study.

There were no treatment-related changes in clinical signs, body weight or feed consumption. Two females in the 20 000 ppm group were euthanized when they showed poor condition at parturition and before terminal kill. Fetuses were observed in utero in these rats. As similar deaths were observed at all dose levels, including controls in a subsequent two-generation study, these deaths were considered not to be due to treatment.

Reproductive parameters, including mating, fertility and gestation indices, in the treated groups were comparable with those in the control group. No external malformations in offspring were observed. No effects on the number of stillborn pups, sex ratio, pup survival or physical condition of the pups during lactation were observed. Mean body weights of pups were comparable between the control and treated groups during the 14-day lactation period.

The NOAELs for maternal, reproductive and offspring toxicity in the single-generation reproductive toxicity study in rats were 20 000 ppm (equal to 1327 mg/kg bw per day), the highest dose tested (O'Meara, Serrone & Douglass, 1996).

In a two-generation reproductive toxicity study, cyazofamid (lot no. 9506; purity 95.5%) was administered in the diet to 30 Sprague-Dawley rats of each sex per group at a concentration of 0, 200, 2000 or 20 000 ppm. The parental animals of each generation were treated continuously throughout the growth, mating, gestation, lactation and resting phases of the study, and the offspring were exposed to the test diets throughout the lactation period. The growth period for the F_0 parental animals was 10 weeks. The animals were then mated for 2 weeks to produce the F_{1a} litters. After the F_{1a} litters were weaned, the parents were given a rest period of at least 1 week prior to mating for the F_{1b} litters. Selection for the F_2 generation was made from the weaned F_{1a} litters. The growth period for the F_{1a} generation was 12 weeks. The F_{1a} parental animals then went through a reproduction sequence similar to that for the F_0 animals to produce the F_{2a} and F_{2b} litters. On day 4 of lactation, each litter size was adjusted to eight pups. All animals, including pups, were monitored at least twice daily for mortality and clinical signs of toxicity. For parental animals, physical examinations, body weights and feed consumption were recorded at regular intervals. F_0 and F_1 parental males were necropsied following mating for the F_{1b} or F_{2b} litters, respectively. Females were necropsied on day 21 of the F_{1b} or F_{2b} lactation period. Representative organs and tissues fixed from 10 rats of each sex per group were examined microscopically from the control and high-dose animals.

During the reproductive phase, evidence of mating, date of delivery, number, weight and sex of pups, and any abnormalities observed in the offspring were recorded. Number, weight and sex of pups were recorded at regular intervals during lactation. All animals on study were necropsied. A complete set of tissues (above) was collected and preserved from 10 F_{2a} males and 10 F_{2a} females from each group at weaning. No histopathology was conducted on these tissues.

Average chemical intakes in the F_0 and F_1 generations are shown in Table 14.

Table 14. Chemical intake in two-generation reproductive toxicity study in rats

	Dose (mg/kg bw per day)		
Generation	**200 ppm**	**2000 ppm**	**20 000 ppm**
F_0 males	9.5	94.2	958
F_0 females	13.4	134	1 340
F_1 males	8.9	89.2	936
F_1 females	13.7	138	1 400

bw: body weight; F_0: first parental generation; F_1: second parental generation; ppm: parts per million
Source: O'Meara & Watson (1998)

In parental animals, there were no treatment-related effects on mortality, clinical observations, feed consumption or gross necropsy observations for either the F_0 or F_1 animals. Microscopically, there were no findings in tissues examined that were considered to be related to treatment. During the growth phase, body weights of treated F_0 and F_1 males were comparable with those of the control group animals.

Body weights for the F_0 females and the low- and mid-dose F_1 females were comparable with those of the control group animals. The mean body weights for the F_1 females at 20 000 ppm were slightly, but statistically significantly, lower after weaning up to mating compared with the mean weights in the control group (Table 15). The change in body weight observed just after weaning in the F_1 generation was considered to be a slight effect on offspring. During the gestation and lactation periods, body weights in F_0 and F_1 females at 20 000 ppm were slightly, but statistically significantly, depressed at many time points (Table 16). Findings similar to those seen in the F_0 females were noted in the F_1 females – that is, the mean body weights for the F_1 females at 20 000 ppm were slightly, but statistically significantly, lower during pregnancy/lactation compared with the control mean weights (Table 16). These continuous body weight decreases in F_0 and F_1 females during the gestation and lactation periods were considered to be a treatment-related effect on the parent. During the growth phase, the body weights of treated F_0 and F_1 males were similar to those of the control group animals. Body weights of the F_0 females and the low- and mid-dose F_1 females were comparable with the mean body weight of the control group.

In reproductive parameters, mating and fertility indices and gestation length were not affected by treatment with cyazofamid. No malformations were increased by the treatment in either generation. No effects on the pup sex ratio, pup survival or physical condition of the pups during lactation were observed. The body weights of pups on day 21 in all generations at 20 000 ppm were slightly (approximately 10%), but statistically significantly, lower than those in the control group (Table 17). This common change in all generations was considered to be a treatment-related adverse effect. The number of stillborn pups showed a slightly increased tendency at 20 000 ppm through the F_{1a}, F_{1b} and F_{2a} generations. This difference from control was not apparent for the F_{2b} generation. The survivability of pups in the treated groups from days 1 to 21 was comparable with that of the control group for all litters. Therefore, these changes were not considered to be a biologically significant effect. Although the numbers of pups and litters with dilated pelvis were increased in the F_{1a} generation at 20 000 ppm, the number in other generations was varied, indicating that the increase was not toxicologically significant. No findings that were considered to be treatment related were observed at the necropsy of the pups.

In this two-generation reproductive toxicity study in rats, the NOAEL for parental toxicity was 2000 ppm (equal to 134 mg/kg bw per day), based on reduced body weights in F_0 females at 20 000 ppm (equal to 1340 mg/kg bw per day). The NOAEL for reproductive toxicity was 20 000 ppm (equal to 936 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 2000 ppm (equal to 138 mg/kg bw per day), based on reduced body weights in F_1 females at 20 000 ppm (equal to 1400 mg/kg bw per day) (O'Meara & Watson, 1998).

(b) Developmental toxicity

Rats

Cyazofamid (lot no. 9506; purity 95.5%) was administered by gavage to six pregnant female Sprague-Dawley rats per group at 0, 20, 100, 500 or 1000 mg/kg bw per day during gestation days (GDs) 0–19. Animals were checked twice daily for mortality and for obvious signs of toxicity. They were also subjected to a detailed physical examination on days 0–20 of gestation. Body weights and feed consumption were measured on days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation. All females were necropsied on GD 20, and gravid uterine weights, number of corpora lutea, number of implantation sites, number of live fetuses and number of resorptions were recorded. Each fetus was weighed, sexed and individually identified. A gross external examination for defects, including observations of the palate, and for external sex determination was performed on each fetus. All fetuses were sacrificed and discarded.

Table 15. F_1 female body weights during non-reproduction periods

Dietary concentration (ppm)	Body weight (g)														
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 21	Week 22
0	72.4	107.7	144.4	167.9	192.7	213.9	228.8	241.8	257.6	264.6	267.2	273.4	279.9	313.0	313.1
200	70.8	106.4	142.2	167.0	190.4	211.5	228.7	240.0	255.0	265.0	267.8	275.6	281.7	312.7	312.6
2 000	71.5	108.0	145.9	172.7	197.2	219.3	233.9	245.1	258.7	269.1	272.6	280.9	286.1	313.6	315.2
20 000	66.2*	100.1*	135.6**	160.6	183.2*	203.5*	220.8	231.7	244.4*	252.9	257.5	263.5	269.0	294.8*	296.0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: O'Meara & Watson (1998)

Table 16. Female body weights during gestation and lactation in two-generation reproductive toxicity study in rats

Dietary concentration (ppm)	Body weight (g)							
	Gestation				Lactation			
	Day 0	Day 7	Day 14	Day 20	Day 0	Day 7	Day 14	Day 21
F_0 females for F_{1a} litters								
0	259.0	289.2	319.7	386.1	302.4	310.0	323.0	309.1
200	261.6	294.5	323.7	383.1	301.0	313.8	323.0	304.1
2 000	251.8	281.5	313.6	370.7	296.1	305.6	317.1	306.7
20 000	251.9	280.9	308.3	371.6	286.8	298.8	309.4	296.9
F_0 females for F_{1b} litters								
0	300.4	330.8	362.1	425.5	336.2	344.9	353.6	334.2
200	302.6	333.9	359.9	425.6	338.4	344.2	349.2	328.1
2 000	296.5	323.4	349.5	418.0	334.2	342.9	346.0	330.5
20 000	287.8	310.0*	338.4*	407.2	317.7	330.6	336.7*	325.7

Dietary concentration (ppm)	Body weight (g)							
	Gestation				Lactation			
	Day 0	Day 7	Day 14	Day 20	Day 0	Day 7	Day 14	Day 21
F_{1a} females for F_{2a} litters								
0	275.3	306.3	332.1	390.3	309.8	323.1	333.0	315.6
200	274.6	304.1	332.7	393.9	316.0	330.1	334.5	318.6
2 000	279.9	306.1	332.9	395.4	312.2	324.8	337.0	320.0
20 000	260.5*	286.7*	313.6*	375.0	291.5*	305.0**	312.1**	302.6
F_{1a} females for F_{2b} litters								
0	310.2	336.7	372.0	439.6	351.6	357.7	361.8	337.2
200	307.1	334.9	369.1	439.4	349.7	355.5	356.0	335.0
2 000	307.4	330.5	362.3	429.3	347.4	348.5	356.8	339.1
20 000	294.7*	318.5*	351.5	416.7	333.4	335.6**	342.3*	327.1

F_1: first filial generation; F_2: second filial generation; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: O'Meara & Watson (1998)

Table 17. Body weight of pups in the two-generation reproductive toxicity study in rats

Day	Body weight (g)															
	F_{1a}				F_{1b}				F_{2a}				F_{2b}			
	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm
0	6.2	6.5	6.6*	6.3	6.3	6.3	6.4	6.4	6.1	6.5*	6.3	6.2	6.2	6.4	6.1	6.3
7	16.2	17.3	17.3	15.8	16.8	17.2	17.0	16.2	16.2	17.1	16.3	15.8	16.7	17.5	16.5	16.5
21	52.1	53.5	54.1	48.2*	54.1	52.5	54.5	49.4*	52.9	55.1	52.5	49.3*	55.1	56.5	54.3	31.5*

F_1: first filial generation; F_2: second filial generation; ppm: parts per million; *: $P < 0.05$

Source: O'Meara & Watson (1998)

No treatment-related clinical signs were observed. One dam at 100 mg/kg bw per day showing lower body weight and feed consumption was noted to be emaciated and had laboured breathing from GDs 15 to 20. Macroscopic findings of the dam, such as red discoloration of the lungs and a fluid-filled thoracic cavity with adhesions of thoracic organs, suggested an administration error. Similar abnormalities were not observed in other dams at the same dose. Therefore, the abnormalities found in the dam at 100 mg/kg bw per day were not considered to be related to the chemical treatment. The mean number of corpora lutea, uterine implantation sites, live fetuses and resorptions per pregnant female were not affected by the treatment at any dose.

Cyazofamid had no effect on fetal weights except for the decreased weights of fetuses whose dam was affected by an administration error. The decrease was a secondary effect on the dam and was not treatment related. The sex distribution of the fetuses was comparable between the control and treatment groups. No malformations or variations were noted during the external examinations. No external malformations or variations were seen in the control group fetuses.

The NOAELs for maternal and embryo/fetal toxicity in rats were 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1999a).

Rabbits

Cyazofamid (lot no. 9506; purity 95.5%) was administered to six mature, time-mated female New Zealand White rabbits per group by gavage at 0, 20, 100, 500 or 1000 mg/kg bw per day during GDs 4–28. Does were checked twice daily for mortality and for obvious signs of toxicity. They were also subjected to a detailed physical examination daily from receipt through GD 29. Body weights and feed consumption were measured on GDs 0, 4, 6, 9, 12, 15, 18, 21, 24, 27 and 29. All does were necropsied on GD 29, and gravid uterine weights were recorded. The number of corpora lutea was counted, and implantation parameters were examined. A gross external examination for defects, including observations of the palate, was performed on each fetus.

No treatment-related mortality occurred in the course of the study. One female at 1000 mg/kg bw per day died on GD 9 due to an intubation error, but all other animals survived to termination. No treatment-related abnormalities or changes in body weight or feed consumption were noted. At necropsy, there were no treatment-related changes in maternal macroscopic postmortem findings. White fluid in the trachea and white discoloured lungs were observed in the animal that died following dosing on GD 9. No treatment-related effects on uterine implantation parameters, including the numbers of corpora lutea, implantation sites, preimplantation and postimplantation losses, live and dead fetuses, and early and late resorptions, were noted in any treated group. In the findings on fetuses, no effects of treatment with cyazofamid on fetal weight, malformations or variations were observed.

The NOAELs for maternal and embryo/fetal toxicity in rabbits were 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1999b).

2.6 Special studies

(a) Acute neurotoxicity

Cyazofamid (lot no. 9507; purity 95.7%) was administered by single dose gavage to 10 young Sprague-Dawley rats of each sex per group at 0, 80, 400 or 2000 mg/kg bw. After the treatment, mortality and morbidity were checked once a day. The neurobehavioural assessment, such as a functional observational battery and a motor activity test, was conducted before initiation of treatment and approximately 30–60 minutes, 7 days and 14 days after dosing. Fourteen days after dosing, the surviving animals were necropsied. After necropsy, six rats of each sex per group were fixed by perfusion for neuropathological examination.

No treatment-related changes in mortality, body weight, feed consumption, cage-side observations, parameters of neurobehavioural assessment, or macroscopic and microscopic neuropathological analysis were observed at any time point examined.

The NOAEL for acute neurotoxicity in rats was 2000 mg/kg bw, the highest dose tested (Ridder, 2000).

(b) Subchronic neurotoxicity

Cyazofamid (lot no. 9801-2; purity 94.9%) was administered to 12 Sprague-Dawley rats of each sex per group for 13 weeks at a dietary concentration of 0, 500, 2000 or 20 000 ppm (equal to 0, 34, 134 and 1356 mg/kg bw per day for males and 0, 39, 156 and 1539 mg/kg bw per day for females, respectively). All animals were observed twice daily for mortality and moribundity. Detailed physical examinations, body weights and feed consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for all animals during pretreatment and at weeks 3, 7 and 12. The functional observational battery, including handling, open-field, sensory, neuromuscular and physiological observations, was conducted on all animals. At the termination of the study, all animals were necropsied and fixed with buffered 4.0% paraformaldehyde solution. Fixed brain weight and brain dimensions (length [excluding olfactory bulbs] and width) were recorded. Neuropathological examination was performed using six selected rats of each sex in the control and 20 000 ppm groups.

There were no treatment-related changes in mortality or in handling, open-field, sensory, neuromuscular and physiological observations in the functional observational battery or macroscopic and microscopic findings. Males in the 20 000 ppm group showed slightly, but statistically significantly, lower (approximately 15% lower than that in the control group) body weight gain in the first week of treatment. The mean body weight gain in males at 20 000 ppm before treatment was significantly higher (3.4%) than that in the control group. Feed consumption was not affected during the first week of treatment. Therefore, the decrease was considered to be a treatment-related adverse effect, but it was not a neurotoxicity indicator. At 10 weeks, the body weight gain in females at 20 000 ppm was slightly (9 g at 0 ppm and 2 g at 20 000 ppm) lower, without a change in feed consumption. The transient change was not considered to be treatment related, but was an incidental effect. The only statistically significant difference in locomotor activity between the control and test substance–exposed groups during the exposure period when the six subintervals were evaluated was a lower mean ambulatory count in females at 20 000 ppm at week 12. The mean overall ambulatory count was not affected. No other changes indicating neurotoxicity were observed. Therefore, this transient difference was considered to be incidental.

The NOAEL for general toxicity was 2000 ppm (equal to 156 mg/kg bw per day), based on slightly lower body weight gain during the early phase of treatment at 20 000 ppm (equal to 1356 mg/kg bw per day). The NOAEL for subchronic neurotoxicity in rats was 20 000 ppm (equal to 1356 mg/kg bw per day), the highest dose tested (Toot, 2012).

(c) Immunotoxicity

Seven-week-old female CD-1 mice (10 mice per group) were administered cyazofamid (lot no. 9506; purity 95.0%) in the diet at 0, 600, 3000 or 6000 ppm (equal to 0, 136, 599 and 1381 mg/kg bw per day, respectively) for 28 days. All animals were immunized with an intravenous injection of sheep red blood cells on day 24. The animals in the positive control group were administered an intraperitoneal injection of cyclophosphamide monohydrate (50 mg/kg bw per day) once daily for 4 consecutive days (study days 24–27). All animals were checked for mortality, moribundity and clinical signs. Detailed physical examinations were performed approximately weekly. Body weights and feed consumption were recorded approximately twice weekly. Blood samples for possible immunoglobulin M (IgM) antibody analysis were withdrawn from all animals at termination (on day 28). After necropsy, thymus and spleen weights were measured. The thymus was fixed for possible future microscopic examination, and the spleen was subjected to immunotoxicological evaluation, the IgM antibody-forming cell (AFC) assay.

All animals survived to termination. There were no treatment-related clinical signs or findings. Although occasional slightly, but statistically significantly, lower mean body weight gains were noted

in the 3000 and 6000 ppm groups, these differences were not considered to be treatment related owing to the lack of a dose–response relationship. No abnormalities in macroscopic findings or changes in thymus weights or spleen weights were observed in the cyazofamid-treated groups.

In the AFC assay, cyazofamid treatment showed no significant suppression of the humoral immune response. Statistically significantly lower spleen cell numbers, mean specific activity and mean total spleen activity values were noted in the positive control group.

The NOAEL for immunotoxicity in mice was 6000 ppm (equal to 1381 mg/kg bw per day), the highest dose tested (Wasil, 2012).

(d) Studies on metabolites

Data on the acute toxicity in rats and the in vitro genotoxicity of CCIM (a first intermediate metabolite in rats, metabolite in goat muscle and fat and plants), CCIM-AM (large animals, plants), CCBA (a major urinary metabolite in rats) and CTCA (plants and soil) were provided.

CCIM

Acute toxicity. CCIM (lot no. 9809; purity 99.0%), a major intermediate metabolite of cyazofamid in animals, was administered to Sprague-Dawley rats (five rats of each sex per group) at 100, 160, 256, 410 or 656 mg/kg bw by gavage. All rats were monitored for toxic signs 30 minutes and 1, 3 and 6 hours after dosing and daily for 14 days. Body weights were recorded weekly. Each animal was subjected to necropsy either immediately after being found dead or 14 days after treatment. LD_{50} values were calculated from the mortality rates.

Deaths occurred 3 hours after test material administration in males and from 3 hours to 1 day in females. A summary of their mortality is shown in Table 18. No animals died at 100 or 160 mg/kg bw. Clinical signs included a decrease in spontaneous motor activity, prone position and sedation, tremors, ptosis and salivation. The prone position was observed at all dose levels, and the incidences of clinical signs were increased in a dose-dependent manner (Table 19). For animals found dead during the study, the most predominant necropsy observations included red-coloured lungs and digestive tracts and oedema in the lungs and small intestines. All surviving animals gained weight throughout the study, and the body weights in the treated groups were comparable with those in the control group. There were no abnormalities noted in animals surviving to study termination.

All surviving animals gained weight throughout the study. The LD_{50}s for CCIM in rats were 324 mg/kg bw in males and 443 mg/kg bw in females (Shutoh, 1999a).

Genotoxicity. An in vitro genotoxicity study of CCIM (lot no. 9809; purity 99.0%) was conducted. Reverse mutation tests were performed in *Escherichia coli* WP2 uvrA and four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537). The treatment doses were 20, 39, 78, 156, 313, 625 and 1250 μg/plate, with and without metabolic activation (S9 mix). After incubation in the presence or absence of S9 mix, the number of revertant colonies was counted.

The mean number of revertant colonies in plates treated with CCIM was similar to that of the corresponding solvent control in all strains, with or without S9 mix. Toxicity to the tester strains was observed at 625 μg/plate and higher in the TA strains and at 1250 μg/plate in the WP2 strain. The mean number of revertant colonies in positive controls was at least 3-fold higher than the solvent control numbers, indicating that CCIM was non-mutagenic to bacteria under the conditions of this study.

CCIM was concluded to be non-mutagenic in vitro (Matsumoto, 1999a).

Table 18. Mortality in rats treated with a single dose of CCIM

	Mortality													
	Males							Females						
Dose (mg/kg bw)	**30 min**	**1 h**	**3 h**	**6 h**	**Day 1**	**Days 2–14**	**Final mortality**	**30 min**	**1 h**	**3 h**	**6 h**	**Day 1**	**Days 2–14**	**Final mortality**
100	0	0	0	0	0	0	0/5	0	0	0	0	0	0	0/5
160	0	0	0	0	0	0	0/5	0	0	0	0	0	0	0/5
256	0	0	3	0	0	0	3/5	0	0	1	0	0	0	1/5
410	0	0	2	0	0	0	2/5	0	0	2	0	0	0	2/5
656	0	0	5	0	0	0	5/5	0	0	3	0	1	0	4/5

bw: body weight
Source: Shutoh (1999a)

Table 19. Summary of clinical signs in rats treated with a single dose of CCIM

	100 mg/kg bw		160 mg/kg bw		256 mg/kg bw		410 mg/kg bw		656 mg/kg bw	
Clinical signs	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**
Males										
Number of rats	0	5	0	5	3	2	2	3	5	0
No abnormalities detected	–	3	–	1	0	1	1	0	0	–
Prone position	–	2	–	4	3	0	1	3	2	–
Decreased spontaneous motor activity	–	0	–	0	0	1	0	2	0	–
Crawling	–	0	–	1	3	0	0	1	2	–
Deep respiration	–	0	–	0	1	0	0	1	0	–
Sedation	–	0	–	4	2	1	0	3	5	–
Tremor	–	0	–	0	0	0	0	1	0	–
Ptosis	–	0	–	0	0	1	0	3	0	–

	100 mg/kg bw		160 mg/kg bw		256 mg/kg bw		410 mg/kg bw		656 mg/kg bw	
Clinical signs	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**	**Fd**	**Tk**
Salivation	–	0	–	0	1	0	0	0	0	–
Soiled fur in the nasorostal region	–	0	–	0	0	0	0	1	0	–
Females										
Number of rats	0	5	0	5	1	4	2	3	4	1
No abnormalities detected	–	4	–	1	0	1	0	0	0	0
Prone position	–	1	–	4	1	3	2	2	3	0
Decreased spontaneous motor activity	–	0	–	1	0	2	1	3	1	0
Crawling	–	0	–	1	1	1	1	1	0	0
Deep respiration	–	0	–	0	1	0	1	2	0	0
Sedation	–	0	–	3	1	2	1	3	4	0
Tremor	–	0	–	0	1	0	0	0	0	1
Ptosis	–	0	–	0	1	0	1	2	1	0
Salivation	–	0	–	0	1	0	1	0	0	0
Soiled fur in the nasorostal region	–	0	–	0	0	0	0	1	0	0
Hunched position	–	0	–	0	0	0	0	0	1	1

bw: body weight; Fd: found dead; Tk: terminal kill
Source: Shutoh (1999a)

CCIM-AM

Acute toxicity. CCIM-AM (lot no. 9809; purity 99.6%) was administered to Sprague-Dawley rats (five of each sex per group) at 3000 mg/kg bw by gavage. All rats were monitored for toxic signs 30 minutes and 1, 3 and 6 hours after dosing and daily for 14 days. Body weights were recorded weekly. Each animal was subjected to necropsy either immediately after being found dead or 14 days after treatment. LD_{50} values were calculated from the mortality rates.

No deaths or clinical signs were observed. The treatment did not affect body weight or macroscopic examination. The LD_{50}s for CCIM-AM in both sexes were greater than 3000 mg/kg bw (Shutoh, 1999b).

Genotoxicity. An in vitro genotoxicity study of CCIM-AM (lot no. 9809; purity 99.6%) was conducted. Reverse mutation tests were performed in *Escherichia coli* WP2 uvrA and four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537). The treatment doses were 78, 156, 313, 625 and 1250 µg/plate, with and without metabolic activation (S9 mix). After incubation in the presence or absence of S9 mix, the number of revertant colonies was counted.

The mean number of revertant colonies in plates treated with CCIM-AM was similar to that of the corresponding solvent control in all strains, with or without S9 mix. Toxicity to the tester strains was not observed. A 2-fold or greater increase in the mean number of revertant colonies compared with the solvent control was not observed in any strain at any dose of CCIM-AM in the reverse mutation tests, with and without metabolic activation, indicating that CCIM-AM was non-mutagenic to bacteria under the conditions of this study.

CCIM-AM was concluded to be non-mutagenic in vitro (Matsumoto, 1999b).

CTCA

Acute toxicity. CTCA (lot no. 9809; purity 99.7%) was administered to Crj:CD rats (five of each sex per group) at 763, 1221, 1953, 3125 or 5000 mg/kg bw by gavage. All rats were monitored for toxic signs 30 minutes and 1, 3 and 6 hours after dosing and daily for 14 days. Body weights were recorded weekly. Each animal was subjected to necropsy either immediately after being found dead or 14 days after treatment. LD_{50} values were calculated from the mortality rates.

Mortalities in the 763, 1221, 1953, 3125 and 5000 mg/kg bw groups were 0/5, 0/5, 0/5, 4/5 and 4/5 in males and 0/5, 1/5, 2/5, 5/5 and 5/5 in females, respectively. The deaths occurred from day 3 to day 8 after administration in males and from day 1 to day 7 in females. Various clinical signs, such as emaciation, hunchback position, decreases in or loss of spontaneous motor activity, bradypnoea, sedation, stupor, hypothermy, ptosis, soiled fur in the naso-orbital region, and soiled fur in the perianal region, were observed in many animals at dose levels of 1953 mg/kg bw and above in males and 1221 mg/kg bw and above in females. These signs began to appear from 3 hours after administration and disappeared by day 11. The body weights of survivors were reduced in one male and two females on day 7. At necropsy of surviving animals, smaller sizes of the thymus and testes were noted.

Based on the mortalities observed, the LD_{50}s for CTCA were considered to be 2947 and 1963 mg/kg bw for males and females, respectively (Shutoh, 1999c).

Genotoxicity. An in vitro genotoxicity study of CTCA (lot no. 9809; purity 99.6%) was conducted. Reverse mutation tests were performed in *Escherichia coli* WP2 uvrA and four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537). The treatment doses were 78, 156, 313, 625, 1250, 2500 and 5000 µg/plate, with and without metabolic activation (S9 mix). After incubation in the presence or absence of S9 mix, the number of revertant colonies was counted.

The mean number of revertant colonies in plates treated with CTCA was similar to that of the corresponding solvent control in all strains, with or without S9 mix. Toxicity to the tester strains was not observed. A 2-fold or greater increase in the mean number of revertant colonies compared with

the solvent control was not observed in any strain at any dose of CTCA in the reverse mutation tests, with and without metabolic activation, indicating that CTCA was non-mutagenic to bacteria under the conditions of this study.

CTCA was concluded to be non-mutagenic in vitro (Matsumoto, 1999c).

Dimethylsulfamic acid

Acute toxicity. Dimethylsulfamic acid (a predictive metabolite produced during the pathway from cyazofamid to the first metabolite CCIM in animals, plants, soil and water; lot no. 9902; purity 99.0%) was administered at 1000, 1600, 2560, 4096 or 6554 mg/kg bw by gavage to Crj:CD rats (five of each sex per group). All rats were monitored for toxic signs 30 minutes and 1, 3 and 6 hours after dosing and daily for 14 days. Body weights were recorded weekly. Each animal was subjected to necropsy either immediately after being found dead or 14 days after treatment. LD_{50} values were calculated from the mortality rates.

Mortalities in the 1000, 1600, 2560, 4096 and 6554 mg/kg bw groups were 0/5, 0/5, 0/5, 5/5 and 5/5 for males and 0/5, 0/5, 1/5, 5/5 and 5/5 for females, respectively. The animals died 1 hour after administration in males and from 1 hour after administration to day 2 in females. Many animals of both sexes showed clinical signs suggesting general poor condition, such as prone position, decrease in or loss of spontaneous motor activity and sedation. Bradypnoea, stupor, hypothermy, lacrimation and ptosis were also noted in the rats killed when moribund. All survivors gained weight on days 7 and 14, showing recovery. No macroscopic abnormalities were noted in surviving animals at terminal kill.

Based on the mortalities observed, the LD_{50}s for dimethylsulfamic acid were considered to be 3238 and 2948 mg/kg bw for males and females, respectively (Shutoh, 1999d).

Genotoxicity. An in vitro genotoxicity study of dimethylsulfamic acid (lot no. 9809; purity 99.6%) was conducted. Reverse mutation tests were performed in *Escherichia coli* WP2 uvrA and four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537). The treatment doses were 313, 625, 1250, 2500 and 5000 μg/plate, with and without metabolic activation (S9 mix). After incubation in the presence or absence of S9 mix, the number of revertant colonies was counted.

The mean number of revertant colonies in plates treated with dimethylsulfamic acid was similar to that of the corresponding solvent control in all strains, with or without S9 mix. Toxicity to the tester strains was not observed. A 2-fold or greater increase in the mean number of revertant colonies compared with the solvent control was not observed in any strain at any dose of dimethylsulfamic acid in the reverse mutation tests with and without metabolic activation, indicating that dimethylsulfamic acid was non-mutagenic to bacteria under the conditions of this study.

Dimethylsulfamic acid was concluded to be non-mutagenic in vitro (Matsumoto, 1999d).

3. Observations in humans

Based on information from manufacturing plants, as well as a review of published literature, no reported incidents of cyazofamid poisoning in humans have been found. No epidemiological studies involving cyazofamid were found in a review of published literature.

Comments

Biochemical aspects

Cyazofamid was dose-dependently absorbed in rats: up to 84% at a low dose (0.5 mg/kg bw) and up to 6% at a high dose (1000 mg/kg bw). Peak plasma and tissue concentrations of radiolabelled cyazofamid were achieved within 1 hour after oral administration at the low and high doses. The

radioactive dose was distributed primarily to the kidney and liver at the low dose and was more widely distributed at the high dose. The metabolic pathway of cyazofamid is rapid hydrolysis to form dimethylsulfamic acid and 4-chloro-5-*p*-tolylimidazole-2-carbonitrile (CCIM). CCIM is then either oxidized at the benzonyl methyl group, resulting in 4-(4-chloro-2-cyanoimidazole-5-yl) benzoic acid (CCBA), the major urinary metabolite, or conjugated with glutathione and further metabolized to form CH_3-SO-CCIM and CH_3SO_2-CCIM, which are also excreted in the urine. Cyazofamid and its metabolites are rapidly excreted in urine at the low dose: greater than 90% excretion within 24 hours (Huhtanen & Savides, 1998a,b,c; McFadden, Yoshida & Huhtanen, 1999; Murray & Savides, 1999).

Toxicological data

The oral LD_{50} for cyazofamid was greater than 5000 mg/kg bw in mice and rats. The dermal LD_{50} was greater than 2000 mg/kg bw in rats (Yoshida, Brock & Watson, 1998a,b; Yoshida & Watson, 1999). The inhalation LC_{50} was greater than 5.5 mg/L in rats (Ulrich, 1998). Cyazofamid was slightly irritating to the skin and eyes of rabbits. Cyazofamid was not sensitizing in the guinea-pig maximization test (Yoshida, Brock & Watson, 1998c,d,e).

The kidney was the main target organ of cyazofamid toxicity in short- and long-term studies in rats. Cyazofamid at higher doses also decreased body weight gain in rats.

In a 6-week toxicity study in mice administered cyazofamid in the diet at a concentration of 0, 40, 200, 1000, 3500 or 7000 ppm (equal to 0, 8, 38, 193, 653 and 1419 mg/kg bw per day for males and 0, 9, 47, 248, 854 and 1796 mg/kg bw per day for females, respectively), the NOAEL was 7000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested (O'Meara, Serrone & Lorence, 1996).

In a 90-day toxicity study in rats administered cyazofamid in the diet at a concentration of 0, 10, 50, 500 or 5000 ppm (equal to 0, 0.597, 2.91, 29.5 and 295 mg/kg bw per day, respectively) for males and 0, 50, 500, 5000 or 20 000 ppm (equal to 0, 3.30, 33.3, 338 and 1360 mg/kg bw per day, respectively) for females, the NOAEL was 500 ppm (equal to 29.5 mg/kg bw per day), based on effects on the kidney (i.e. basophilic tubules, increased urinary protein and pH) in males at 5000 ppm (equal to 295 mg/kg bw per day) (Nakashima, 1999b).

In a 90-day toxicity study in dogs administered cyazofamid by capsule at 0, 40, 200 or 1000 mg/kg bw per day (both sexes), the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Savides, Lucas & Watson, 1998).

In a 1-year toxicity study in dogs administered cyazofamid by capsule at 0, 40, 200 or 1000 mg/kg bw per day (both sexes), the NOAEL was 200 mg/kg bw per day, based on decreased spleen weight in both sexes and atrophy of the thymus in females at 1000 mg/kg bw (Savides & Watson, 1999).

The Meeting concluded that the overall NOAEL for oral toxicity in dogs was 200 mg/kg bw per day, and the overall LOAEL was 1000 mg/kg bw per day.

In an 18-month toxicity and carcinogenicity study in mice administered cyazofamid in the diet at a concentration of 0, 70, 700 or 7000 ppm (equal to 0, 9.5, 94.8 and 985 mg/kg bw per day for males and 0, 12.2, 124 and 1203 mg/kg bw per day for females, respectively), the NOAEL was 7000 ppm (equal to 985 mg/kg bw per day), the highest dose tested. No treatment-related tumours were observed in this study (O'Meara & Watson, 1999).

In a 2-year study of toxicity and carcinogenicity in rats administered cyazofamid in the diet at a concentration of 0, 10, 50, 500 or 5000 ppm (equal to 0, 0.336, 1.68, 17.1 and 171 mg/kg bw per day, respectively) for males and 0, 50, 500, 5000 or 20 000 ppm (equal to 0, 2.01, 20.2, 208 and 856 mg/kg bw per day, respectively) for females, the NOAEL was 500 ppm (equal to 17.1 mg/kg bw per day), based on kidney effects (i.e. increases in blood urea nitrogen and urinary volume) in males at 5000 ppm (equal to 171 mg/kg bw per day). No treatment-related tumours were observed in this study (Nakashima, 1999c).

The Meeting concluded that cyazofamid is not carcinogenic in mice or rats.

Cyazofamid was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found (Akanuma, 1998; Akhurst, 1998; Kirkpatrick, 1998; Kitching, 1998; Proudlock & Dawe, 1998).

The Meeting concluded that cyazofamid is unlikely to be genotoxic.

On the basis of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that cyazofamid is unlikely to pose a carcinogenic risk to humans.

In a single-generation reproductive toxicity study in rats administered cyazofamid in the diet at a concentration of 0, 1000, 3000, 7000 or 20 000 ppm (equal to 0, 66.5, 200, 450 and 1327 mg/kg bw per day for males and 0, 77.5, 252, 562 and 1613 mg/kg bw per day for females, respectively), the NOAELs for maternal, reproductive and offspring toxicity were 20 000 ppm (equal to 1327 mg/kg bw per day), the highest dose tested (O'Meara, Serrone & Douglass, 1996).

In a two-generation reproductive toxicity study in rats administered cyazofamid in the diet at a concentration of 0, 200, 2000 or 20 000 ppm (equal to 0, 9.5, 94.2 and 958 mg/kg bw per day for F_0 males; 0, 13.4, 134 and 1340 mg/kg bw per day for F_0 females; 0, 8.9, 89.2 and 936 mg/kg bw per day for F_1 males; and 0, 13.7, 138 and 1400 mg/kg bw per day for F_1 females, respectively), the NOAEL for parental toxicity was 2000 ppm (equal to 134 mg/kg bw per day), based on reduced body weights in F_0 females at 20 000 ppm (equal to 1340 mg/kg bw per day). The NOAEL for reproductive toxicity was 20 000 ppm (equal to 936 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 2000 ppm (equal to 138 mg/kg bw per day), based on reduced body weights in F_1 females at 20 000 ppm (equal to 1400 mg/kg bw per day) (O'Meara & Watson, 1998).

In a developmental toxicity study in rats administered cyazofamid by gavage at a dose of 0, 20, 100, 500 or 1000 mg/kg bw per day, the NOAELs for maternal and embryo/fetal toxicity in rats were 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1999a).

In a developmental toxicity study in rabbits administered cyazofamid by gavage at a dose of 0, 20, 100, 500 or 1000 mg/kg bw per day, the NOAELs for maternal and embryo/fetal toxicity in rabbits were 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1999b).

The Meeting concluded that cyazofamid is not teratogenic.

In an acute neurotoxicity study in rats administered cyazofamid by a single gavage dose at 0, 80, 400 or 2000 mg/kg bw, the NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested (Ridder, 2000).

In a 90-day neurotoxicity study in rats administered cyazofamid in the diet at a concentration of 0, 500, 2000 or 20 000 ppm (equal to 0, 34, 134 and 1356 mg/kg bw per day for males and 0, 39, 156 and 1539 mg/kg bw per day for females, respectively), the NOAEL for subchronic neurotoxicity was 20 000 ppm (equal to 1356 mg/kg bw per day), the highest dose tested (Toot, 2012).

The Meeting concluded that cyazofamid is not neurotoxic.

In an immunotoxicity study in mice administered cyazofamid in the diet at 0, 600, 3000 or 6000 ppm (equal to 0, 136, 599 and 1381 mg/kg bw per day, respectively), the NOAEL for immunotoxicity was 6000 ppm (equal to 1381 mg/kg bw per day), the highest dose tested (Wasil, 2012).

The Meeting concluded that cyazofamid is not immunotoxic.

Biochemical and toxicological data on metabolites and/or degradates

Studies with radiolabelled CCIM demonstrated that this metabolite was more rapidly absorbed than cyazofamid itself (Murray & Savides, 1999).

Acute toxicity studies on CCIM (the first metabolite in rodents and in muscle and fat of large animals such as goats) were conducted in rats. Results of an in vitro genotoxicity test on this compound were also provided.

CCIM was more acutely toxic than the parent, with an oral LD_{50} in rats of 324 mg/kg bw. In this study, no deaths were observed at 100 and 160 mg/kg bw. Clinical signs were seen at all doses, but these were slight and occurred in only some animals at 100 mg/kg bw (Shutoh, 1999a). CCIM did not show evidence of genotoxicity in vitro (Matsumoto, 1999a).

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.2 mg/kg bw on the basis of the NOAEL of 17.1 mg/kg bw per day in a 2-year study in rats, based on kidney effects in males at 171 mg/kg bw per day. A safety factor of 100 was applied.

The ADI also applies to CCIM, as CCIM is quickly formed and as plasma or liver concentrations are quickly decomposed to CCBA, the major urinary metabolite, or are conjugated with glutathione at low doses. The ADI for the sum of cyazofamid and CCIM is expressed as cyazofamid.

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

The Meeting established an ARfD for CCIM of 0.2 mg/kg bw, on the basis of a LOAEL of 100 mg/kg bw for clinical signs identified in an acute toxicity study in rats. A safety factor of 500 was applied, including an additional factor of 5 to account for the use of a LOAEL instead of a NOAEL.

Levels relevant to risk assessment of cyazofamid

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	7 000 ppm, equal to 985 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 985 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	500 ppm, equal to 17.1 mg/kg bw per day	5 000 ppm, equal to 171 mg/kg bw per day
		Carcinogenicity	5 000 ppm, equal to 171 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 000 ppm, equal to 936 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
		Parental toxicity	2 000 ppm, equal to 134 mg/kg bw per day	20 000 ppm, equal to 1 340 mg/kg bw per day
		Offspring toxicity	2 000 ppm, equal to 138 mg/kg bw per day	20 000 ppm, equal to 1 400 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Dog	Thirteen-week and 1-year studies of toxicity[d,e]	Toxicity	200 mg/kg bw per day	1 000 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI) for sum of cyazofamid and CCIM, expressed as cyazofamid

0–0.2 mg/kg bw

Estimate of acute reference dose (ARfD) for cyazofamid

Unnecessary

Estimate of acute reference dose (ARfD) for CCIM

0.2 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 cyazofamid

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly but dose-dependently absorbed (T_{max} < 1 h; absorption < 84% at low dose and < 6% at high dose)
Dermal absorption	No data
Distribution	Liver and kidney at low dose; more widely distributed at high dose
Potential for accumulation	No significant tissue accumulation

Rate and extent of excretion	Rapidly excreted (> 90% within 24 h)
Metabolism in animals	Hydrolysis, oxidation and conjugation with glutathione
Toxicologically significant compounds in animals and plants	Cyazofamid, CCIM
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.5 mg/L
Rabbit, dermal irritation	Slightly irritating to skin
Rabbit, ocular irritation	Slightly irritating to eye
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Kidney/basophilic tubule (rat)
Lowest relevant oral NOAEL	29.5 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 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	Kidney/increased blood urea nitrogen, urine volume and kidney weight (rat)
Lowest relevant NOAEL	17.1 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	Reduced body weight
Lowest relevant parental NOAEL	134 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	138 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	936 mg/kg bw per day (highest dose tested; rat)
Developmental toxicity	
Target/critical effect	No toxic effect
Lowest relevant maternal NOAEL	1 000 mg/kg bw per day (highest dose tested; rat, rabbit)
Lowest relevant embryo/fetal NOAEL	1 000 mg/kg bw per day (highest dose tested; rat, rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	1 356 mg/kg bw (highest dose tested; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 381 mg/kg bw (highest dose tested; mouse)

Studies on toxicologically relevant metabolites	*CCIM* Oral LD_{50}: 324 mg/kg bw (rat) LOAEL: 100 mg/kg bw on the basis of clinical signs (rat) Not genotoxic in vitro
Medical data	
	No adverse effects noted in medical surveillance reports on manufacturing plant personnel

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

Summary

	Value	Study	Safety factor
Cyazofamid			
ADI	0–0.2 mg/kg bw	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	Unnecessary	–	–
CCIM			
ADI	Covered by ADI for parent	–	–
ARfD	0.2 mg/kg bw	Acute toxicity study (rat)	500

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ETHEPHON

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Explanation

Ethephon is the International Organization for Standardization (ISO)–approved common name for 2-chloroethylphosphonic acid (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 16672-87-0. Ethephon is a plant growth regulator that acts by release of ethylene, directly influencing several physiological processes, such as ripening and maturation, and stimulating the production of endogenous ethylene. Ethephon is used on a variety of crops, including fruits, vegetables, cereals and oilseed crops.

Ethephon was previously evaluated for toxicology by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1977, 1978, 1993, 1995, 1997 and 2002. In 1993, the Meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg body weight (bw) on the basis of a no-observed-adverse-effect level (NOAEL) of 0.5 mg/kg bw per day in studies in humans given repeated ethephon doses and application of a 10-fold safety factor. In 2002, the Meeting established an acute reference dose (ARfD) of 0.05 mg/kg bw on the basis of human data.

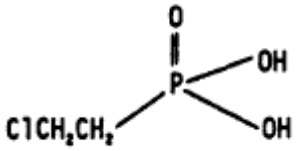
Ethephon was re-evaluated by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues. Both new toxicity studies with ethephon in dogs and with the ethephon metabolite 2-hydroxyethyl phosphonic acid (or 2-hydroxyethephon; HEPA) in rats and previously submitted studies were considered by the present Meeting.

Some of the critical studies do not comply with good laboratory practice (GLP), as the data were generated before the implementation of GLP regulations. Overall, however, the Meeting considered that the database was adequate for the risk assessment.

Available studies with ethephon in humans were performed in accordance with the ethical standards at the time and were compliant with the Declaration of Helsinki.

The chemical structure of ethephon is shown in Fig. 1.

Fig. 1. Structure of ethephon



Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

The absorption, distribution and excretion of [U-^{14}C-ethyl]ethephon (radiochemical purity > 96%; batch no. CFQ.5611) dissolved in physiological saline adjusted to pH 3–4 using lactic acid were studied in groups of five male and five female Crl:CD(SD)BR rats. The experimental procedures are presented in Table 1.

At termination, blood and bone marrow, fat, heart, lungs, skeletal muscle, testes/ovaries, uterus, bone (femur), brain, kidney, liver, spleen and residual carcass were collected from the animals of groups A–D. Expired radioactivity was captured by a mercuric perchlorate and 2-ethoxyethanol:ethanolamine-containing trapping system. Bile was not collected. Clinical signs were observed daily, and body weights were measured at the start and termination of the study. The identification of ethephon and its metabolites in tissues and excreta is described in section 1.2 (Savage, 1990).

Recovery was 84–91%. At 120 hours after a single intravenous dose of 50 mg/kg bw, 67–71%, 0.9–1.7% and 8.9–10.8% of the radioactivity were recovered from urine, faeces and expired air, respectively. At 120 hours after a single oral dose of 50 and 1000 mg/kg bw or after an oral dose of 50 mg/kg bw following 14 oral daily pretreatments with unlabelled ethephon at 50 mg/kg bw, 47–60%, 4.0–6.5% and 18–22% of the radioactivity were recovered from urine, faeces and expired air, respectively. In all studies, radioactivity in expired air was mainly excreted as ethylene (96–98%) and a small fraction (2–4%) as carbon dioxide. Within 24 hours, excretion in urine and through expiration was 70–79% after a dose of 50 mg/kg bw and 62–68% after a dose of 1000 mg/kg bw, indicating that excretion occurred predominantly within the first 24 hours after dose administration. There were no remarkable differences in absorption and excretion between sexes and oral dosing regimens. Tissues and carcass contained only 0.05–0.06% (studies A and B), 0.4–0.5% (study C) or 0.01% (study D) of the administered radioactivity. Highest concentrations were found in bone, liver, blood and kidney. Radioactivity concentrations in brain and bone marrow were low. After single oral doses of 50 and 1000 mg/kg bw, peak blood concentrations were reached after 1.0–1.3 and 1.9–2.5 hours, respectively. Peak blood concentrations at 1000 mg/kg bw (63–66 mg equivalents/kg) were less than

Table 1. Study designs for absorption, distribution and excretion investigations in rats

Study	No. of animals of each sex	Treatment	Sampling times (hours after last dose)	Termination (hours after last dose)
A	5	Single intravenous dose at 50 mg/kg bw [U-^{14}C-ethyl]ethephon	0–4, 4–8, 8–12, 12–16, 16–24, 24–48, 48–72, 72–96 and 96–120: urine, faeces and cage debris 0–4, 4–8, 8–12, 12–16, 16–24, 24–36, 36–48, 48–72, 72–96 and 96–120: volatiles and CO_2 in expired air 120: cage wash 120: blood, selected organs and tissues	120
B	5	Single oral dose at 50 mg/kg bw [U-^{14}C-ethyl]ethephon	See study A	120
C	5	14 daily oral pretreatments of unlabelled ethephon (50 mg/kg bw per day, purity 96.1%) followed by one oral dose of 50 mg/kg bw [U-^{14}C-ethyl]ethephon	See study A	120
D	5	Single oral dose at 1 000 mg/kg bw [U-^{14}C-ethyl]ethephon	See study A	120
E	5	Single oral dose at 50 mg/kg bw [U-^{14}C-ethyl]ethephon	0.5, 1, 2, 3, 4, 6, 12, 24, 48, 72, 96, 120, 144 and 168: blood	168
F	5	Single oral dose at 1 000 mg/kg bw [U-^{14}C-ethyl]ethephon	0.5, 1, 2, 3, 4, 6, 12, 24, 48, 72, 96, 120, 144 and 168: blood	168

Source: Savage (1990)

proportional to dose, compared with peak concentrations at 50 mg/kg bw (10 mg equivalents/kg) (Savage, 1990).

Dogs

The absorption, distribution and excretion of [^{14}C]ethephon (radiochemical purity and batch number unknown) dissolved in a methanolic solution were studied in three male Beagle dogs dosed orally by gavage at a single dose of 180 mg/kg bw. Ethylene and carbon dioxide were collected from the expired air by a carbon dioxide and ethylene trapping system. Blood samples were collected at 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 48 hours after dosing. Urine and faeces were collected at intervals of 0–24, 24–48 and 48–72 hours. Blood samples were also used for the determination of erythrocyte and plasma cholinesterase levels. The animals were killed 72 hours after dosing, and radioactivity levels in brain, heart, lungs, liver, kidneys, spleen, small intestine, large intestine, stomach, testes, spinal cord, muscle and fat were determined.

Two of the three dogs vomited 15–30 minutes after dosing; thus, the actual dose received is unknown. About 35% of the dose appeared in the urine of two dogs within 24 hours after dosing. The radioactivity in urine dropped within 72 hours to levels below 1%. In one dog receiving the full dose,

4%, 0.1% and 0.03% of the dose were found in faeces at 24, 48 and 72 hours after dosing, respectively. In this dog, 30% of the dose was recovered as ethylene in the expired air, and only traces of carbon dioxide were recovered. At sacrifice, total selected organs retained a maximum of 0.25% of the administered dose. Peak plasma and red blood cell concentrations were observed 2 hours after dosing. Only traces were observed after 22 hours. Highest radioactivity concentrations were observed in liver, kidneys and spleen. Plasma cholinesterase levels were reduced at 2 hours, with recovery starting within a few hours. Erythrocyte cholinesterase levels responded more slowly, with few signs of recovery over the 72-hour period. In view of the uncertainties regarding the doses received, this study is considered to be of limited value (Stephen & Walker, 1971).

1.2 Biotransformation

Rats

The metabolism of [U-^{14}C-ethyl]ethephon (radiochemical purity > 96%; batch no. CFQ.5611) was studied in groups of five male and five female Crl:CD(SD)BR rats dosed intravenously with a single dose of 50 mg/kg bw or orally by gavage at a single dose of 50 or 1000 mg/kg bw or a single oral dose of 50 mg/kg bw after 14 daily pretreatments with unlabelled ethephon at the same dose. The study design and toxicokinetics are described in section 1.1 (Savage, 1990). Identification of metabolites in urine and faeces was performed using thin-layer chromatography (TLC) followed by liquid chromatography/mass spectrometry and nuclear magnetic resonance.

In urine and faeces, 10 regions of radioactivity were discernible from the autoradiograms. These could be grouped into three sets of closely migrating regions that were not fully resolved and a separate region. The fraction containing the disodium salt of ethephon constituted the major component in urine and faeces (about 64% and 3% of the administered radioactivity, respectively, in study A and about 38–47% and 2–8% of the administered radioactivity, respectively, in studies B, C and D). Up to 6% of the total radioactivity (urine and faeces combined) represented the monosodium salt of ethephon. The toxicokinetics data (Savage, 1990; see section 1.1) showed that about 10% (study A) and 18–22% (studies B, C and D) of the dose were excreted as ethylene in expired air, with small amounts exhaled as carbon dioxide (Hardy et al., 1990; Savage, 1990).

The metabolism of [U-^{14}C]ethephon (radiochemical purity 99.7%; batch no. CFQ12839) in dissolved saline was studied in groups of five male Sprague-Dawley CD rats dosed orally by gavage at a single dose of 50 mg/kg bw. At 1 hour after dosing, the animals were killed, and liver and kidney were collected. Identification of metabolites was performed using TLC and high-performance liquid chromatography.

Ethephon was the major radioactive component in the kidneys (87% of radioactivity recovered in kidney) and the liver (58% of radioactivity recovered in liver). The metabolite HEPA was also found in the kidneys (13% of radioactivity recovered in kidney) and liver (37% of radioactivity recovered in liver) (Odin-Feurtet, 2002).

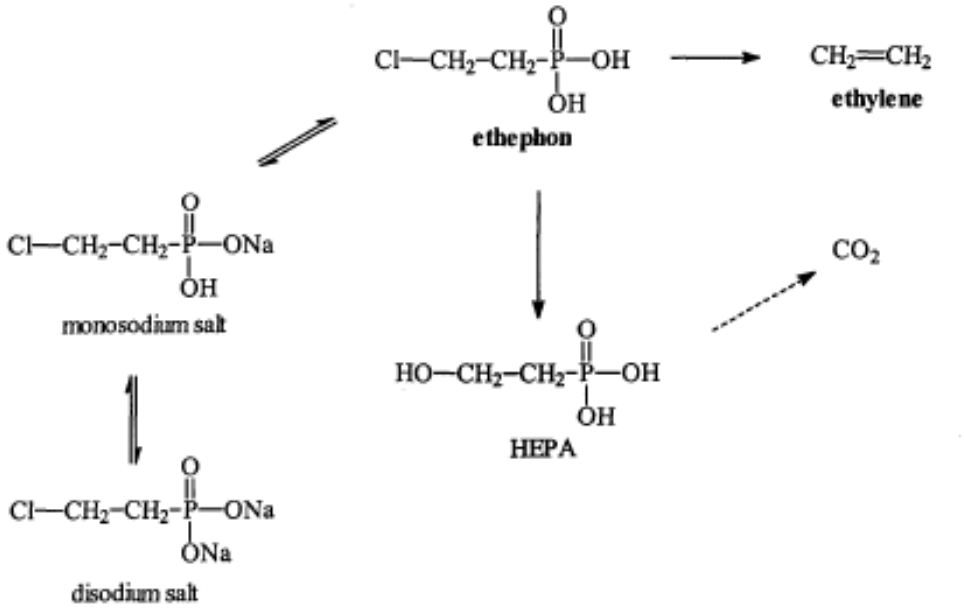
The proposed metabolic pathway of ethephon in rats is shown in Fig. 2.

Dogs

The metabolism of [^{14}C-ethyl]ethephon (radiochemical purity and batch number unknown) was studied in three male Beagle dogs dosed by gavage with a single dose of 180 mg/kg bw. The study design and toxicokinetics are described in section 1.1 (Stephen & Walker, 1971). Identification of metabolites in urine was performed using TLC and gas chromatography.

In the urine, unchanged ethephon was identified (Stephen & Stanovick, 1971).

Fig. 2. Proposed metabolic pathway of ethephon in rats



2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with ethephon are summarized in Table 2.

An acute oral toxicity study in rabbits (Weatherholtz, 1980) was not included, as the purity of the test material was not reported and only two females per dose were used.

(b) Dermal irritation

In an acute dermal irritation study, the intact skin of three male and three female New Zealand White rabbits was exposed for 1 or 4 hours under occlusion to 0.5 mL ethephon (purity 70%; batch no. RTC 2839AA; a slightly viscous liquid). Dermal irritation was scored at 24 and 72 hours after patch removal.

Contact with 0.5 mL of ethephon for 4 hours resulted in slight to well-defined erythema on 6/6 rabbits. Moderate to severe oedema was noted in 4/6 rabbits. Also evident on these four animals was necrosis, varying from one or two spots to a large area covering most of the dosed surface. Therefore, ethephon is considered to be corrosive. It is noted that the study was terminated after the 48-hour observation period. After 1 hour of exposure, less severe irritation was recorded. Five out of six rabbits had slight to well-defined erythema, but none developed oedema or necrosis (Myers, 1983).

(c) Ocular irritation

No studies were conducted, because the pH of ethephon is less than 2. Therefore, testing for eye irritation is not required.

Table 2. Results of studies of acute toxicity with ethephon

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50}/LC_{50}	Reference
Mouse	Carbia	M	Oral	Not reported	100	1 920 mg/kg bw[a]	Holsing (1969)[b]
Rat	Hilltop-Wistar	M + F	Oral	Water	70.75	2 639 mg/kg bw[a] (M) 1 564 mg/kg bw[a] (F)	Myers (1989)[c]
Rabbit	New Zealand White	M + F	Dermal	Water	70.75	1 210 mg/kg bw[a] (M) 983 mg/kg bw[a] (F)	Myers (1983)[d]
Rat	Sprague-Dawley	M + F	Inhalation	–	72.2	3.26 mg/L[a] (M/F)	Nachreiner & Klonne (1989)[e]

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

[a] Doses/concentrations are corrected for purity of the test material.

[b] At 2150 mg/kg bw, 2/5 mice died. At doses of 3160–10 000 mg/kg bw, all mice died. Depression, laboured respiration and prostrate appearance were noted at 2150, 3160 and 4640 mg/kg bw. At 6810 and 10 000 mg/kg bw, depression was noted 1 hour prior to mortality.

[c] The study design resembles Organisation for Economic Co-operation and Development (OECD) Test Guideline 401. The male rats were given a dose of 1738, 3475 or 6950 mg/kg bw, and the female rats were given a dose of 1738, 2460 or 3475 mg/kg bw. Signs of toxicity were observed at doses of 3475 mg/kg bw and higher in males and at doses of 2460 mg/kg bw and higher in females and included sluggishness, piloerection, emaciation (in one rat) and prostration. No mortality and no macroscopic changes at necropsy were observed at 1738 mg/kg bw. At higher doses, death occurred at 1 hour to 1 day. Survivors recovered at 1–3 days. At necropsy, the visceral surfaces of livers were mottled tan and brown, the glandular portions of stomachs were black and the lungs of two animals were red. Batch no. 47-20.

[d] The study design resembles OECD Test Guideline 402. All rabbits (five males and five females) at 2000 mg/kg bw and one male and two female rabbits at 1000 mg/kg bw died. Pinpoint pupils, salivation, unsteady gait and prostration were observed, followed by death at 1–3 days. Batch number was not reported.

[e] The study design resembles OECD Test Guideline 403. Rats were exposed (whole body) to actual ethephon concentrations of 6.12, 3.34 or 2.11 mg/L. At the highest concentration, all rats died. All rats displayed ocular and respiratory irritation and bright red extremities. Additional signs observed in the 6.12 and 3.34 mg/L groups included hypothermia (6.12 mg/L group only), tremors (6.12 mg/L group males only), a slow surface righting reflex and an absent tail pinch reflex. Depressed body weight gains were observed for some animals in the 3.34 mg/L group and for one female rat in the 2.11 mg/L group during the first week of the post-exposure period, but not during the second week after exposure. Treatment-related macroscopic lesions were found only in animals that died and included discoloration of the lungs, liver, salivary glands and thymic region, brain haemorrhage, and gaseous stomach and intestines. Mass mass median aerodynamic diameter ranged from 3.3 to 6.0 µm. The sample bore the reference number 4022193 and was assigned BRRC Sample No. 52-226.

(d) Dermal sensitization

In a dermal sensitization study using the Buehler test, performed in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 406, ethephon (purity 72.2%; batch no. 4022193) was tested in five male and five female Dunkin-Hartley guinea-pigs. The vehicle control group consisted of five males and five females. The dose levels were based on the results of a range-finding study in which two animals of each sex were treated topically with 1%, 5%, 10% or 25% weight per volume (w/v) of the test substance in distilled water. Slight patchy erythema was found after 24 hours at all test substance concentrations. In the induction phase, the animals received three topical inductions with 25% ethephon in distilled water once per week, followed by a topical challenge with 10% ethephon in distilled water after 2 weeks (6-hour exposure under occlusion). Dinitrochlorobenzene (DNCB) was used as a positive control.

Topical induction with ethephon at 25% w/v caused no dermal responses in any of the three inductions, with the exception of two animals that showed slight patchy erythema, one after the second induction and one after the third induction. Following challenge with 10% w/v, slight patchy erythema was noted after 24 hours in all animals of the treatment group and in 7/10 animals of the negative control group. After 48 hours, four animals of the treatment group and one animal of the negative control group showed slight patchy erythema. Sensitization of this strain was positively tested with DNCB. According to the reaction in the challenge phase, ethephon is not a skin sensitizer

(Rush, 1989). However, in view of the minimal reaction during the induction phase and the small number of animals in the test group, this study is considered to be of limited value.

In a dermal sensitization study using the Magnusson and Kligman maximization test, performed in accordance with OECD Test Guideline 406, ethephon (purity 72.4%; batch no. DA 588) was tested in 20 female Dunkin-Hartley guinea-pigs. The vehicle control group consisted of 10 males and 10 females. In the induction phase, the animals received intradermal injections of 0.75% ethephon followed by epidermal treatment with 50% ethephon on day 9 (48-hour exposure under occlusion). The challenge on day 22 was performed with epidermal application of 35% ethephon. DNCB was used as a positive control.

Equivocal macroscopic reactions were observed in 15/20 ethephon-treated animals. Histopathological examination of these lesions showed images of orthoergic irritation in nine animals. No reactions of cutaneous sensitization were observed in the 20 ethephon-treated guinea-pigs, but the lesions of orthoergic irritation noted in nine of them may have hidden possible reactions of cutaneous sensitization (Clement, 1989).

In a dermal sensitization study using the Magnusson and Kligman maximization test, performed in accordance with OECD Test Guideline 406, ethephon (purity 74.1%; batch no. 4120181) was tested in 10 male and 10 female Hartley Crl: (HA) BR guinea-pigs. The vehicle control group consisted of five males and five females. In the induction phase, the animals received intradermal injections of 0.5% ethephon followed by epidermal treatment with 50% ethephon on day 8 (48-hour exposure under occlusion). The challenge on day 22 was performed with epidermal application of 25% ethephon. In a contemporaneous study, the sensitivity of the experimental technique was demonstrated using mercaptobenzothiazole.

No clinical signs and no deaths related to treatment were noted during the study. After the challenge application, no cutaneous reactions were observed in the animals of the control group. In the treated group, at the 24-hour reading, a discrete erythema was noted in 5/20 animals. At the 48-hour reading, skin reactions faded, and discrete erythema (grade 1) persisted in 1/20 animals only. Dryness of the skin, which could have masked scoring of the cutaneous reactions in one animal, was observed at the 24- and 48-hour readings in 5/20 and 7/20 animals of the treated group, respectively. As the cutaneous reactions observed in the animals of the treated group were non-persistent and of low incidence and severity, they were attributed to the known irritating properties of the test substance, but not to delayed-contact hypersensitivity (Griffon, 2000).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day dietary study, ethephon (purity 71.3%; batch no. A51563) was administered to groups of 10 male and 10 female CD-1 mice at 0, 30, 100, 300, 1000 or 3000 parts per million (ppm) (equal to 0, 5.3, 18, 51, 181 and 546 mg/kg bw per day for males and 0, 6.5, 22, 69, 210 and 635 mg/kg bw per day for females, respectively). An additional five males and five females were included in the 0, 300 and 1000 ppm groups for cholinesterase activity determinations after 2 weeks of treatment. Mice were observed daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. At day 14, blood was sampled from five rats of each sex (fasted overnight), after which the animals were killed and the brains were removed. Cholinesterase activity was determined in erythrocytes, plasma and brain. The same was done for the remaining animals on day 28. In addition, levels of aspartate aminotransferase (ASAT), sorbitol dehydrogenase, alkaline

Table 3. Cholinesterase activity in the erythrocytes, brain and plasma of mice treated with ethephon for 28 days

	% reduction compared with controls									
	30 ppm		100 ppm		300 ppm		1 000 ppm		3 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Week 2										
Plasma ChE	–	–	–	–	18*	29*	43*	49*	–	–
Erythrocyte AChE	–	–	–	–	9	23*	29*	38*	–	–
Brain AChE	–	–	–	–	+	+	3	+	–	–
Week 4										
Plasma ChE	3	2	4	4	22*	23*	44*	46*	63*	55*
Erythrocyte AChE	3	+	6	5	18*	14*	34*	41*	62*	56*
Brain AChE	14	+	9	3	11	+	14	+	13	+

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ppm: parts per million; +: ChE activity was equivalent to or slightly greater than control values; –: not determined; *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller & Troup (1986a)

phosphatase (ALP) and alanine aminotransferase (ALAT) were determined in blood. No haematology, ophthalmoscopy or histopathology were performed. At termination, the heart, lungs, liver, kidneys and spleen of each animal were weighed.

No effects of treatment on mortality, clinical signs, feed consumption, body weight, organ weights or clinical chemistry were observed.

The effects of ethephon on cholinesterase activity are presented in Table 3.

The NOAEL was 100 ppm (equal to 22 mg/kg bw per day), based on 23% reduction of erythrocyte acetylcholinesterase (AChE) activity observed in females at 300 ppm (equal to 69 mg/kg bw per day) (Van Miller & Troup, 1986a).

In a 28-day dietary study, ethephon (purity 71.3%; batch no. A51563) was administered to groups of 15 male and 15 female CD-1 mice at 0, 3000, 10 000, 25 000 or 50 000 ppm (equal to 0, 530, 1800, 4500 and 10 000 mg/kg bw per day for males and 0, 630, 2200, 5900 and 15 000 mg/kg bw per day for females, respectively). Mice were observed daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. At day 14, blood was sampled from five rats of each sex (fasted overnight), after which the animals were killed and the brains were removed for analysis of brain cholinesterase activity. Cholinesterase activity was determined in erythrocytes, plasma and brain. No other clinical chemistry measurements, haematology, ophthalmoscopy or histopathology was performed. At termination, the heart, lungs, liver, kidneys and spleen of each animal were weighed.

No effects of treatment on mortality or clinical signs were observed. Feed consumption was significantly reduced (19–23%) for males and females during the first week of treatment at 50 000 ppm. Weight loss (15–16%) was observed for males and females at 50 000 ppm in week 1, followed by weight gains in subsequent treatment weeks. Compared with control mice, terminal body weight was lower in males (14%, statistically significant) and females (8%, not statistically significant). After 1 week of treatment, body weight at 25 000 ppm was slightly (6–9%), but statistically significantly, reduced compared with controls.

The effects of ethephon on cholinesterase activity are presented in Table 4.

Table 4. Cholinesterase activity in the erythrocytes, brain and plasma of mice treated with ethephon for 28 days

	% reduction compared with controls							
	3 000 ppm		10 000 ppm		25 000 ppm		50 000 ppm	
	M	F	M	F	M	F	M	F
Week 2								
Plasma ChE	55*	55*	54*	66*	77*	80*	79*	82*
Erythrocyte AChE	54*	62*	68*	80*	88*	88*	90*	92*
Brain AChE	6	1	4	+	4	8	13	8
Week 4								
Plasma ChE	55*	57*	60*	65*	71*	73*	77*	78*
Erythrocyte AChE	57*	62*	67*	76*	82*	82*	87*	89*
Brain AChE	9	6	12*	+	14*	13	19*	1

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ppm: parts per million; +: AChE activity was equivalent to or slightly greater than control values; *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller & Troup (1986b)

A NOAEL could not be identified. The lowest-observed-adverse-effect level (LOAEL) was 3000 ppm (equal to 530 mg/kg bw per day), based on reductions in erythrocyte AChE activity (Van Miller & Troup, 1986b).

Rats

In a 2-week range-finding toxicity study (preliminary for the subchronic toxicity study), ethephon (purity 72.4%; batch no. A4051511) was administered to groups of six male and six female Sprague-Dawley Crl:CD(SD)BR rats by gavage at 0, 100, 300, 600 or 1000 mg/kg bw per day. Animals were checked daily for clinical signs of toxicity. A detailed physical examination was performed weekly. Body weights were measured twice per week, and feed consumption was measured weekly. Before the treatment was started and prior to dosing on days 2, 8 and 15, the rats were subjected to a functional observational battery, and blood was sampled. At termination on day 15, all animals were necropsied.

At 600 mg/kg bw per day, two males and four females were found dead. At 1000 mg/kg bw per day, all males and five females were found dead or were killed in a moribund condition. In most of the rats at 600 and 1000 mg/kg bw per day, fur staining, skin pallor, abnormal breathing, respiratory sounds, dehydration, cold to touch, decreased activity, weak appearance and abdominal distension were observed. In the lower-dose groups (i.e. 100 and 300 mg/kg bw per day), no treatment-related clinical signs were observed. Body weight loss and reduced feed consumption were observed at 600 and 1000 mg/kg bw per day.

In the functional observational battery, abnormal breathing, myosis, muzzle staining, diarrhoea and impaired gait were observed in a few rats at 300 mg/kg bw per day and above. Erythrocyte AChE levels were not affected by treatment. Dose-dependent decreases in plasma cholinesterase levels were observed in all treatment groups (Beyrouty, 1997a).

In a 28-day dietary range-finding study, ethephon (purity 71.3%; batch no. A51563) was administered to groups of 10 male and 10 female Sprague-Dawley CD rats at 0, 625, 1250, 2500,

Table 5. Cholinesterase activity in the erythrocytes, brain and plasma of rats treated with ethephon for 28 days

	% reduction compared with controls									
	625 ppm		1 250 ppm		2 500 ppm		5 000 ppm		10 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Week 2										
Plasma ChE	ND	ND	21*	44	18*	45	ND	ND	ND	ND
Erythrocyte AChE	ND	ND	16	14*	36*	32*	ND	ND	ND	ND
Brain AChE	ND	ND	7	+	9	+	ND	ND	ND	ND
Week 4										
Plasma ChE	13*	29*	27*	50*	16*	50*	30*	49*	35*	63*
Erythrocyte AChE	9*	19*	22*	35*	41*	50*	58*	67*	73*	78*
Brain AChE	+	1	+	4	+	1	13	4	1	4

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ND: not determined; ppm: parts per million; +: AChE activity was equivalent to or slightly greater than control values; *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller & Troup (1986c)

5000 or 10 000 ppm (equal to 0, 52, 106, 214, 431 and 831 mg/kg bw per day for males and 0, 59, 120, 251, 487 and 980 mg/kg bw per day for females, respectively). An additional five males and five females were included in the 0, 1250 and 2500 ppm groups for cholinesterase activity determinations after 2 weeks of treatment. The rats were observed daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. At day 14, blood was sampled from five rats of each sex (fasted overnight), after which the animals were killed and the brains were removed. Cholinesterase activity was determined in erythrocytes, plasma and brain. The same was done for the remaining animals on day 28. In addition, haematology was performed, and levels of ASAT, ALAT, sorbitol dehydrogenase and ALP were determined in blood. At termination, the heart, lungs, liver, kidneys and spleen of each animal were weighed. No ophthalmoscopy or histopathology was performed.

No effects of treatment on mortality, clinical signs, feed consumption, haematology or clinical chemistry were observed. Slight reductions (up to 9%) in net body weight gains in males at 2500 and 10 000 ppm were considered to be not toxicologically relevant. Organ weights were not affected by treatment. The effects of ethephon on cholinesterase activity are presented in Table 5.

The NOAEL was 625 ppm (equal to 52 mg/kg bw per day), based on 22% reduction of erythrocyte AChE observed in males at 1250 ppm (equal to 106 mg/kg bw per day) (Van Miller & Troup, 1986c).

In a 28-day dietary range-finding toxicity study, ethephon (purity 71.3%; batch no. A51563) was administered to groups of 15 male and 15 female Sprague-Dawley CD rats at 0, 10 000, 25 000 or 50 000 ppm (equal to 0, 962, 2300 and 4673 mg/kg bw per day for males and 0, 996, 2488 and 4900 mg/kg bw per day for females, respectively). Groups of 14 male and 14 female rats received basal diet. Animals were checked daily for clinical signs of toxicity. Body weights and feed consumption were measured weekly. At day 14, blood was sampled from five rats of each sex, after which the animals were killed and the brains were removed. Cholinesterase activity was determined in erythrocytes, plasma and brain. The same was done for the remaining animals on day 28. No other clinical chemistry measurements, haematology, ophthalmoscopy or histopathology was performed. At termination, the heart, lungs, liver, kidneys and spleen of each animal were weighed.

Table 6. Cholinesterase activity in the erythrocytes, brain and plasma of rats treated with ethephon for 28 days

	% reduction compared with controls					
	10 000 ppm		25 000 ppm		50 000 ppm	
	M	F	M	F	M	F
Week 2						
Plasma ChE	23*	66	31*	66*	42*	74*
Erythrocyte AChE	69*	72*	84*	82*	91*	91*
Brain AChE	11	4	4	2	15*	13*
Week 4						
Plasma ChE	27*	46*	34*	58*	45*	61*
Erythrocyte AChE	72*	73*	82*	80*	91*	89*
Brain AChE	8	7	+	3	14	3

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ppm: parts per million; +: AChE activity was greater than control values; *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller & Troup (1986d)

No mortality was observed. Loose faeces were observed in males and females at 50 000 ppm from day 10 onward. Feed consumption was statistically significantly reduced for males at 25 000 and 50 000 ppm in weeks 1 (10–24%) and 2 (7–8%), for females at 10 000 ppm in weeks 3 and 4 (10–11%), for females at 25 000 ppm in weeks 1, 3 and 4 (8–11%) and for females at 50 000 ppm throughout treatment (9–24%). Statistically significantly lower body weights compared with controls were recorded for males and females at 50 000 ppm throughout treatment, leading to total weight gain deficits relative to controls of approximately 27% and 34% for males and females, respectively. Statistically significant total weight gain deficits were approximately 15% and 18% for males and females, respectively, at 25 000 ppm and approximately 18% for females at 10 000 ppm. No effect on body weight development was noted for males at 10 000 ppm. Observed changes in organ weights at doses of 10 000 ppm or higher were attributed to differences in terminal body weights. The effects of ethephon on cholinesterase activity are presented in Table 6.

The LOAEL was 10 000 ppm (equal to 962 mg/kg bw per day), based on reduction of erythrocyte AChE activity in both sexes (Van Miller & Troup, 1986d).

Dogs

In a 1-year study, five male and five female Beagle dogs per dose group received ethephon (purity 71.1%; lot no. A62833) at a dietary concentration of 0, 100, 300, 1000 or 2000 ppm (equal to 0, 2.8, 8.1, 27 and 54 mg/kg bw per day for males and 0, 2.6, 8.4, 30 and 50 mg/kg bw per day for females, respectively) for 52 weeks. All animals were checked daily for mortality, moribundity and clinical signs. Feed consumption and body weights were measured weekly. Daily water consumption was measured at weeks 12, 25 and 51 (males) or 52 (females). Haematology, clinical chemistry and urine analysis were performed pretreatment (week −2) and at weeks 13, 26 and 52. Ophthalmology was tested pretreatment and at week 52. All dogs were necropsied, and weights of adrenals, brain, testes with epididymides/ovaries, heart, kidneys, liver with gallbladder drained, spleen, thyroid with parathyroid, and pituitary were recorded. Macroscopic and histopathological examinations were performed in all groups at termination. Reduction of erythrocyte, plasma and brain cholinesterase activities was not measured in this study.

There were no treatment-related effects on mortalities. The incidences of soft and/or mucoid stools were higher in both sexes in all treatment groups than in the controls, but the increases were not dose dependent, indicating that soft and/or mucoid stools are not treatment related. Frothy emesis, ataxia, tremors, head tilt and temporal high body temperature were observed from week 41 in one female at 1000 ppm without major abnormality in a neurological evaluation and X-ray examination. These symptoms, except for tremors, recovered essentially by termination after medication with sodium pentobarbital, suggesting that they were not caused by inhibition of cholinesterase activity. In the treated groups, mean weekly body weight gains in both sexes and terminal body weights in females did not show statistically significant changes compared with the controls. Statistically significantly lower terminal body weight in males in the 2000 ppm group (11.4 kg in the controls versus 9.3 kg in the 2000 ppm group) and slight decreases in mean body weight gain for 0–52 weeks at 2000 ppm in both sexes (males, 2.1 kg in the controls versus 1.7 kg in the 2000 ppm group; females, 1.3 kg in the controls versus 0.84 kg in the 2000 ppm group) were considered to be treatment related. There were no significant treatment-related effects on feed and water consumption, haematology, clinical chemistry, urine analysis or ophthalmological parameters. At 2000 ppm, spleen weight was decreased in both sexes. The statistically significantly lower absolute and relative (to body weight) spleen weights in males in the 2000 ppm group were considered to be treatment related (29.6 g and 0.26 g in the controls versus 16.5 g and 0.18 g in the 2000 ppm group for absolute and relative weights, respectively). In females of the 2000 ppm group, relative spleen weight was lower than the control value, with statistical significance (0.28 g in the controls versus 0.21 g in the 2000 ppm group). However, this finding was due to one female losing body weight and was considered not to be treatment related. As for other organ weights, statistically significant decreases in absolute heart and thyroid/parathyroid weights in males in the 2000 ppm group were caused by a statistically significant decrease in terminal body weight and were not a direct effect of treatment. In females at 2000 ppm, a statistically significant increase in kidney weight relative to brain weight was caused by two animals showing low relative brain weight and high relative kidney weight accompanied by slight mineralization and tubular regeneration, respectively, which are not related to the treatment. There were no treatment-related macroscopic or histopathological findings in any organs, including the spleen, in all treated groups.

The NOAEL was 1000 ppm (equal to 27 mg/kg bw per day), based on a lower body weight gain at 52 weeks in both sexes and low absolute and relative spleen weights in males at 2000 ppm (equal to 54 mg/kg bw per day) (Hamada, 1989).

In a 2-year study, six male and six female Beagle dogs per dose group received ethephon (purity 75.6%; batch no. AL 1030-42; for Source A) at a dietary concentration of 0, 30, 300 or 3000/2000/1000/1500 ppm (equal to 0, 0.86, 7.6 and 42.2 mg/kg bw per day for males and 0, 0.86, 8.4 and 47.8 mg/kg bw per day for females, respectively) for 104 weeks. In the high-dose group, the dose was 3000 ppm in the first 3 weeks and was then changed to 2000 ppm (weeks 4–5), then to 1000 ppm (weeks 6–24) and finally to 1500 ppm (from week 25) owing to the persistent decrease in body weight gain in the first 3 weeks. In this study, another source of ethephon (purity 73.6%; batch no. AL-3096; for Source B) at a dietary concentration of 300 ppm (equal to 5.9 and 6.3 mg/kg bw per day for males and females, respectively) was administered to six dogs of each sex in the same manner. All animals were checked daily for mortality, moribundity and clinical signs. Feed consumption and body weights were measured weekly in the first 4 weeks and every 4 weeks after week 5. Haematology and clinical chemistry, including plasma and erythrocyte cholinesterase activities, were performed in weeks 0, 13, 26, 52, 78 and 104. Fasting blood glucose was also determined at weeks 31 (in the controls and 1500 ppm group) and 39 (for all dogs). After a 104-week treatment, all animals were maintained at the appropriate dietary level for 10 days before sacrifice. All dogs were necropsied, and weights of thyroids, heart, liver, spleen, kidneys, adrenals and testes with epididymides were recorded. Histopathological examination was performed in all groups. The brain cholinesterase activity of ethephon was measured at termination. This study was not conducted in compliance with GLP.

There were no treatment-related effects on mortality. In the high-dose group, a high incidence of soft stools was recorded in the first 4 weeks (at 3000 or 2000 ppm) and observed persistently in

males or sporadically in females during the study. A high incidence of intermittent emesis was also observed in both sexes in the high-dose group. There were no statistically significant and treatment-related effects on body weight from week 25 or feed consumption in all treated groups. For haematology, clinical chemistry and absolute and relative organ weights, no significant treatment-related changes were observed in either sex in any treated group.

The effects of ethephon treatment on cholinesterase activity are presented in Table 7. From week 6, erythrocyte AChE activity was statistically significantly reduced by more than 20% (42–56% in males and 47–56% in females at 300 ppm and 68–79% in males and 59–79% in females at 1500 ppm) in both sexes at 300 ppm and higher, compared with the corresponding controls and the values at week 0. Brain AChE activity was not affected in either sex at any dose.

Morphologically, smooth muscle hypertrophy in the duodenum was observed in two and three females at 300 ppm and 1500 ppm, respectively. In addition to the duodenal lesion, each female at 300 ppm had smooth muscle hypertrophy in the stomach or in both jejunum and ileum, respectively. One of three females bearing a duodenal lesion at 1500 ppm also had smooth muscle hypertrophy in the stomach and jejunum. In males, smooth muscle hypertrophy of the duodenum was observed in one animal at 1500 ppm. However, the lesion was not observed in other parts of the small intestine in the same animal or other treated groups of males. In the stomach and small intestine, other findings, such as chronic gastritis and congestion in the duodenum, were noted at 300 and 1500 ppm in males and/or females. However, the affected animals were different from the ones showing smooth muscle hypertrophy, indicating that the smooth muscle hypertrophy was not related to the observed gastrointestinal lesions. It is reported that smooth muscle hypertrophy in the intestine is caused by obstruction, diverticulum, inflammation or infection or may occur spontaneously in animals (Bettini et al., 2003; Murakami et al., 2010; Liu et al., 2014). Although the cause of smooth muscle hypertrophy in the stomach and small intestine could not be determined in this 2-year study, the increased incidence of the smooth muscle hypertrophy is considered to be treatment related. However, the biological significance of this effect is unclear, and it is not considered to be toxicologically adverse. There were no other treatment-related findings in other examined organs.

The NOAEL was 30 ppm (equal to 0.86 mg/kg bw per day), based on greater than 20% reduction of erythrocyte AChE activity in both sexes at 300 ppm (equal to 7.6 mg/kg bw per day) (Reno & Voelker, 1977).

(b) Dermal application

Rabbits

A 21-day toxicity test was carried out with dermal application of ethephon (purity 39.5%; batch number unknown) in rabbits. Groups of 5–10 male and female adult New Zealand White rabbits received ethephon at a dose of 0, 119 or 237 mg/kg bw per day (doses corrected for purity), 5 days/week, for 3 weeks, on the abdominal skin clipped free of hair. After 6–8 hours of exposure, the abdomen was washed with water. The general appearance, behaviour, body weight, clinical chemistry, signs of dermal irritation, gross pathology and histopathology were studied.

No systemic toxicity of ethephon was demonstrated at the two dose levels, apart from a severe dermal irritation characterized by subepidermal fibrosis, acanthosis, hyperkeratosis and ulceration of the epidermis (Holsing, 1969).

In a 21-day dermal toxicity test, ethephon (purity 72.2%; batch no. 4022193) was applied to the skin of groups of 10 male and 10 female Hra:(NZW)SPF rabbits at an ethephon dose of 0, 18, 53 or 107 mg/kg bw per day (corrected for purity), 5 days/week, for 3 weeks. After at least 6 hours of exposure, the abdomen was washed with water. Dermal irritation was scored immediately before each

Table 7. Cholinesterase activity in the erythrocytes, brain and plasma of dogs treated with ethephon for 2 years

	% reduction compared with controls					
Dietary concentration (ppm)	**Week 6**	**Week 13**	**Week 26**	**Week 52**	**Week 78**	**Week 104**
Erythrocyte AChE						
Males						
30 ppm (Source A)	−9	−8	−5	−10	−10	−11
300 ppm (Source A)	−47*	−48*	−42*	−46*	−56*	−46*
1 500 ppm (Source A)	−79*	−70*	−68*	−71*	−79*	−73*
300 ppm (Source B)	−54*	−56*	−48*	−55*	−53*	−47*
Females						
30 ppm (Source A)	−9	2	−8	0	10	−13
300 ppm (Source A)	−54*	−53*	−50*	−48*	−47*	−56*
1 500 ppm (Source A)	−79*	−70*	−71*	−59*	−59*	−74*
300 ppm (Source B)	−45*	−53*	−39*	−52*	−36*	−49*
Brain AChE						
Males						
30 ppm (Source A)	ND	ND	ND	ND	ND	53
300 ppm (Source A)	ND	ND	ND	ND	ND	42
1 500 ppm (Source A)	ND	ND	ND	ND	ND	20
300 ppm (Source B)	ND	ND	ND	ND	ND	40
Females						
30 ppm (Source A)	ND	ND	ND	ND	ND	39
300 ppm (Source A)	ND	ND	ND	ND	ND	9
1 500 ppm (Source A)	ND	ND	ND	ND	ND	17
300 ppm (Source B)	ND	ND	ND	ND	ND	4
Plasma ChE						
Males						
30 ppm (Source A)	−24*	−22*	−26*	−22*	−31*	−31*
300 ppm (Source A)	−48*	−46*	−51*	−48*	−62*	−56*
1 500 ppm (Source A)	−54*	−45*	−54*	−53*	−67*	−63*
300 ppm (Source B)	−52*	−46*	−51*	−51*	−59*	−57*
Females						
30 ppm (Source A)	−34*	−24*	−38*	−31*	−29*	−30*
300 ppm (Source A)	−52*	−51*	−57*	−53*	−58*	−58*
1 500 ppm (Source A)	−61*	−59*	−63*	−65*	−61*	−66*
300 ppm (Source B)	−53*	−55*	−61*	−57*	−54*	−58*

AChE: acetylcholinesterase; ChE: cholinesterase; ND: not determined; ppm: parts per million; *: $P < 0.05$ (Scheffe's method)
Source: Reno & Voelker (1977)

application (except day 0) and on the day of necropsy. The animals were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded weekly. Haematology and clinical chemistry parameters were evaluated on the day prior to termination. All animals were examined macroscopically, and selected organs were weighed. Selected tissues from all animals in the 0 and 107 mg/kg bw per day groups were examined microscopically.

There were no treatment-related effects on behaviour, body weight, feed consumption, haematology, clinical chemistry or organ weights. In the low- and mid-dose groups, slight to moderate erythema and desquamation, slight oedema and slight fissuring in some of the animals were observed. In the high-dose group, there was slight to moderate erythema, slight to moderate oedema, slight to moderate desquamation and slight to moderate fissuring. Microscopic examination revealed acanthosis and chronic active inflammation of the skin in high-dose animals.

The NOAEL for systemic toxicity was 107 mg/kg bw per day, the highest dose tested (Henwood, 1989a).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 78-week dietary carcinogenicity study performed according to OECD Test Guideline 451, ethephon (purity 75%; batch no. X00782) was administered to groups of 85 male and 85 female CD-1 mice at 0, 30, 300 or 1000 ppm (equivalent to 0, 4.5, 45 and 150 mg/kg bw per day, respectively). The mice were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Group feed consumption and individual body weights were recorded weekly for the first 26 weeks and every month thereafter. Haematology and cholinesterase determinations in plasma, erythrocytes and brain were performed on five mice of each sex per dose in weeks 26, 52 and 78. No macroscopic or histopathological examinations were done on these animals. The remaining rats were killed in week 78. All animals were examined macroscopically. The brain, liver, kidneys, spleen, heart, thyroid gland with parathyroid, adrenal glands, pituitary, testes, epididymides, seminal vesicles, prostate, ovaries and uterus were weighed. Histological examinations were performed on a wide range of organs and tissues. It is noted that because of a mistake in the allocation of animals of different sexes together, five females (one from the control group and four from the top-dose group) became pregnant. These animals were not included in analyses of group mortality, group mean body weight, group mean absolute and relative organ weights, or group mean feed consumption data (cages bearing these animals were eliminated). They were, however, included in necropsy and histopathological evaluations.

Statistically significant decreases in survival were noted in mid- and high-dose males beginning in weeks 64 and 72, respectively. The increased mortality was most likely related to the higher incidence of genitourinary infections, dermatitis and haematopoietic tumours in the mid- and high-dose animals during this period. As none of the above pathological entities was considered to be related to administration of the test compound, the increased mortality in male animals at the 300 and 1000 ppm levels was not considered to be compound related. Clinical signs, body weight gain and feed consumption were not affected by treatment. A statistically significant increase in total leukocytes in high-dose females at week 26 was not considered to be a treatment-related effect because it was an isolated finding and the value was within the accepted normal range for this parameter. No other differences in haematological parameters were found.

The effects of ethephon on cholinesterase activity are presented in Table 8.

No toxicologically relevant differences in organ weight, macroscopic findings or histopathological findings were observed. There was no indication of a neoplastic effect of the test compound on any organ in either sex.

Table 8. Cholinesterase activity in the erythrocytes, brain and plasma of mice treated with ethephon for 78 weeks

	% reduction compared with controls					
	30 ppm		300 ppm		1 000 ppm	
	M	F	M	F	M	F
Week 26						
Plasma ChE	+	2	9	13*	40*	33*
Erythrocyte AChE	+	0	+	18	13	45*
Brain AChE	+	+	+	9	+	8
Week 52						
Plasma ChE	2	+	23*	19*	50*	39*
Erythrocyte AChE	1	17	25	36*	32	47*
Brain AChE	+	+	6	3	+	+
Week 78						
Plasma ChE	+	+	34*	28*	61*	64*
Erythrocyte AChE	+	31	11	56*	21	51*
Brain AChE	+	+	+	4	+	9

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ppm: parts per million; +: ChE activity was greater than control values; *: $P < 0.05$ (least significant difference test)
Source: Voss & Becci (1985)

The NOAEL was 30 ppm (equivalent to 4.5 mg/kg bw per day), based on a statistically significant reduction of erythrocyte AChE activity by more than 20% observed in females at 300 ppm (equivalent to 45 mg/kg bw per day) at weeks 52 and 78. No treatment-related tumours were observed in CD-1 mice under the conditions of the study (Voss & Becci, 1985).

In a 78-week dietary carcinogenicity study performed according to OECD Test Guideline 451, ethephon (purity 70.6–72.0%; batch nos A51563, HTS5841AA, A6041;18, 803A13-LJH, A62534, A70073) was administered to groups of 70 male and 70 female CD-1 mice at 0, 100, 1000 or 10 000 ppm (equal to 0, 14, 139 and 1477 mg/kg bw per day for males and 0, 17, 173 and 1782 mg/kg bw per day for females, respectively). Although there was initially a fifth group of males and females treated with 50 000 ppm, high incidences of mortality and morbidity were observed within 2 days of treatment. Therefore, this group was terminated, and the study proceeded with the four remaining groups. The mice were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Group feed consumption and individual body weights were recorded weekly for the first 13 weeks and every other week thereafter. Water consumption was measured over a 24-hour period at weeks 12, 25, 50, 51 (12-month interim sacrifice animals) and 55 (recovery animals). Ten animals of each sex per dose were used for haematology and urine analysis, and another 10 of each sex per dose were used for clinical chemistry and cholinesterase determinations pretest and in weeks 52 and 77/78. Twenty animals of each sex per dose were killed after 12 months for interim examination. The remaining rats were killed in week 78. Ophthalmoscopy was performed before treatment and before termination. All animals were examined macroscopically. The brain, liver, kidneys, spleen, heart, thyroid gland, adrenal glands and gonads were weighed. The brain of animals selected for brain cholinesterase activity measurement was divided in half. One half of the brain was retained for histological examination, and the other half was used for AChE activity measurement. Histological examinations were performed on a wide range of organs and tissues from all control and

Table 9. Cholinesterase activity in the erythrocytes, brain and plasma of mice treated with ethephon for 2 years

	% reduction compared with controls					
	100 ppm		1 000 ppm		10 000 ppm	
	M	F	M	F	M	F
Week 52						
Plasma ChE	+	18*	35*	41*	65*	76*
Erythrocyte AChE	+	17	36*	36*	70*	74*
Brain AChE	+	8	+	4	+	18
Week 78						
Plasma ChE	2	24	41*	61*	71*	74*
Erythrocyte AChE	+	14	35*	21	72*	60
Brain AChE	+	+	+	+	+	+

AChE: acetylcholinesterase; ChE: cholinesterase; F: females; M: males; ppm: parts per million; +: ChE activity was greater than control values *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller (1988)

10 000 ppm mice and from all mice from the 100 and 1000 ppm groups that died or were killed in a moribund condition.

There were no effects of treatment on mortality, clinical signs, haematology, clinical chemistry or ophthalomoscopy. Slightly increased feed consumption occasionally seen in mice at 1000 and 10 000 ppm was attributed to increased spillage of test diets. Females at 10 000 ppm showed slightly (up to 6%), but statistically significantly, lower body weights in weeks 61, 63, 67 and 75, and body weight gains were significantly lower (up to 14%) in weeks 39, 47, 55, 57, 59, 61, 63, 67, 69, 71 and 75. The urinary pH was significantly lower in males at 1000 and 10 000 ppm after 77 weeks, which was attributed to the acidity of the test substance.

The effects of ethephon on cholinesterase activity are presented in Table 9.

A statistically significant increase in lung adenomas was observed in males at 1000 ppm. However, as no dose–response relationship was observed, and as this is a common finding in this strain of mice, this increase was not considered to be related to treatment.

The NOAEL was 100 ppm (equal to 14 mg/kg bw per day), based on a statistically significant reduction of erythrocyte AChE activity by more than 20% in males and females at 1000 ppm (equal to 139 mg/kg bw per day). No treatment-related tumours were observed in CD-1 mice under the conditions of the study (Van Miller, 1988).

Rats

In a 2-year dietary toxicity study, ethephon (purity 75.6%; batch no. AL-1030-42) was administered to groups of 55 male and 55 female Sprague-Dawley CD rats at 0, 30, 300 or 3000 ppm (equal to 0, 1.2, 13 and 129 mg/kg bw per day for males and 0, 1.6, 16 and 171 mg/kg bw per day for females, respectively). The rats were checked daily for mortality and clinical signs. Every fourth week, a detailed clinical examination was performed, and body weights and feed consumption were measured. Five animals of each sex per dose were used for haematology, clinical chemistry and cholinesterase determinations in weeks 13, 26, 52, 78 and 104. Brain cholinesterase determinations were performed on five animals of each sex per group killed at week 52 and on all surviving animals

at 104 weeks. All animals were necropsied, and the liver, kidneys, spleen, heart, thyroid gland, adrenal glands, and testes with epididymides were weighed. Histological examinations were performed on a wide range of organs and tissues of 20 males and 20 females in the control and high-dose groups. In addition, all gross tissue masses and suspected tumours from all dose groups were examined.

There were no effects of treatment on mortality or clinical signs. Slightly lower net body weight gains (7–8%) were recorded for males at 300 ppm and for both sexes at 3000 ppm. Feed consumption was not affected by treatment. Brain AChE activity was not affected by ethephon. AChE activity in erythrocytes was significantly reduced (> 20%) at 3000 ppm in both sexes. Cholinesterase activity in plasma was significantly reduced (> 20%) at 300 and 3000 ppm in both sexes. No other toxicologically relevant changes in clinical chemistry parameters or organ weights were observed. Macroscopic and histopathological examination did not reveal any findings that could be attributed to the test material. There were no treatment-related increases in the incidence of neoplastic lesions.

The NOAEL was 300 ppm (equal to 13 mg/kg bw per day), based on reduction of erythrocyte AChE activity in both sexes at 3000 ppm (equal to 129 mg/kg bw per day). No treatment-related tumours were observed in Sprague-Dawley CD rats under the conditions of the study (Reno, Serota & Voelker, 1978).

In a 2-year dietary toxicity study, ethephon (purity 70.6–72.1%; batch nos A51563, HTS5841AA, A6041;18, 803A13-LJH, A62534, A70073, A70471) was administered to groups of 90–100 male and 90–100 female Sprague-Dawley CD rats at 0, 300, 3000, 10 000 or 30 000 ppm (equal to 0, 13, 131, 446 and 1416 mg/kg bw per day for males and 0, 16, 161, 543 and 1794 mg/kg bw per day for females, respectively). The rats were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Group feed consumption and individual body weights were recorded weekly for the first 13 weeks and every other week thereafter. Water consumption was measured over a 24-hour period at weeks 12, 25, 50, 51 (12-month interim sacrifice animals) and 55 (recovery animals). Ten animals of each sex per dose were used for haematology and urine analysis, and another 10 of each sex per dose were used for clinical chemistry and cholinesterase determinations in weeks 12/13, 25/26, 50/51, 77/78, 97 (males) and 103/104 (females). In weeks 13, 26, 51 and 78, only plasma and erythrocyte cholinesterase activities were determined. Ten animals of each sex per dose were killed after 12 months for interim examination. At 12 months, 10 animals of each sex in the control, 10 000 ppm and 30 000 ppm groups were placed on control diet for 1 month and served as recovery groups. The recovery animals were used for haematology, urine analysis, clinical chemistry and cholinesterase determinations during weeks 51/52 and 55/56. The remaining rats (50 and 30 animals of each sex for oncogenicity and chronic toxicity investigations, respectively) were killed in week 97 (males) or in week 104 (females). Ophthalmoscopy was performed before treatment and before termination. All animals were examined macroscopically. The brain, liver, kidneys, spleen, heart, thyroid gland, adrenal glands and gonads were weighed. The brains of animals selected for brain cholinesterase activity measurement were divided in half. One half of each brain was retained for histological examination, and the other half was used for AChE activity measurement. Histological examinations were performed on a wide range of organs and tissues from all rats.

There were no effects of treatment on mortality or ophthalmoscopy. In the high-dose rats, an increased incidence of loose faeces was observed from day 13 onward. An increased incidence of red and thickened ears was observed in females at 3000 ppm or higher. Histological examination showed increased incidences of aurocular chondropathy, the cause of which could not be established. This effect was considered to be not biologically significant. Statistically significantly lower body weights were recorded for males and females at 30 000 ppm throughout treatment, but particularly in the first year of treatment. A 28–31% reduction in body weight gain was observed in male and female rats at 30 000 ppm after the first week of treatment. The weight gain deficits at the end of the first year of the study (relative to untreated controls) at 30 000 ppm were 17% and 27% for males and females,

respectively. In the recovery period following 52 weeks of exposure, body weights remained significantly lower for males at 30 000 ppm. Males at 10 000 ppm had a weight gain deficit of 6% at the end of the first year of the study. Feed consumption was statistically significantly reduced (up to 11%) in males and females at 30 000 ppm in the majority of measurements in the first year of treatment, and the reduction was observed as early as in the first week of treatment. In the 4-week recovery period following 52 weeks of exposure, males and females at 30 000 ppm returned to normal feed consumption levels. The efficiency of feed utilization (body weight gain/feed consumed) in the first 13 weeks of treatment was significantly lower for males and females at 30 000 ppm.

Serum glucose level was significantly lower for males and females at 30 000 ppm after 26 weeks and for males at 30 000 ppm after 52 weeks. These differences were considered to be related to the reduced feed consumption and body weights observed for these animals. Serum phosphorus level was statistically significantly reduced (10%) for males at 30 000 ppm after 13 and 26 weeks. In males at 30 000 ppm, erythrocyte counts were up to 6% higher after 13 and 26 weeks, haematocrit was 7% higher after 13 weeks, and mean corpuscular haemoglobin concentration was 2–4% lower after 13, 26, 51 and 78 weeks. Prothrombin time was up to 5% lower for females at 30 000 ppm after 51 and 104 weeks. In view of the small magnitude of these haematological changes, they were not considered to be toxicologically relevant. Urinary pH was significantly lower throughout the study for males and females at 10 000 and 30 000 ppm, with a dose-related trend. A significantly lower urinary pH was also recorded for females at 300 ppm after 103 weeks and for females at 3000 ppm after 77 and 103 weeks. Under control conditions for 4 weeks following the 52-week exposure, the urinary pH returned to normal levels for males and females at 10 000 and 30 000 ppm. This effect is likely to be related to the acidity of the test substance. The specific gravity of urine was significantly higher for females at 10 000 and 30 000 ppm after 50 (high dose only) and 77 weeks. In the investigations during week 77, females at 10 000 and 30 000 ppm showed a strong decrease in urinary volume. As this effect was incidental, it was considered not to be treatment related.

The effects of ethephon on cholinesterase activity are presented in Table 10.

In the interim group at 52 weeks, relative kidney weights were significantly higher for males (27%) and females (22%) at 30 000 ppm. After 2 years of treatment, relative kidney weights were significantly higher for males at 3000 (31%) and 10 000 ppm (35%) and for males (25%) and females (31%) at 30 000 ppm. All other deviations in absolute or relative organ weights at 10 000 or 30 000 ppm at the interim and terminal kills reflected lower terminal body weights. In the kidneys of high-dose females at termination, the incidence of glomerulosclerosis (34/46) was significantly increased compared with controls (16/42). In the liver, the incidence of biliary hyperplasia was significantly higher in males at 30 000 ppm (35/48 versus 21/44 in controls). In females at 3000 ppm and higher, increased incidences of aurocular chondropathy were observed.

There were no treatment-related increases in the incidence of neoplastic lesions.

The NOAEL was 300 ppm (equal to 13 mg/kg bw per day), based on a statistically significant reduction by more than 20% in erythrocyte AChE activity observed in males and females at 3000 ppm (equal to 131 mg/kg bw per day). No treatment-related tumours were observed in Sprague-Dawley CD rats under the conditions of the study (Van Miller, 1989).

2.4 Genotoxicity

Ethephon was tested for genotoxicity in a range of assays. A number of these studies, although indicating no genotoxic effect of ethephon, were considered as supportive evidence only, as the purity of the test material and often batch number were not reported. An unscheduled DNA synthesis test in vitro (Cifone, 1988) that showed negative results was considered supportive only, as no doses with appropriate cytotoxicity were tested. Ethephon induced a positive response in a gene mutation test (Ames test). In the absence of metabolic activation, ethephon induced up to 4-fold

Table 10. Cholinesterase activity in the erythrocytes, brain and plasma of rats treated with ethephon for 2 years

	% reduction compared with controls							
	300 ppm		3 000 ppm		10 000 ppm		30 000 ppm	
	M	F	M	F	M	F	M	F
Week 13								
Plasma ChE	18*	23*	27*	59*	38*	61*	45*	72*
Erythrocyte AChE	9*	13*	45*	55*	65*	72*	83*	82*
Week 26								
Plasma ChE	17	24	36*	58*	42*	65*	51*	69*
Erythrocyte AChE	10*	11*	42*	58*	72*	79*	86*	86*
Week 51								
Plasma ChE	29*	15	47*	53*	48*	64*	56*	61*
Erythrocyte AChE	6	19*	47*	63*	78*	78*	84*	85*
Week 52								
Plasma ChE	12	22	35*	48*	46*	62*	62*	71*
Erythrocyte AChE	2	11*	47*	50*	70*	75*	86*	88*
Brain AChE	+	+	6	+	+	+	7	+
Week 56 recovery								
Plasma ChE	ND	ND	ND	ND	3	9	24	13
Erythrocyte AChE	ND	ND	ND	ND	15*	16*	22*	24*
Brain AChE	ND	ND	ND	ND	+	+	8	+
Week 78								
Plasma ChE	26*	28	35*	47*	41*	59*	48*	57*
Erythrocyte AChE	4	8*	47*	59*	72*	77*	87*	83*
Weeks 97/104								
Plasma ChE	44	22	32	37*	67*	47*	56*	86*
Erythrocyte AChE	8	9	39*	43*	81*	73*	86*	86*
Brain ChE	2	+	4	+	+	2	+	2

AChE: acetylcholinesterase; ChE: cholinesterase; ND: not determined; F: females; M: males; ppm: parts per million; +: ChE activity was greater than control values; *: $P < 0.05$ (Student's *t*-test)
Source: Van Miller (1989)

increases in the number of revertant colonies of the *Salmonella typhimurium* tester strain TA1535. In the presence of metabolic activation, the test substances induced up to 9-fold increases in the number of revertant colonies. Ethephon did not induce point mutations in *S. typhimurium* in the absence or presence of metabolic activation in tester strains TA98, TA100, TA1537 or TA1538. In all other available studies, ethephon gave negative results (Table 11).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation dietary reproductive toxicity study performed according to OECD Test Guideline 416, Sprague-Dawley Crl:CD(SD)BR rats (28 of each sex per group for the F_0 and F_1 generations) were fed ethephon (purity 71.14–72.14%; batch nos A6041, 18,803A, 13-LJH, A62534, A70073, A70471) at a dietary concentration of 0, 300, 3000 or 30 000 ppm (equal to 0, 22, 220 and 2260 mg/kg bw per day for F_0 males and 0, 25, 260 and 2570 mg/kg bw per day for F_0 females; and 0, 20, 200 and 2220 mg/kg bw per day for F_{1b} males and 0, 24, 245 and 2520 mg/kg bw per day for F_{1b} females, respectively). F_0 adults were treated over a 10-week premating period and throughout the 3-

Table 11. Overview of genotoxicity tests with ethephon[a]

Test	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Gene mutations	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538	0.1–50 µg/plate (±S9)	72.3	Positive	Jagannath (1987)[b]
Gene mutations	Chinese hamster ovary cells, HPRT test	500–2 500 µg/mL (±S9)	Not reported	Negative *Study considered supportive*	Godek, Naismith & Matthews (1983)[c]
Gene mutations	Chinese hamster ovary cells, HPRT test	166–5 000 µg/mL (±S9)	Not reported	Negative *Study considered supportive*	Godek, Naismith & Matthews (1984)[d]
Gene mutations	Chinese hamster ovary cells, HPRT test	500–2 500 µg/mL (−S9); 500–2 600 µg/mL (+S9)	72.3	Negative	Young (1988)[e]
Chromosomal aberrations	Chinese hamster ovary cells	753–2 010 µg/mL (−S9); 502–2 010 µg/mL (+S9)	71.3	Negative	Murli (1988)[f]
Unscheduled DNA synthesis	Male Fischer 344 rat hepatocytes	10–1 000 µg/well	Not reported	Negative *Study considered supportive*	Barfknecht, Naismith & Matthews (1984)[g]
Unscheduled DNA synthesis	Male Fischer 344 rat hepatocytes	Experiment 1: 25–1 000 µg/mL Experiment 2: 500–2 000 µg/mL	71.3	Negative *Study considered supportive*	Cifone (1988)[h]
In vivo					
Micronucleus test	Male and female CD-1 mice, bone marrow	Experiment 1: intraperitoneal dose of 200 mg/kg bw, harvesting after 30 h Experiment 2: intraperitoneal dose of 200 mg/kg bw, harvesting after 480 h Experiment 3: two intraperitoneal doses of 200 mg/kg bw, separated by 24 h, harvesting 48 h after first dose Experiment 4: two intraperitoneal doses of 200 mg/kg bw, separated by 24 h, harvesting 72 h after first dose	Not reported	Negative *Study considered supportive*	Sorg, Naismith & Matthews (1981)[i]

Table 11 (continued)

Test	Test object	Concentration	Purity (%)	Results	Reference
Dominant lethal mutation	Sprague-Dawley COBS CD (SD) rats	Oral dose of 250, 500 or 1 000 mg/kg bw per day for 5 days	Not reported	Negative *Study considered supportive*	Naismith & Matthews (1979)[j]
Unscheduled DNA synthesis	Male Han Wistar rat hepatocytes	Experiment 1: oral dose of 800 or 2 000 mg/kg bw, hepatocyte harvesting after 12–14 h Experiment 2: oral dose of 800 or 2 000 mg/kg bw, hepatocyte harvesting after 2–4 h	71.14	Negative	Howe (2002)[k]

bw: body weight; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

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

[b] Test substance: Ethephon; active ingredient 72.3%. Batch number not reported. In the absence of metabolic activation, the test substance induced up to 4-fold increases in the number of revertant colonies of the tester strain TA1535. In the presence of metabolic activation, the test substances induced up to 9-fold increases in the number of revertant colonies. Ethephon did not induce point mutations in *S. typhimurium* in the absence or presence of metabolic activation in tester strains TA98, TA100, TA1537 or TA1538. The *Escherichia coli* strain WP2uvrA was not included in the present study.

[c] Test substance: Ethephon. Batch number not reported. Study design resembles OECD Test Guideline 476. Vehicle was culture medium with 5% fetal calf serum. Post-treatment survival at 2500 µg/mL was 25–30% in the absence of S9 and 14–15% in the presence of S9. Ethephon did not induce gene mutations in mammalian cells.

[d] Test substance: Ethephon. Batch number not reported. Study design resembles OECD Test Guideline 476. Vehicle was culture medium with 5% fetal calf serum. Cytotoxicity was observed at 5 mg/mL in the absence and presence of metabolic activation. Ethephon did not induce gene mutations in mammalian cells.

[e] Test substance: Ethephon, purity 72.3%. Batch number not reported. The study was performed in accordance with OECD Test Guideline 476. Cytotoxicity below 50% was observed only in the first experiment without metabolic activation and in the second experiment with metabolic activation.

[f] Test substance: Ethephon. Batch number not reported. Study design resembles OECD Test Guideline 473. Solvent was McCoys 5a culture medium. Doses used in this assay were based on results from cytotoxicity assays. Cytotoxicity was observed at 1990 µg/mL (−S9). Ethephon did not induce chromosomal aberrations in Chinese hamster ovary cells.

[g] Test substance: Ethephon, batch no. A-41213. Study design resembles OECD Test Guideline 482. Vehicle was deionized water. Cytotoxicity was observed at 3333 and 10 000 µg/well.

[h] Test substance: Ethephon. Study design resembles OECD Test Guideline 482. Vehicle was WME culture medium. No dose levels with appropriate toxicity were tested. At the highest dose of 1000 µg/mL in the first experiment, survival was 89.5%. At the highest dose of 2000 µg/mL in the second experiment, survival was 83.2%. Only five dose levels in the first experiment and two dose levels in the second experiment were used for counting grains.

[i] Purity and batch number not reported. Doses were based on a range-finding test. Following a single dose of 200 mg/kg bw, the mice exhibited writhing, decreased activity, abnormal gait, ptosis and a decreased body tone. No increases in micronuclei were observed in ethephon-treated mice. Polychromatic erythrocyte/normochromatic erythrocyte ratio was not reported. A toxicokinetic study indicates that following an oral or intravenous dose of radiolabelled ethephon to rats, radioactivity concentrations in bone marrow are low (Savage, 1990).

[j] Purity not reported. Batch no. 56375. Three groups of 10 male rats were administered ethephon orally at a dose of 250, 500 or 1000 mg/kg bw per day for 5 consecutive days. Twenty-four hours after the fifth dose, each male was co-housed with two virgin females for 7 days. The matings were repeated weekly with two virgin females for a total of 8 weeks. The females were killed 14 days from mid-week of co-housing, and the numbers of corpora lutea and live and dead implantations were counted and recorded. Ethephon did not produce dominant lethal effects in the male rats at the doses administered, as measured by preimplantation and postimplantation losses. Postimplantation fetal deaths were significantly increased after treatment with the positive control, triethylenemelamine.

[k] Test substance: Ethephon, batch no. C1045. Test performed according to OECD Test Guideline 486. Groups of four male rats were treated once with the solvent (purified water), ethephon (at 800 or 2000 mg/kg bw) or the required positive control, by gavage, at a dosing volume of 10 mL/kg bw. The positive controls used were 2-acetamidofluorene (75 mg/kg bw) suspended in corn oil (12- to 14-hour experiment) and dimethylnitrosamine (10 mg/kg bw) dissolved in purified water (2- to 4-hour experiment).

week mating period, gestation and 21-day lactation of two litters (F_{1a} and F_{1b}). The second mating was made specifically with males and females that failed at the first mating (so-called alternative pairing). F_0 parents were killed after weaning of the F_{1b} pups.

Twenty-eight rats of each sex (predominantly F_{1b} pups) selected to produce the next generation followed the same protocol. Each litter (F_{1a}, F_{1b}, F_{2a}, F_{2b}) was randomly culled to eight pups on postnatal day (PND) 4. Parental (F_0) rats were exposed from 10 weeks before mating until termination, and F_1 rats were exposed from postnatal week 3 until termination. Clinical examination was performed daily. Feed consumption was recorded weekly during the premating period. Body weights of parental rats were recorded weekly; in addition, females were weighed on gestation days (GDs) 0, 4, 7, 14 and 20 and PNDs 0, 4, 7, 14 and 21. At termination, necropsy was performed on all F_0 and F_{1b} parental rats, and weights of brain, ovaries and testes were recorded. Histological examination was performed on gross lesions of all F_0 and F_{1b} parental rats. In addition, in F_0 and F_{1b} control and high-dose adults, histopathology was extended to the reproductive tract (ovaries, uterus, vagina/prostate with seminal vesicles, testes with epididymides) and pituitary. All litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and gross external anomalies. Survival indices were calculated at 0, 4, 7 and 14 days after birth and at weaning. All litters were kept until the youngest litter was 28 days old. Pups were weighed on PNDs 0, 4, 7, 14, 21 (at weaning) and 28. Ten pups of each sex per dose of the F_{1a}, F_{1b}, F_{2a} and F_{2b} generations were necropsied. The remaining offspring were examined for gross external abnormalities. Cholinesterase activity measurements were not performed.

No treatment-related mortality was observed in the F_0 or F_1 parental animals. At 30 000 ppm, increased incidences of unkempt appearance and urinary stains were observed in F_0 male rats, and loose faeces were observed in F_0 and F_1 parental animals of both sexes. Loose faeces were also observed in F_{1b} males at 3000 ppm.

At the end of the premating period, F_0 males and females at 30 000 ppm exhibited a lower body weight (up to 10% in males and 12% in females) and body weight gain (15% in males and 25% in females) compared with controls. The largest reduction in body weight gain (32%) was observed in the first week of treatment. A slight reduction in feed consumption (generally less than 10%) was observed at 30 000 ppm. At the F_0 breeding to produce F_{1a} litters, gestational parameters were unaffected by treatment. Although high-dose F_0 females had a lower body weight than controls throughout gestation and lactation of the F_{1a} and F_{1b} generations, the body weight gain throughout these periods was not lower than that of controls. At birth, the F_{1a} and F_{1b} pups at 30 000 ppm had an 8% lower body weight compared with control pups. During lactation, the weight gain in high-dose pups was lower, so that at weaning, the body weights of these pups were 26–30% lower than those of controls. A similar reduction in body weight was observed at PND 28. In the F_{1b} generation, an increased number of stillborn pups was observed at 30 000 ppm. The number of deaths of F_{1b} pups was increased at 30 000 ppm on PNDs 0–4 (19 versus 2 in the control group) and on PNDs 4–7 at 3000 ppm (8 versus 0 in the control group). Necropsy of F_0 adults or F_{1a} and F_{1b} pups revealed no treatment-related findings. At histopathological examination of high-dose F_0 adults, no treatment-related lesions were observed. Whereas terminal body weights were reduced in F_0 males and females at 30 000 ppm, absolute organ weights were unaffected by treatment. There were also no differences in organ weights relative to brain weight.

During the 10-week premating exposure, the F_{1a} and F_{1b} parental animals at 30 000 ppm had a 15–17% lower body weight gain compared with controls. At 3000 ppm, body weight gain was slightly decreased (4–7%). Feed consumption was decreased at 30 000 ppm.

At the F_{1b} breeding to produce F_{2a} litters, gestational parameters were unaffected by treatment. In the F_{2a} pups, perinatal deaths and lactational survival were unaffected by treatment. The number of stillborn F_{2b} pups (n = 15) and deaths from PNDs 1 to 4 (n = 9) were increased at 30 000 ppm compared with controls (n = 2 and 1, respectively). At 30 000 ppm, maternal body weight was about

15% lower than that of control dams throughout gestation and lactation of the F_{2a} and F_{2b} generations. However, body weight gain during these periods was similar. At birth, the F_{2a} and F_{2b} pups at 30 000 ppm had a 10–11% lower body weight compared with control pups. During lactation, the weight gain in high-dose pups was lower, so that at weaning, the body weights of these pups were 26–30% lower than those of controls. A similar reduction in body weight was observed at PND 28. A slight reduction in body weight gain during lactation (up to 10%) was observed in F_{2b} pups at 3000 ppm.

There were no treatment-related lesions observed in the necropsy of F_{2a} and F_{2b} pups or F_{1b} adults. There were also no treatment-related lesions observed in the histopathological examination of selected organs from high-dose and control F_{1b} adults. Terminal body weights were reduced in F_{1b} males (18%) and females (16%) at 30 000 ppm.

The NOAEL for parental toxicity was 300 ppm (equal to 20 mg/kg bw per day), based on an increased incidence of loose faeces in F_{1b} males at 3000 ppm (equal to 200 mg/kg bw per day).

The NOAEL for offspring toxicity was 300 ppm (equal to 22 mg/kg bw per day), based on an increased mortality in F_{1b} pups from PNDs 4 to 7 and a reduction in body weight gain during lactation in F_{2b} pups at 3000 ppm (equal to 220 mg/kg bw per day).

The NOAEL for reproductive toxicity was 30 000 ppm (equal to 2220 mg/kg bw per day), the highest dose tested (Neeper-Bradley & Tyl, 1990).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 25 pregnant Charles River COBS CD rats were treated orally, by gavage, with ethephon (purity and batch number not reported) in 0.5% aqueous Methocel at a dose level of 0, 200, 600 or 1800 mg/kg bw per day from days 6 through 15 of gestation (day 0 = day on which sperm were detected in the vaginal smear). Clinical signs and mortality were recorded daily from GDs 6 to 20. Body weight was measured on GDs 0, 6, 9, 12, 16 and 20. All females were killed on day 20 of gestation and subjected to gross examination. The uterus was examined, and the numbers 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 two thirds of the fetuses from each litter were selected for skeletal examinations, and one third for visceral examinations. Cholinesterase activity measurements were not performed.

At 1800 mg/kg bw per day, 14/25 dams died during the treatment period, and only nine litters with viable fetuses were available for evaluation. Gastroenteritis, respiratory disease and necrotic hepatitis were noted as the immediate causes of death in several of these animals. Mortality was first observed on GD 10 in four dams. Dry red matter around the mouth and/or nose and laboured breathing were noted in the majority of animals that died at this dose. Several rats in this treatment group had excessive salivation and matting and/or staining of the anogenital region towards the end of the treatment period. In the high-dose dams, a body weight loss (4 g) was observed from GD 6 to GD 9, compared with a body weight gain of 7 g in control dams. In the high-dose dams surviving to termination, net body weight gain minus uterine weight (13 g) was lower than in control dams (39 g). In these high-dose dams, necropsy revealed hydronephrosis, distension of the stomach and intestines with gas, enlarged spleen with white coloration on the outer surface, yellowish-brown discoloration of the kidneys and a dark red depressed area on the quadrate lobe of the liver. Histopathological examination of the high-dose dams revealed focal lymphoid hyperplasia of the spleen and focal parenchymal fibrosis of the liver. No effects of treatment on mortality, clinical signs, body weight, macroscopy or histopathology were observed at 200 and 600 mg/kg bw per day. The number of implantations, number of early and late resorptions, number of live fetuses, sex ratio and uterus weight were not affected by treatment. A slightly lower fetal weight (−6%) at 1800 mg/kg bw per day may be due to maternal toxicity or an increased number of viable fetuses at the high dose (14.4 versus 11.9 in controls). Visceral and skeletal examination revealed no treatment-related findings.

The NOAEL for maternal toxicity was 600 mg/kg bw per day, based on increased mortality, clinical signs (salivation), reduced body weight gain, and various macroscopic findings and histological changes (focal lymphoid hyperplasia of the spleen and focal parenchymal fibrosis of the liver) at 1800 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 1800 mg/kg bw per day, the highest dose tested (Rodwell, 1980).

In a developmental toxicity study, groups of 25 mated female Crl:CD(SD)BR rats were treated orally, by gavage, with ethephon (purity 71.7%; batch no. A70471) in distilled water at a dose of 0, 125, 250 or 500 mg/kg bw per day from days 6 through 15 of gestation (day 0 = day on which sperm were detected in the vaginal smear). Clinical signs and mortality were recorded daily. Body weight was measured on GDs 0, 6, 9, 12, 16 and 20. All females were killed on day 20 of gestation and subjected to gross examination. The uterus was examined, and the numbers 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 cross-sectional visceral examinations. Cholinesterase activity measurements were not performed.

No treatment-related effects on mortality, clinical signs, abortions or body weight were observed. Necropsy of the dams revealed no effect of ethephon. The number of implantations, number of early and late resorptions, number of live fetuses, fetal weight, sex ratio and uterus weight were not affected by treatment. Visceral and skeletal examination revealed no treatment-related findings.

The NOAEL for maternal toxicity was 500 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Henwood, 1989b).

Rabbits

In a developmental toxicity study, groups of 17 pregnant female New Zealand White rabbits were treated orally, by gavage, with ethephon (purity unknown; batch no. aa) in deionized water at a dose of 0, 50, 100 or 250 mg/kg bw per day from days 6 through 19 of gestation. Clinical signs and mortality were recorded daily. Body weight was measured on GDs 0, 6, 11, 15, 19 and 29. Feed consumption was measured daily. All females were killed on day 29 of gestation. The uterus was examined, and the numbers of live and dead fetuses, corpora lutea, implantations and resorptions were counted. Body weight and length and sex of the fetuses were recorded. All fetuses were examined for skeletal and visceral anomalies. Cholinesterase activity measurements were not performed.

One control animal, two low-dose animals, four mid-dose animals and eight high-dose animals were found dead or killed in extremis during the study. An increased incidence of inactive animals was noted in the maternal 250 mg/kg bw per day group during the treatment and post-treatment phases. At the middle and high doses, body weight losses of 105 and 187 g, respectively, were observed from GD 6 to GD 11, whereas control and low-dose rabbits gained weight during this period. At the high dose, feed consumption was reduced by about 23% during treatment. Necropsy revealed no effect of treatment. The mean number of resorptions was higher at the middle (2.4) and high doses (1.8) compared with controls (0.8). It is not indicated whether these were early or late resorptions. At 100 and 250 mg/kg bw per day, the mean number of live fetuses (4.7 and 3.1, respectively) and fetal viability (63% and 47%, respectively) were lower than in controls (mean number of live fetuses 5.9, viability 79%), although the differences were not statistically significant. The mean fetal weights and lengths were comparable between the control and treated groups. Visceral and skeletal examination revealed no effect of treatment.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on a body weight reduction from GD 6 to GD 11 and increased number of resorptions at 100 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on the reduced number of live fetuses and viability of fetuses at 100 mg/kg bw per day (Weatherholtz, Wolfe & Durloo, 1981).

In a developmental toxicity study, groups of 22 artificially inseminated female Hra(NZW)SPF rabbits were treated orally, by gavage, with ethephon (purity 72.2%; batch no. 4022193) in deionized water at a dose of 0, 62.5, 125 or 250 mg/kg bw per day from days 7 through 19 of gestation. Clinical signs and mortality were recorded daily. Body weight was measured on GDs 0, 7, 10, 13, 16, 20, 24 and 29. All females were killed on day 29 of gestation. The uterus was examined, and the numbers 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 cross-sectional visceral examinations. Cholinesterase activity measurements were not performed.

At 250 mg/kg bw per day, three does died and 14 does were killed in a moribund condition. Additionally, one dam at 250 mg/kg bw was euthanized because of an eye lesion (buphthalmos and corneal opacity). At the high dose, the majority of the does showed ataxia, reduced activity, prostration and/or yellow-stained anogenital area. The high-dose females lost weight (about 8% on GD 13), whereas the other groups showed a slight increase in body weight (1–2%) during this period. In the two remaining dams at 250 mg/kg bw, the incidences of early resorptions (2 versus 0.5 in controls) and postimplantation loss (43% versus 12% in controls) were high, and the number of live fetuses per litter was low. Visceral and skeletal examination revealed no treatment-related findings.

The NOAEL for maternal toxicity was 125 mg/kg bw per day, based on mortality, clinical signs of toxicity and body weight loss at 250 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 125 mg/kg bw per day (Henwood, 1990). The Meeting considered that the number of fetuses in the high-dose group was insufficient to conclude on the effects of ethephon at 250 mg/kg bw per day on prenatal development.

2.6 Special studies

(a) Mechanistic studies on butyrylcholinesterase inhibition

The mechanistic basis for inhibition of butyrylcholinesterase (cholinesterase) was investigated in vitro. The sensitivity of plasma cholinesterase to ethephon (90-minute preincubation at 25 °C) was greatest for humans, dogs and mice (median inhibitory concentration [IC_{50}] 6–23 µmol/L), intermediate for chickens, rabbits, rats and guinea-pigs (IC_{50} 26–53 µmol/L) and lowest for pigs and horses (IC_{50} 92–172 µmol/L). The IC_{50} decreased linearly with time on a log–log scale to values of 0.15–0.3 µmol/L for human, dog and horse cholinesterase at 24 hours. The inhibition rate was generally related to ethephon concentration, consistent with a bimolecular reaction (e.g. phosphorylation). The extent of inhibition of the esteratic activity of cholinesterase by ethephon was directly proportional to the extent of inhibition of [^{3}H]diisopropyl phosphorofluoridate postlabelling, which is not reversible on removing the ethephon, either directly or after further incubation for 24 hours at 25 °C. These observations strongly suggest that ethephon, similar to diisopropyl phosphorofluoridate, phosphorylates human plasma cholinesterase at the Ser-198 of the esteratic site, leading to the formation of a phosphobutyrylcholinesterase (Haux, 2000).

The metabolism and reactivity as ethylene generators (on the basis of plant growth regulator activity), in vitro inhibition of plasma cholinesterase, and alkylating and phosphorylating properties of the phosphorus-containing components in technical ethephon were investigated. Urinary products of technical ethephon in rats were the parent compound, $HOP(O)(OH)_2$ and unmetabolized $(HO)_2P(O)CH_2CH_2P(O)(OH)_2$. Ethephon was more potent than the impurities present in the technical-grade material as plant growth regulators (tomato epinasty assay), in vitro inhibitors of plasma

cholinesterase and phosphorylating agents. The ethephon metabolite HEPA did not cause inhibition of plasma cholinesterase activity. The authors concluded that the biological activity of technical-grade ethephon appears to be associated with the reactions of its principal component, particularly ethylene liberation, and possibly phosphorylating activity (Segall et al., 1991).

Ethephon as the dianion phosphorylates cholinesterase at its active site. To define the structure–activity relationships and mechanism of cholinesterase inhibition by ethephon (2-chloroethylphosphonic acid), this compound and substituted phenyl moieties (3- and 4-nitrophenyl-, 3- and 4-dimethylaminophenyl- and 3- and 4-trimethylammoniumphenylethylphosphonic acid) were investigated. The study showed that the substituted phenyl moieties decompose under basic conditions about 100-fold faster than ethephon to yield the corresponding styrene derivatives. Electron-withdrawing substituents on the phenyl ring decrease the hydrolysis rate, whereas electron-donating substituents increase the rate. The 4-trimethylammonium analogue had the highest affinity (dissociation constant [K_i] = 180 mmol/L) and potency (IC_{50} = 19 mmol/L) in first binding reversibly at the substrate site (possibly with stabilization in a dianion–monoanion environment) and then progressively and irreversibly inhibiting the enzyme activity. The authors concluded that it is likely that dissociation of chloride is the first and rate-limiting step both in the hydrolysis and, by analogy, in the phosphorylation of cholinesterase by ethephon bound at the active site (Zhang & Casida, 2002).

(a) Acute and subchronic neurotoxicity

Rats

In a pilot study aimed at finding the time to peak effect after a single dose of ethephon, groups of 18 male and 18 female Sprague-Dawley Crl:CDR(SD)BR rats were given ethephon (purity 72.4%; batch no. 4051511) by gavage at 0, 250, 500, 1000 or 2000 mg/kg bw. The animals were observed daily for mortality and clinical signs. Body weight was measured on days 1 and 7. Three rats of each sex per dose were subjected to a functional observational battery 0.5, 1, 2, 4, 6, 8 and 24 hours or on day 7 after dosing. The remaining rats were bled pre-dosing (via the tail vein) for plasma and erythrocyte cholinesterase levels, and from these, three rats of each sex per group were killed 0.5, 1, 2, 4 or 8 hours post-dosing for plasma, erythrocyte and brain cholinesterase determinations.

No treatment-related mortality and no clinical signs were observed for animals treated with ethephon. Moderate to large body weight losses (at least 14 g) were observed for all males and one female in the 2000 mg/kg bw group and for one male in the 1000 mg/kg bw group at the first day after treatment. These same animals tended to gain more weight from days 1 to 7 compared with the controls.

Erythrocyte and brain AChE levels were not affected by treatment. Plasma cholinesterase levels were decreased for both males and females of all treated groups and in a dose-related manner from 0.5 hour post-dosing for the 1000 and 2000 mg/kg bw groups and from 1 hour post-dosing for the other treated groups. The maximum suppression for all groups was at 4–8 hours following treatment (Beyrouty, 1996a).

In an acute neurotoxicity study, performed according to OECD Test Guideline 424, groups of 12 male and 12 female Sprague-Dawley Crl:CDR(SD)BR rats were given ethephon (purity 72.4%; batch no. 4051511) by gavage at 0, 500, 1000 or 2000 mg/kg bw. The animals were observed daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and feed consumption were measured weekly. The rats were subjected to a functional observational battery and a motor activity test on day 0 at about 5–5.5 hours after dosing and on days 7 and 14. At study completion on day 15, all rats were necropsied. Neurohistological examination of the brain, ganglions, nerves, eyes and gastrocnemius muscle was performed on six rats of each sex per dose.

Two females dosed at 2000 mg/kg bw died within 2 days after dosing, and one female at 1000 mg/kg bw was found dead on day 5. At 2000 mg/kg bw, laboured breathing, piloerection, prominent backbone, thin body condition, swollen abdominal region, cold to touch, reduced activity and fur staining were observed. At 2000 mg/kg bw, body weight was 6–7% lower than in controls at day 7 after dosing. Feed consumption during the first week after treatment was 9% lower than in controls in mid-dose females and high-dose males and females. Increased incidences of myosis were observed at 2000 mg/kg bw (6/12 in males; 10/12 in females), 1000 mg/kg bw (3/12 males; 5/12 females) and 500 mg/kg bw (3/12 males; 5/12 females), compared with controls (1/12 males and females). The myosis persisted up to day 14 in a few treated animals. Hypothermia was observed in high-dose rats on day 0. A statistically significant increase in urination was noted for males at 2000 mg/kg bw on day 0, and reduced motor activity was observed in males at 1000 and 2000 mg/kg bw and females at 2000 mg/kg bw on day 0. Brain weight, length and width were not affected. Macroscopic postmortem examination and histopathological examination of nervous tissues did not reveal any abnormalities.

A NOAEL could not be identified. The LOAEL was 500 mg/kg bw, based on increased incidences of myosis at all doses (Beyrouty, 1996b).

In a 2-week range-finding toxicity study, ethephon (purity 72.4%; batch no. A4051511) was administered to groups of six male and six female Sprague-Dawley Crl:CD(SD)BR rats by gavage at 0, 100, 300, 600 or 1000 mg/kg bw per day (doses corrected for purity). Animals were checked daily for clinical signs of toxicity. A detailed physical examination was performed weekly. Body weights were measured twice per week, and feed consumption was measured weekly. Before the treatment was started and prior to dosing on days 2, 8 and 15, the rats were subjected to a functional observational battery, and blood was sampled. At termination on day 15, all animals were necropsied.

At 600 mg/kg bw per day, two males and four females were found dead. At 1000 mg/kg bw per day, all males and five females were found dead or were killed in a moribund condition. In most of the rats at 600 and 1000 mg/kg bw per day, fur staining, skin pallor, abnormal breathing, respiratory sounds, dehydration, cold to touch, decreased activity, weak appearance and abdominal distension were observed. In the lower-dose groups (i.e. 100 and 300 mg/kg bw per day), no treatment-related clinical signs were observed. Body weight loss and reduced feed consumption were observed at 600 and 1000 mg/kg bw per day.

In the functional observational battery, abnormal breathing, myosis, muzzle staining, diarrhoea and impaired gait were observed in a few rats at 300 mg/kg bw per day and above. Red blood cell cholinesterase levels were not affected by treatment. Dose-dependent decreases in plasma cholinesterase levels were observed in all treatment groups (Beyrouty, 1997a).

In a 13-week neurotoxicity study, ethephon (purity 72.4%; batch no. A4051511) was administered to groups of 22 male and 22 female Sprague-Dawley Crl:CD(SD)BR rats by gavage at 0, 75, 150 or 400 mg/kg bw per day. The high dose level was decreased to 300 mg/kg bw per day during week 10/11 of treatment. Animals were checked daily for clinical signs of toxicity. A detailed physical examination was performed weekly. Body weights and feed consumption were measured weekly. Before the treatment was started and prior to dosing in weeks 4, 8 and 13, 12 rats of each sex per dose were subjected to a functional observational battery and motor activity test. In the remaining 10 animals of each sex per dose, blood was sampled for cholinesterase measurements in weeks 4 and 8 and at termination in week 13. At termination, all animals were necropsied. The brains of 10 animals of each sex per dose, with the exception of nine females at the top dose, were sampled for AChE measurements. Neuropathological examination was performed on six rats of each sex per dose.

At 400 mg/kg bw per day, three males and three females were found dead on week 5 (one male and one female) and week 10 of treatment (two males and two females). Abnormal breathing was shown by 13 males and 11 females at 400/300 mg/kg bw per day. Six high-dose males were cold to touch, and five males and one female at this dose showed a weak/thin/dehydrated appearance. Slightly reduced body weight gains (up to 9%) were noted in rats at 400/300 mg/kg bw per day. There

Table 12. Cholinesterase activity in the erythrocytes, brain and plasma of rats treated with ethephon for 13 weeks

	% reduction compared with controls					
	75 mg/kg bw per day		150 mg/kg bw per day		400/300 mg/kg bw per day	
	M	F	M	F	M	F
Week 4						
Plasma ChE	18.6*	21.2*	19.0*	34.7*	32.3*	53.5*
Erythrocyte AChE	4.6	16.7*	9.6	21.7*	16.8*	23.6*
Week 8						
Plasma ChE	15.1*	34.6	17.1*	48.2*	31.5*	62.6*
Erythrocyte AChE	2.5	19.1*	7.6	24.7*	15.5*	30.2*
Week 13						
Plasma ChE	20.3*	43.3	20.6*	56.0*	25.2*	63.9*
Erythrocyte AChE	8.1*	10.0	12.3*	9.1	21.8*	18.6*
Brain AChE	+	4.5	+	5.5	+	8.5*

AChE: acetylcholinesterase; bw: body weight; ChE: cholinesterase; F: females; M: males; +: AChE activity was greater than control values; *: $P < 0.05$ (Dunnett's)
Source: Beyrouty (1997b)

were no behavioural changes observed in the functional observational battery and motor activity tests that were indicative of neurotoxicity.

The effects of ethephon on cholinesterase activity in this study are presented in Table 12.

Macroscopic and histopathological examination of nervous tissues did not reveal any abnormalities.

The NOAEL was 75 mg/kg bw per day, based on a statistically significant reduction of erythrocyte AChE activity in females at 150 mg/kg bw per day (Beyrouty, 1997b).

Dogs

To determine a NOAEL for reduction of cholinesterase activity, three female Beagle dogs per dose group received ethephon (purity 71.3%; batch no. 2250197) at a dietary concentration of 0, 250 or 750 ppm (equal to 0, 6 and 14 mg/kg bw per day, respectively) for 28 days (doses corrected for purity). All animals were checked daily for clinical signs. Detailed clinical examinations were performed weekly. Feed consumption was measured daily and body weights were measured weekly to calculate the intake of ethephon. Plasma cholinesterase and erythrocyte AChE activity were measured for all animals once prior to administration of the test substance and during days 7, 14, 21 and 28, and brain AChE activity was determined at study termination. Other examinations, including haematology, clinical chemistry and pathological analysis, were not performed.

There were no animals found dead or moribund and no compound-related clinical observations. The effects of ethephon on cholinesterase activity are presented in Table 13. It is noted that the cholinesterase measurements were not performed at the time of peak plasma cholinesterase inhibition after a gavage dose (4–8 hours post-dosing; see Beyrouti, 1996a).

The NOAEL for cholinesterase inhibition was 250 ppm (equal to 6 mg/kg bw per day), based on greater than 20% inhibition of erythrocyte AChE activity with statistical significance at 750 ppm (equal to 14 mg/kg bw per day) (Eigenberg, 2006a).

Table 13. Cholinesterase activity in the erythrocytes, brain and plasma of female dogs treated with ethephon for 28 days

	% reduction relative to controls[a]			
Dietary concentration (ppm)	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Erythrocyte AChE				
250	+	+	+	5
750	19	39	50*	58*
Brain AChE				
250	ND	ND	ND	4
750	ND	ND	ND	+
Plasma ChE				
250	30	46*	48*	49*
750	56*	63*	62*	60*

AChE: acetylcholinesterase; ChE: cholinesterase; ND: not determined; ppm: parts per million; +: AChE activity was greater than control values; *: $P < 0.05$ (ANOVA + Student's *t*-tests, two-sided)
Source: Eigenberg (2006a)

In a 91-day toxicity study to evaluate the effects of ethephon on blood and brain cholinesterase inhibition, four male and four female Beagle dogs per dose group received ethephon (purity 71.9%; batch no. 040201) at a dietary concentration of 0, 70, 140 or 525 ppm (equal to 0, 2, 4 and 15 mg/kg bw per day for males and 0, 2, 4 and 18 mg/kg bw per day for females, respectively). All animals were checked daily for clinical signs. Detailed clinical examinations were performed weekly. Feed consumption was measured daily, and body weights were measured weekly. Plasma cholinesterase and erythrocyte AChE activity were measured on all animals twice prior to administration of the test substance and during days 3, 10, 25, 53, 70 and 87. At study termination, brain AChE activity was measured. Other examinations, including haematology, clinical chemistry and pathological analysis, were not performed.

There was no mortality or treatment-related clinical signs. There was no compound-related effect on body weight or feed consumption. The effects of ethephon on cholinesterase activity are presented in Table 14. Erythrocyte AChE activity was statistically significantly inhibited at 525 ppm from day 10 in males and at 140 ppm from day 53 and 525 ppm from day 25 in females, compared with the mean pretreatment values for each treated group. In the female 140 ppm and male and female 525 ppm groups, these AChE inhibitions were greater than 20% compared with the corresponding control groups at the time points when the cholinesterase inhibition was statistically significant compared with the pretreatment values. Brain AChE activity was not affected by the treatment in males.

The NOAEL for cholinesterase inhibition was 70 ppm (equal to 2 mg/kg bw per day), based on greater than 20% reduction of erythrocyte AChE activity with statistical significance in females at 140 ppm (equal to 4 mg/kg bw per day) (Eigenberg, 2006b)

(c) Delayed neurotoxicity

Groups of 10 white Vantress chickens received ethephon by intubation at 1000 mg/kg bw per day (purity 88%; batch number not reported) on days 1 through 5 or at 1000 mg/kg bw on day 1 and thereafter at 500 mg/kg bw per day on days 2 through 10. Two positive control groups received tri-*o*-cresyl phosphate at 60 mg/kg bw per day. Two negative control groups received olive oil at 300 mg/kg bw per day.

Table 14. Cholinesterase activity in erythrocytes, plasma and brain of dogs treated with ethephon for 91 days

	% reduction compared with controls[a]					
	70 ppm		140 ppm		525 ppm	
	Males	Females	Males	Females	Males	Females
Erythrocyte AChE						
Day 3	6	22	10	+	9	28
Day 10	6	29	16	7	22*	37
Day 25	13	34	35	16	58*	59*
Day 53	18	36	35	31*	73*	70*
Day 70	21	39	40	32*	75*	72*
Day 87	3	37	40	29*	72*	70*
Brain AChE						
Day 91	+	8#	+	10#	+	14#
Plasma ChE						
Day 3	9*	42	23*	45	50*	52*
Day 10	33*	62*	38*	65*	46*	65*
Day 25	35*	57*	43*	57*	62*	63*
Day 53	34*	60*	39*	56*	61*	61*
Day 70	28*	60*	41*	58*	61*	63*
Day 87	34*	58*	37*	55*	58*	60*

AChE: acetylcholinesterase; ChE: cholinesterase; ppm: parts per million; +: AChE activity was greater than control values; *: $P < 0.05$ (ANOVA + Dunnett's tests) versus the pretreatment value; #: $P < 0.05$ (ANOVA + Student's *t*-tests) versus the control

Source: Eigenberg (2006b)

No clinical signs of neurotoxicity and no gross pathology were observed in any of the necropsied chickens treated with ethephon. Microscopic examination showed no cytopathological changes in the spinal cord or sciatic nerve of the animals receiving ethephon. Administration of tri-*o*-cresyl phosphate caused clinical signs of neurotoxicity and some spinal axonal dystrophy in 10 chickens and sciatic neuropathy in one chicken.

There was no evidence of delayed neurotoxicity induced by ethephon (Weatherholtz & Shott, 1970).

Groups of 15–30 white leghorn chickens received a single oral dose of ethephon (purity 71%; batch number not reported) of 0, 3160 or 3850 mg/kg bw. A positive control group received tri-*o*-tolyl phosphate orally at 500 mg/kg bw. Twenty-one days following dosing, all surviving birds ($n = 21$) were treated orally with a single dose of ethephon at 2370 mg/kg bw.

Twenty-eight of 30 birds in the 3850 mg/kg bw group were found dead within 24 hours after the first dose. One additional mortality was recorded in this group on test day 8. Ten of 30 birds in the 3160 mg/kg bw group were found dead within 24 hours after the first dose. One bird from this group was found dead within 48 hours after the second dose. Signs of lethargy and anorexia were present

following dosing. Complete recovery of all surviving birds was seen during both 21-day test periods. There were no signs of locomotor disturbances or other clinical signs of delayed neurotoxicity among any of the ethephon-treated chickens during the 42-day test period. Ethephon-treated birds exhibited decreased feed intake and body weight loss during the test period. The positive control birds lost weight and exhibited behavioural signs of neurotoxicity by day 9 of the investigation. All positive control birds were killed in extremis on test day 17 or 18. Gross pathological examination of birds found dead within 24 hours after dosing revealed diffuse red discoloration with severe dilatation of the vessels in the intestinal tract and diffuse light grey discoloration with transparent gel circumscribing the crop area in all birds. Histopathology of neural tissues from the ethephon-treated birds revealed no changes. Treatment-related lesions were noted with respect to the positive control birds.

No evidence of delayed neurotoxicity was observed in this study at 3160 mg/kg bw. The 3850 mg/kg bw dose could not be evaluated, as only one bird survived the observation period at this high dose (Fletcher, 1983).

In a delayed neurotoxicity study performed according to OECD Test Guideline 418, a group of 20 Hyline hens received ethephon (purity 71.3%; batch no. 040201) at a single oral dose of 2000 mg/kg bw. Sixteen control hens received water. Observations for mortality, adverse clinical signs, assessment of delayed locomotor ataxia and body weight were performed at scheduled intervals during the study. Twenty-four and 48 hours after dosing, three birds from each treatment group at each time point were killed, and brain and spinal cord tissues were examined for AChE and neurotoxic esterase activities. Positive control data using tri-*o*-cresyl phosphate were generated and reported as a separate study.

No clinical signs of delayed locomotor ataxia were observed in any treated or control birds during the study. There was no sign of any enzymatic inhibition of either AChE or neurotoxic esterase in either the group dosed with ethephon or the control group at any sampling time. No treatment-related findings on body weight gain or macroscopic or histological examination were detected (Rodgers, 2005).

(d) Studies with metabolites

Acute and short-term toxicity and genotoxicity studies with HEPA, the major metabolite of ethephon, were available. HEPA is a major metabolite in kidneys of male rats and also the main plant metabolite.

Acute toxicity

The results of studies of acute toxicity with HEPA are summarized in Table 15.

Table 15. Results of acute toxicity studies with HEPA

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50} (mg/kg bw)	Reference[a]
Rat	Crl:WI(Glx/BRL/Han)BR)	M + F	Oral	Water	95.7	> 2 000 (M/F)	Denton (2001)

bw: body weight; F: female; LD_{50}: median lethal dose; M: male

[a] Statements of adherence to quality assurance and GLP were included in all studies.

[b] Batch no. B907. Study was performed according to OECD Test Guideline 423. A dose of 2000 mg/kg bw was administered by gavage to three male and three female rats. Vehicle was water. There were no deaths. Clinical signs were diarrhoea and lethargy. A slight body weight loss was observed in all rats 1 day after treatment and in one male and one female 1 week after treatment. Necropsy revealed no macroscopic changes.

Short-term studies of toxicity

In a 2-week range-finding toxicity study, HEPA (purity 95.9%; batch no. B960/LJ33246) was administered to groups of five male and five female Sprague-Dawley ICO: OFA. SD. (IOPS Caw) rats by gavage at 0, 125, 250 or 500 mg/kg bw per day. Animals were checked daily for clinical signs of toxicity. Body weights were measured before the start of treatment and on days 1, 7 and 15. Feed consumption was measured weekly. On day 16, blood was sampled for haematology and clinical chemistry. Urine analysis was performed on day 14. After termination on day 16, all animals were necropsied, and brain, liver, kidneys, spleen, ovaries/testes and thyroids were weighed.

At 500 mg/kg bw per day, liver weights were increased (15–16%). No other treatment-related effects were observed (Bigot, 2003a).

In a 28-day toxicity study, HEPA (purity 95.9%; batch no. B960/LJ33246) was administered to groups of 10 male and 10 female Sprague-Dawley ICO: OFA. SD. (IOPS Caw) rats by gavage at 0, 125, 350 or 1000/700 mg/kg bw per day. The highest dose level was reduced from 1000 to 700 mg/kg bw per day from day 5 onwards, as a result of mortality. Animals were checked daily for clinical signs of toxicity. Detailed physical examinations were performed at least weekly. Body weights were measured before the start of treatment, on days 1, 4, 8, 15, 22 and 28 and before necropsy. Feed consumption was measured weekly. Ophthalmoscopy was performed before treatment and on day 28. On day 24 or 25, blood was sampled for haematology and clinical chemistry. Urine analysis was performed on day 29, 30 or 31. After termination on day 29, 30 or 31, all animals were necropsied, and adrenal glands, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed. Histopathological examination of a wide range of organs and tissues was performed on all control and high-dose rats, as well as all animals that died before termination in the intermediate-dose group.

At 1000 mg/kg bw per day, one male and two females were found dead on day 4, and one female was killed for humane reasons on day 4. After lowering the dose to 700 mg/kg bw per day on day 5, one male was found dead on day 21. Seven animals showed body weight loss from days 1 to 4, and on day 8, after reduction of the dose from 1000 to 700 mg/kg bw per day, the mean body weight gain was reduced by 28–42% when compared with controls. Feed consumption was reduced (−12%) in females during the first week of treatment. At 1000/700 mg/kg bw per day, wasted appearance, laboured/noisy respiration, piloerection, few/soft/mucoid faeces, reduced motor activity, increased salivation, cold to touch, skin lesions, hair loss and scabs were observed. No changes were noted during the neurotoxicity assessment and at ophthalmological examination. Haematology and clinical chemistry showed no effect of treatment. Urine analysis showed lower pH and ketone levels in males with fewer crystals than usually observed. In animals found dead or killed for humane reasons, macroscopic findings were gaseous distension of stomach/intestines, pale/small spleen, red foci/mottled thymus, dark red lung, dark liver, small prostate gland and small seminal vesicles. Microscopic examination of these animals revealed epithelial necrosis and intraluminal inflammatory exudates in trachea.

The NOAEL was 350 mg/kg bw per day, based on mortality, clinical signs, reduced body weight gain and feed consumption (females only), changes in urinary parameters and various macroscopic findings and histological changes (epithelial necrosis and intraluminal inflammatory exudates in trachea) observed at 1000/700 mg/kg bw per day. The effects observed at the high dose are considered related to the gavage administration and the physicochemical properties of HEPA (Bigot, 2003b).

Table 16. Results of studies on the genotoxicity of HEPA

End-point	Test object	Concentration	Purity (%)	Results	Reference[a]
In vitro					
Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538	1.6–5 000 µg/plate (±S9)	95.7	Negative	Johnson (2002)[b]
Gene mutation	Mouse lymphoma L5178Y (*TK*) locus	40–1 260 µg/mL (−S9), 2.5–40 µg/mL (±S9), experiment 1 200–1260 µg/mL (±S9), experiment 2	95.7	Negative	Ballantyne (2002)[c]
Chromosomal aberrations	Human peripheral blood lymphocytes	516.5–1 261 µg/mL (−S9, 3 h); 404.3–1 261 µg/mL (−S9, 20 h); 807–1 261 µg/mL (+S9, 3 h); 911–1 261 µg/mL (+S9, repeat assay, 3 h)	95.7	Negative	Whitwell (2002)[d]

S9: 9000 × *g* supernatant fraction from rat liver homogenate; *TK*: thymidine kinase

[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. B907. Performed according to OECD Test Guideline 471. Vehicle was purified water. Toxicity was observed at 1000 and 5000 µg/plate. HEPA did not induce point mutations in *S. typhimurium* under the conditions of the test.

[c] Batch no. B907. Performed in accordance with OECD Test Guideline 476. Vehicle was purified water. No cytotoxicity was observed up to the highest tested dose of 1260 µg/mL. HEPA did not induce gene mutations in mouse lymphoma cells under the conditions of the test.

[d] Batch no. B907. Performed in accordance with OECD Test Guideline 473. Vehicle was purified water. Cytotoxicity was observed at a dose level of 1072 µg/mL (−S9, 20-hour exposure). HEPA did not induce chromosomal aberrations in cultured human peripheral blood lymphocytes under the conditions of the test.

Genotoxicity

The results of genotoxicity studies with HEPA are summarized in Table 16.

3. Observations in humans

In a range-finding study with ethephon, two volunteers received ethephon (purity 88%; batch no. 514A) at increasing doses of 5.4–120 mg/day by oral capsule, in three divided doses, one immediately after each meal over a 7-week period. The dose reportedly ranged from 0.06 to 1.25 mg/kg bw per day. Blood was sampled twice per week for assessment of cholinesterase activity. Haematology, clinical chemistry and urine analysis were performed weekly.

No effect of ethephon on haematology and plasma or erythrocyte cholinesterase activity was observed. Transient, subjective feelings of urinary urgency were experienced by both volunteers. No persistent side-effects were observed during the course of the study. A slight elevation in ALAT activity was observed from day 26 to day 46. Laboratory studies performed 2 weeks following the last ethephon administration gave test results within normal biological limits (Reese, 1971).

Five male and five female volunteers received technical ethephon (formulation consisting of 10% ethephon, hydrated silica and cornstarch) at a dose of 124 mg ethephon per day for 28 days by oral capsule, approximately equal to 1.5 mg/kg bw per day for males and 2.2 mg/kg bw per day for females. Three males and three females were given a placebo. Each subject received two capsules postprandially for the first two dosing periods; the third dose (two capsules) was given at the end of the workday. All subjects were monitored constantly during the first 8 hours following ingestion of

the test material for adverse effects or symptoms related to compound administration and once daily thereafter. Cholinesterase activity in plasma and red blood cells was measured before the start of dosing, on days 1, 2, 7, 14 and 28 of the study and 2 weeks following the last dose. Haematology, clinical chemistry and urine analysis were performed weekly.

No changes in plasma or erythrocyte cholinesterase activity and no persistent side-effects were observed. However, transient subjective complaints, such as diarrhoea or urgency of bowel movements, were observed on 1–4 days in the first week of treatment in four volunteers receiving ethephon, but not in control subjects. Urgency or an increased frequency of urination was observed during the course of the study in one control and five treated volunteers. In addition, loose stools, stomach cramps and/or gas, flank pain, and loss or increase of appetite were occasionally reported by some volunteers treated with ethephon. No treatment-related changes in haematology, clinical biochemistry or urine analysis were noted (Reese, 1972).

Ten male and 10 female volunteers received technical ethephon (formulation consisting of 2.5% ethephon, hydrated silica and cornstarch) at a dose of 0.5 mg/kg bw per day by oral capsule, divided over three daily dosages, for 16 days. Six males and four females were given placebo. All subjects were monitored constantly during the first 8 hours following ingestion of the test material for adverse effects or symptoms related to compound administration and once daily thereafter. Cholinesterase activity in plasma and red blood cells was measured before the start of dosing, on days 4, 8, 12 and 16 of treatment, and on days 15 and 29 of a recovery period. Haematology and clinical chemistry were performed before the treatment started, on days 8 and 16 of treatment and on day 29 of the recovery period. Urine analysis was performed on days 1, 9 and 16 of the dosing period.

No treatment-related clinical signs or changes in erythrocyte AChE values, haematology, clinical chemistry or urine analysis were observed. Plasma cholinesterase activity was significantly inhibited (54–62% of pre-dosing levels) in a reversible manner.

The NOAEL was 0.5 mg/kg bw per day, based on lack of inhibition of erythrocyte AChE activity (Weir, 1977a).

In a volunteer study, ethephon (formulation consisting of 21.6% ethephon, hydrated silica and cornstarch) was administered orally for 22 days in capsules to three males and four females at 0.17 mg/kg bw per day and to four males and three females at 0.33 mg/kg bw per day, divided into three doses, for 22 days, followed by a 14-day recovery period. Three males and three females were given placebo. All subjects were monitored constantly during the first 8 hours following ingestion of the test material for adverse effects or symptoms related to compound administration and once daily thereafter. Haematology and clinical chemistry and assessment of cholinesterase activity in plasma and red blood cells were performed before the start of dosing, on days 8, 15 and 22 of treatment and on days 8 and 14 of the recovery period. Urine analysis was performed on days 1, 9 and 16 of the dosing period.

No treatment-related clinical signs or changes in erythrocyte AChE activity, haematology, clinical chemistry or urine analysis were observed. Plasma cholinesterase activity was significantly inhibited (59–74% of pre-dosing levels). The plasma cholinesterase inhibition was not reversible during the 14-day recovery period.

The NOAEL was 0.33 mg/kg bw per day, the highest dose tested (Weir, 1977b).

Comments

Biochemical aspects

In rats, absorption of ethephon was rapid, with a T_{max} of 1.0–1.3 hours after a single oral dose of 50 mg/kg bw and 1.9–2.5 hours after a single oral dose of 1000 mg/kg bw. Peak blood concentrations at 1000 mg/kg bw were less than proportional to dose, compared with those after 50 mg/kg bw. Six days after a single dose, tissues and carcass contained at most 0.06% of the administered radioactivity. Highest concentrations were found in bone, liver, blood and kidney. Radioactivity concentrations in brain were low. Radioactivity was excreted in urine (47–60%), expired air (18–22%, mainly ethylene) and faeces (4.0–6.5%), indicating that at least 65% of the administered dose was absorbed. Excretion was largely complete within the first 24 hours after dose administration (Savage, 1990). Ethephon was mainly recovered as its monosodium and disodium salts, ethylene and, to a lesser extent, HEPA (Hardy et al., 1990; Savage, 1990; Odin-Feurtet, 2002). There were no remarkable differences in absorption and excretion between sexes and between oral dosing regimens (Savage, 1990).

Ethephon inhibits butyrylcholinesterase (cholinesterase) activity in plasma and, to a lesser extent, AChE activity in erythrocytes. Ethephon has virtually no effect on brain AChE activity in vivo. In vitro studies showed that cholinesterase in plasma of dog, human and mouse was more sensitive to ethephon inhibition than cholinesterase in plasma of rabbit, rat, chicken and guinea-pig (Haux, 2000). Mechanistic investigations indicate that ethephon inhibits cholinesterase activity by phosphorylation at Ser-198 of the esteratic site, leading to the formation of a phosphobutyryl-cholinesterase (Haux, 2000).

Toxicological data

The acute toxicity of ethephon is low (rat oral LD_{50} = 1564 mg/kg bw [Myers, 1989]; rabbit dermal LD_{50} = 983 mg/kg bw [Myers, 1983]; rat inhalation LC_{50} = 3.26 mg/L [Nachreiner & Klonne, 1989]). Ethephon was severely irritating to the skin of rabbits (Myers, 1983). No eye irritation study was required, as technical ethephon has a pH of less than 2 and is therefore assumed to be corrosive to the eye. Ethephon was not a skin sensitizer in a Magnusson and Kligman test in guinea-pigs (Griffon, 2000).

In repeated-dose oral toxicity studies with ethephon in mice, rats and dogs, the main effect was reduction of erythrocyte AChE activity.

In a 28-day study in mice administered ethephon at a dietary concentration of 0, 30, 100, 300, 1000 or 3000 ppm (equal to 0, 5.3, 18, 51, 181 and 546 mg/kg bw per day for males and 0, 6.5, 22, 69, 210 and 635 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 22 mg/kg bw per day), based on reduction of erythrocyte AChE activity observed in females at 300 ppm (equal to 69 mg/kg bw per day) (Van Miller & Troup, 1986a). In a second 28-day study in mice administered ethephon at a dietary concentration of 0, 3000, 10 000, 25 000 or 50 000 ppm (equal to 0, 530, 1800, 4500 and 10 000 mg/kg bw per day for males and 0, 630, 2200, 5900 and 15 000 mg/kg bw per day for females, respectively), no NOAEL could be identified, as reductions in erythrocyte AChE activity were observed at all doses (Van Miller & Troup, 1986b).

In a 28-day range-finding study in rats administered ethephon at a dietary concentration of 0, 625, 1250, 2500, 5000 or 10 000 ppm (equal to 0, 52, 106, 214, 431 and 831 mg/kg bw per day for males and 0, 59, 120, 251, 487 and 980 mg/kg bw per day for females, respectively), the NOAEL was 625 ppm (equal to 52 mg/kg bw per day), based on reduction of erythrocyte AChE activity observed in males at 1250 ppm (equal to 106 mg/kg bw per day) (Van Miller & Troup, 1986c). In a second 28-day range-finding study in rats administered ethephon at a dietary concentration of 0, 10 000, 25 000 or 50 000 ppm (equal to 0, 962, 2300 and 4673 mg/kg bw per day for males and 0, 996, 2488 and 4900 mg/kg bw per day for females, respectively), no NOAEL could be identified, as reduction of AChE activity in erythrocytes was observed at all doses (Van Miller & Troup, 1986d).

In a 1-year study in dogs administered ethephon at a dietary concentration of 0, 100, 300, 1000 or 2000 ppm (equal to 0, 2.8, 8.1, 27 and 54 mg/kg bw per day for males and 0, 2.6, 8.4, 30 and 50 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 27 mg/kg bw per day), based on a lower body weight gain at 52 weeks in both sexes and low absolute and relative spleen weights in males at 2000 ppm (equal to 54 mg/kg bw per day). The effect of ethephon treatment on cholinesterase activity was not assessed in this study (Hamada, 1989).

In a 2-year study in dogs administered ethephon at a dietary concentration of 0, 30, 300 or 1500 ppm (equal to 0, 0.86, 7.6 and 42.2 mg/kg bw per day for males and 0, 0.86, 8.4 and 47.8 mg/kg bw per day for females, respectively), the NOAEL was 30 ppm (equal to 0.86 mg/kg bw per day), based on reduction of erythrocyte AChE activity at 300 ppm (equal to 7.6 mg/kg bw per day) (Reno & Voelker, 1977).

In a 78-week carcinogenicity study in mice administered ethephon at a dietary concentration of 0, 30, 300 or 1000 ppm (equivalent to 0, 4.5, 45 and 150 mg/kg bw per day, respectively), the NOAEL was 30 ppm (equivalent to 4.5 mg/kg bw per day), based on reduction of erythrocyte AChE activity observed at weeks 52 and 78 in females at 300 ppm (equivalent to 45 mg/kg bw per day). No treatment-related tumours were observed in mice in this study (Voss & Becci, 1985).

In a second 78-week carcinogenicity study in which mice were administered ethephon at a dietary concentration of 0, 100, 1000 or 10 000 ppm (equal to 0, 14, 139 and 1477 mg/kg bw per day for males and 0, 17, 173 and 1782 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 14 mg/kg bw per day), based on reduction of erythrocyte AChE activity in both sexes at 1000 ppm (equal to 139 mg/kg bw per day). No treatment-related tumours were observed in mice in this study (Van Miller, 1988).

The overall NOAEL for the two 78-week studies in mice was 100 ppm (equal to 14 mg/kg bw per day). The overall LOAEL was 300 ppm (equivalent to 45 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in rats administered ethephon at a dietary concentration of 0, 30, 300 or 3000 ppm (equal to 0, 1.2, 13 and 129 mg/kg bw per day for males and 0, 1.6, 16 and 171 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 13 mg/kg bw per day), based on reduction of erythrocyte AChE activity in both sexes at 3000 ppm (equal to 129 mg/kg bw per day). No treatment-related tumours were observed in rats in this study (Reno, Serota & Voelker, 1978).

In a second 2-year toxicity and carcinogenicity study in which rats were administered ethephon at a dietary concentration of 0, 300, 3000, 10 000 or 30 000 ppm (equal to 0, 13, 131, 446 and 1416 mg/kg bw per day for males and 0, 16, 161, 543 and 1794 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 13 mg/kg bw per day), based on reduction of erythrocyte AChE activity observed in both sexes at 3000 ppm (equal to 131 mg/kg bw per day). No treatment-related tumours were observed in rats in this study (Van Miller, 1989).

The overall NOAEL for the two 2-year studies in rats was 300 ppm (equal to 13 mg/kg bw per day). The overall LOAEL was 3000 ppm (equal to 129 mg/kg bw per day).

The Meeting concluded that ethephon is not carcinogenic in mice or rats.

Ethephon was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. There was no evidence of genotoxicity in vitro (Godek, Naismith & Matthews, 1983, 1984; Barfknecht, Naismith & Matthews, 1984; Cifone, 1988; Murli, 1988; Young, 1988), except for a positive response in *Salmonella typhimurium* strain TA1535 in both the absence and presence of metabolic activation (Jagannath, 1987). There was no evidence of genotoxicity in vivo (Naismith & Matthews, 1979; Sorg, Naismith & Matthews, 1981; Howe, 2002).

Based on the weight of evidence, the Meeting concluded that ethephon is unlikely to be genotoxic in vivo.

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

In a two-generation reproductive toxicity study in rats administered ethephon at a dietary concentration of 0, 300, 3000 or 30 000 ppm (equal to 0, 22, 220 and 2260 mg/kg bw per day for F_0 males and 0, 25, 260 and 2570 mg/kg bw per day for F_0 females, respectively; and 0, 20, 200 and 2220 mg/kg bw per day for F_{1b} males and 0, 24, 245 and 2520 mg/kg bw per day for F_{1b} females, respectively), the NOAEL for parental toxicity was 300 ppm (equal to 20 mg/kg bw per day), based on an increased incidence of loose faeces in F_{1b} males at 3000 ppm (equal to 200 mg/kg bw per day). The NOAEL for offspring toxicity was 300 ppm (equal to 22 mg/kg bw per day), based on an increased mortality in F_{1b} pups from PND 4 to PND 7 and a reduction in body weight gain during lactation in F_{2b} pups at 3000 ppm (equal to 220 mg/kg bw per day). The NOAEL for reproductive toxicity was 30 000 ppm (equal to 2220 mg/kg bw per day), the highest dose tested. The effect of ethephon treatment on cholinesterase activity was not assessed in this study (Neeper-Bradley & Tyl, 1990).

In a developmental toxicity study in rats administered ethephon by gavage at a dose of 0, 200, 600 or 1800 mg/kg bw per day, the NOAEL for maternal toxicity was 600 mg/kg bw per day, based on increased mortality, clinical signs (salivation), reduced body weight gain, and various macroscopic findings and histological changes (focal lymphoid hyperplasia of the spleen and focal parenchymal fibrosis of the liver) at 1800 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 1800 mg/kg bw per day, the highest dose tested. The effect of ethephon treatment on cholinesterase activity was not assessed in this study (Rodwell, 1980).

In a second developmental toxicity study in rats administered ethephon by gavage at a dose of 0, 125, 250 or 500 mg/kg bw per day, the NOAEL for maternal toxicity and for embryo and fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Henwood, 1989b). The effect of ethephon treatment on cholinesterase activity was not assessed in this study.

In a developmental toxicity study in rabbits administered ethephon by gavage at a dose of 0, 50, 100 or 250 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on a body weight reduction from GD 6 to GD 11 and an increased number of resorptions at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on a reduced number of live fetuses and reduced viability of fetuses at 100 mg/kg bw per day. The effect of ethephon treatment on cholinesterase activity was not assessed in this study (Weatherholtz, Wolfe & Durloo, 1981).

In a second developmental toxicity study in rabbits administered ethephon by gavage at a dose of 0, 62.5, 125 or 250 mg/kg bw per day, the NOAEL for maternal toxicity was 125 mg/kg bw per day, based on mortality, clinical signs of toxicity and decreased body weight at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 125 mg/kg bw per day. As three does died and 14 does were killed in a moribund condition at 250 mg/kg bw per day, the number of fetuses in the high-dose group was insufficient to conclude on the effects of ethephon on prenatal development at 250 mg/kg bw per day (Henwood, 1990). The effect of ethephon treatment on cholinesterase activity was not assessed in this study.

In a pilot neurotoxicity study in rats aimed at finding the time to peak effect after a single gavage dose of ethephon of 0, 250, 500, 1000 or 2000 mg/kg bw, the maximum suppression of plasma cholinesterase activity for all groups occurred at 4–8 hours following treatment. Erythrocyte and brain AChE levels were not affected by treatment in this study (Beyrouty, 1996a).

In an acute neurotoxicity study in rats administered ethephon by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw, no NOAEL could be identified, as increased incidences of myosis were observed at all dose levels. The effect of ethephon treatment on cholinesterase activity was not assessed in this study (Beyrouty, 1996b).

In a 13-week neurotoxicity study in rats administered ethephon by gavage at a dose of 0, 75, 150 or 400 mg/kg bw per day (the high dose was decreased to 300 mg/kg bw per day during week

10/11 of treatment), the NOAEL was 75 mg/kg bw per day, based on reduction of erythrocyte AChE activity in females at 150 mg/kg bw per day (Beyrouty, 1997b).

In a 28-day neurotoxicity study in dogs administered ethephon at a dietary concentration of 0, 250 or 750 ppm (equal to 0, 6 and 14 mg/kg bw per day, respectively), the NOAEL was 250 ppm (equal to 6 mg/kg bw per day), based on reduction of AChE activity in erythrocytes at 750 ppm (equal to 14 mg/kg bw per day) (Eigenberg, 2006a).

In a 91-day neurotoxicity study in dogs administered ethephon at a dietary concentration of 0, 70, 140 or 525 ppm (equal to 0, 2, 4 and 15 mg/kg bw per day for males and 0, 2, 4 and 18 mg/kg bw per day for females, respectively), the NOAEL was 70 ppm (equal to 2 mg/kg bw per day), based on reduction of AChE activity in erythrocytes at 140 ppm (equal to 4 mg/kg bw per day) (Eigenberg, 2006b).

The Meeting noted that in the neurotoxicity studies, no clinical signs of neurotoxicity were observed, even though erythrocyte AChE activity was reduced.

No evidence for delayed neurotoxicity was observed in three studies in chickens (Weatherholtz & Shott, 1970; Fletcher, 1983; Rodgers, 2005).

Toxicological data on metabolites and/or degradates

HEPA is a significant metabolite of ethephon in rats (Odin-Feurtet, 2002) and is also the main plant metabolite. Acute and short-term toxicity and genotoxicity studies with HEPA were available. The acute oral toxicity of HEPA was low (rat LD_{50} > 2000 mg/kg bw) (Denton, 2001). HEPA did not cause inhibition of plasma cholinesterase activity in vitro (Segall et al., 1991).

In a 28-day toxicity study in rats administered HEPA by gavage at a dose of 0, 125, 350 or 1000/700 mg/kg bw per day (the highest dose was reduced from 1000 to 700 mg/kg bw per day from day 5 onwards, as a result of mortality), the NOAEL was 350 mg/kg bw per day, based on mortality, clinical signs, reduced body weight gain and feed consumption (females only), changes in urinary parameters, and various macroscopic findings and histological changes (epithelial necrosis and intraluminal inflammatory exudates in trachea) observed at 1000/700 mg/kg bw per day. The effects observed at the high dose are considered related to the gavage administration and the physicochemical properties of HEPA (Bigot, 2003b).

HEPA was negative in a gene mutation test in bacteria and in a gene mutation test and a chromosomal aberration test in mammalian cells in vitro (Ballantyne, 2002; Johnson, 2002; Whitwell, 2002).

In gavage studies in rats, the toxicity of HEPA was similar to that of ethephon. The effects observed in these studies with high doses of HEPA or ethephon are likely the result of a local gastrointestinal effect due to the physicochemical properties of these compounds and are therefore not relevant to the risk assessment. As HEPA does not reduce cholinesterase activity and as the NOAEL for HEPA in a 28-day gavage study is at least 2 orders of magnitude higher than the NOAEL of 0.5 mg/kg bw in humans that forms the basis of the ADI and ARfD (see below), HEPA is not considered to be a toxicologically relevant metabolite.

Human data

In a 28-day study in human volunteers, five males and five females received ethephon at oral (capsule) doses of approximately 1.5 mg/kg bw per day for males and 2.2 mg/kg bw per day for females, divided over three daily dosages. Three males and three females received placebo. Transient, subjective complaints, such as diarrhoea or urgency of bowel movements, were observed on 1–4 days in the first week of treatment in four volunteers receiving ethephon, but not in control subjects.

Urgency or an increased frequency of urination was observed during the course of the study in one control and five treated volunteers. In addition, loose stools, stomach cramps and/or gas, flank pain, and loss or increase of appetite were occasionally reported by some volunteers treated with ethephon. No changes in plasma and erythrocyte cholinesterase activities and no persistent side-effects were observed. No treatment-related changes in haematology, clinical biochemistry or urine analysis parameters were noted (Reese, 1972).

In a 16-day study, volunteers received ethephon orally (by capsule) at a dose of 0 or 0.5 mg/kg bw per day (divided over three daily dosages). Ten males and 10 females received ethephon, and six males and four females received placebo. No treatment-related clinical signs or changes in erythrocyte AChE values or in haematology, clinical chemistry or urine analysis parameters were observed (Weir, 1977a).

In a 22-day volunteer study using ethephon at oral (capsule) doses of 0 (three males and three females), 0.17 (three males and four females) and 0.33 mg/kg bw per day (four males and three females), no treatment-related clinical signs or changes in erythrocyte AChE activities or haematology, clinical chemistry or urine analysis parameters were observed (Weir, 1977b).

The Meeting concluded that the existing database on ethephon 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 0.5 mg/kg bw per day in studies in humans, based on transient, subjective complaints, such as diarrhoea and urgency of bowel movements, loose stools, stomach cramps and/or gas, urgency or an increased frequency of urination, flank pain, and loss or increase of appetite, with the application of a 10-fold safety factor.

The Meeting reaffirmed the ARfD for ethephon of 0.05 mg/kg bw, established on the basis of the overall NOAEL of 0.5 mg/kg bw per day in studies in humans, based on transient, subjective complaints, such as diarrhoea and urgency of bowel movements, loose stools, stomach cramps and/or gas, urgency or an increased frequency of urination, flank pain, and loss or increase of appetite observed during the first week of treatment, with the application of a 10-fold safety factor.

Levels relevant to risk assessment of ethephon

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week studies of toxicity and carcinogenicity[a,b]	Toxicity	100 ppm, equal to 14 mg/kg bw per day	300 ppm, equivalent to 45 mg/kg bw per day
		Carcinogenicity	10 000 ppm, equal to 1 477 mg/kg bw per day[c]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	300 ppm, equal to 13 mg/kg bw per day	3 000 ppm, equal to 129 mg/kg bw per day
		Carcinogenicity	30 000 ppm, equal to 1 416 mg/kg bw per day[c]	–
	Two-generation study of reproductive	Reproductive toxicity	30 000 ppm, equal to 2 220 mg/kg bw per day[c]	–

Species	Study	Effect	NOAEL	LOAEL
	toxicity[a]	Parental toxicity	300 ppm, equal to 20 mg/kg bw per day	3 000 ppm, equal to 200 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 22 mg/kg bw per day	3 000 ppm, equal to 220 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	600 mg/kg bw per day	1 800 mg/kg bw per day
		Embryo and fetal toxicity	1 800 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	50 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	50 mg/kg bw per day	100 mg/kg bw per day
Dog	Thirteen-week study of neurotoxicity[a]	Toxicity	70 ppm, equal to 2 mg/kg bw per day	140 ppm, equivalent to 4 mg/kg bw per day
	Two-year study of toxicity[a]	Toxicity	30 ppm, equal to 0.86 mg/kg bw per day	300 ppm, equal to 7.6 mg/kg bw per day
Human	Sixteen- and 28-day studies of toxicity[b,e]	Toxicity	0.5 mg/kg bw per day	1.5 mg/kg bw per day

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

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 ethephon

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; > 65% at 50 and 1 000 mg/kg bw (rat)
Dermal absorption	No data

Distribution	Widespread distribution, highest concentrations found in bone, liver, blood and kidney; low concentrations in brain (rat)
Potential for accumulation	Low
Rate and extent of excretion	Rapid; largely complete within the first 24 h after dose administration
Metabolism in animals	Converted to its monosodium and disodium salts, ethylene and, to a lesser extent, HEPA
Toxicologically significant compounds in animals and plants	Ethephon
Acute toxicity	
Rat, LD_{50}, oral	1 564 mg/kg bw
Rabbit, LD_{50}, dermal	983 mg/kg bw
Rat, LC_{50}, inhalation	3.26 mg/L
Rabbit, dermal irritation	Severely irritating
Rabbit, ocular irritation	Assumed to be corrosive, pH < 2
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Reduction of erythrocyte AChE activity
Lowest relevant oral NOAEL	0.86 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	237 mg/kg bw per day (highest dose tested); severe dermal irritation at 119 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduction of erythrocyte AChE activity
Lowest relevant NOAEL	13 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in rats or mice[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	No reproductive effect
Lowest relevant parental NOAEL	20 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	22 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	2220 mg/kg bw per day (highest dose tested; rat)
Developmental toxicity	
Target/critical effect	Reduced viability and number of live fetuses
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	50 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity LOAEL	500 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	2 mg/kg bw per day (dog)

Developmental neurotoxicity NOAEL	No data
Delayed neurotoxicity	Negative
Other toxicological studies	
Studies with HEPA	Oral LD_{50} > 2 000 mg/kg bw (rat) 28-day study: NOAEL = 350 mg/kg bw per day (rat) Negative in a gene mutation test in bacteria and in a gene mutation test and a chromosomal aberration test in mammalian cells in vitro
Human data	
	NOAEL 0.5 mg/kg bw per day. Transient, subjective clinical signs were reported in a 28-day oral (capsule) study in human volunteers, using ethephon doses of approximately 1.5–2.2 mg/kg bw per day. No effects on plasma or erythrocyte cholinesterase activities.

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

Summary

	Value	Study	Safety factor
ADI	0–0.05 mg/kg bw	Sixteen-day and 28-day studies in humans	10
ARfD	0.05 mg/kg bw	Sixteen-day and 28-day studies in humans	10

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FLONICAMID

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Explanation

Flonicamid is the International Organization for Standardization (ISO)–approved common name for *N*-cyanomethyl-4-(trifluoromethyl)nicotinamide (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 158062-67-0. It is a novel systemic pyridine carboxamide insecticide with selective activity against hemipterous pests, such as aphids and whiteflies, and thysanopterous pests.

Flonicamid 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.

All critical studies contained statements of compliance with good laboratory practice (GLP).

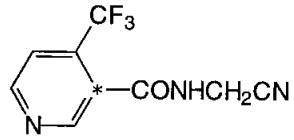
Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion, as well as the toxicokinetics, of flonicamid have been investigated in Sprague-Dawley rats. Summaries of the relevant data are presented below.

The metabolism of flonicamid was investigated using flonicamid labelled at the 3-nicotinamide position (Fig. 1). The test item was a mixture of ^{14}C-labelled flonicamid and unlabelled flonicamid. Radiolabelled flonicamid was administered via oral gavage in 0.75% aqueous methyl cellulose. The study design is summarized in Table 1.

Fig. 1. Structure of flonicamid with radiolabel positions for the metabolism studies



(* = ^{14}C position)

Source: Neal, Savides & Dow (2002a)

Table 1. Dosing groups for metabolism experiments with [^{14}C]flonicamid

Test group	Dose of labelled material (mg/kg bw)	Number of rats of each sex	Remarks	Reference
Pilot study – routes of elimination	0.85	5	A single nominal dose of 0.9 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 168 h.	Neal & Savides (2001a)
Pilot study – routes of elimination	21	5	A single nominal dose of 21 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 168 h.	Neal & Savides (2001a)
Pilot study – pharmacokinetics	2	5	A single nominal dose of 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 72 h.	Neal & Savides (2001a)
Pilot study – pharmacokinetics	50	5	A single nominal dose of 50 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 72 h.	Neal & Savides (2001a)
Single oral low dose in the rat – pharmacokinetics	2	5	A single nominal dose of 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 72 h.	Neal & Savides (2001b)
Single oral high dose in the rat – pharmacokinetics	400	5	A single nominal dose of 400 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 72 h.	Neal & Savides (2001b)

Test group	Dose of labelled material (mg/kg bw)	Number of rats of each sex	Remarks	Reference
Single oral low dose in the rat – elimination and distribution	2	3	A single nominal dose of 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 0.5, 6 or 24 h.	Neal, Savides & Dow (2002a)
Single oral low dose in the rat – elimination and distribution	2	5	A single nominal dose of 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 168 h.	Neal, Savides & Dow (2002a)
Single oral high dose in the rat – elimination and distribution	400	3	A single nominal dose of 400 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Males killed after 3, 14.5 and 24 h, and females killed after 1, 8 and 24 h.	Neal, Savides & Dow (2002a)
Single oral high dose in the rat – elimination and distribution	400	5	A single nominal dose of 400 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 168 h.	Neal, Savides & Dow (2002a)
Repeated dose in the rat	2	3	2 mg/kg bw per day unlabelled flonicamid was administered by oral gavage for 14 days, and 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage on day 15. Killed after 0.5, 6 or 24 h following labelled dose.	Neal, Savides & Dow (2002b)
Repeated dose in the rat	2	5	2 mg/kg bw per day unlabelled flonicamid was administered by oral gavage for 14 days, and 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage on day 15. Killed after 168 h following labelled dose.	Neal, Savides & Dow (2002b)
Single oral low and high dose in the rat – elimination in the bile	2	4	A single nominal dose of 2 mg/kg bw [pyridyl-3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 48 h.	Dow (2002)
	400	4	A single nominal dose of 400 mg/kg bw [pyridyl -3-^{14}C]flonicamid (specific activity 9.08 MBq/mg) was administered by oral gavage. Killed after 48 h.	
Biotransformation in the rat	–	–	Samples of urine, faeces, liver and bile taken from Dow (2002) and Neal, Savides & Dow (2002a,b)	Gupta, Shah & McClanahan (2002)

bw: body weight

1.1 Absorption, distribution and excretion

The data generated indicated that flonicamid is rapidly absorbed and excreted. High-dose males exhibited a decreased rate of elimination relative to other dose groups, with serum

Table 2. Recovery of radioactivity in tissues and excreta of rats after administration of ^{14}C-labelled flonicamid

	% of radioactive dose recovered			
	Single low dose (2 mg/kg bw)		Single high dose (400 mg/kg bw)	
	Males	Females	Males	Females
Tissues[a]	2.094	1.570	1.515	1.260
Urine				
6 h	21.20[b]	36.31[c]	18.05[d]	24.85[b]
12 h	36.76[b]	22.58[c]	27.24[d]	28.41[b]
24 h	12.46[b]	10.08[c]	22.34[d]	19.88[b]
48 h	3.472	2.276	8.316	3.588
72 h	0.416	0.288	1.100	0.450
96 h	0.176	0.164	0.216	0.250
120 h	0.108	0.120	0.128	0.124
144 h	0.082	0.090	0.088	0.114
168 h	0.072	0.100	0.060	0.084
Total urine	74.74	72.01	77.54	77.75
Faeces[a]	6.394	4.970	5.320	3.894
Cage wash[a]	14.77	20.78	9.646	15.80
Total excreted[a]	95.91	97.76	92.51	97.44
Total recovery[a]	98.00	99.33	94.02	98.70

bw: body weight
[a] At 7 days post-dosing.
[b] Ninety-four per cent of urinary excretion in 24 h.
[c] Ninety-six per cent of urinary excretion in 24 h.
[d] Eighty-seven per cent of urinary excretion in 24 h.
Source: Neal, Savides & Dow (2002a)

concentrations reaching a plateau between 0.5–8 hours post-dosing. In all dose groups, radiolabel concentrations in the plasma decreased with time in a manner consistent with first-order kinetics. The predominant route of excretion was in the urine, accounting for 72–78% of the administered radiolabelled dose (Table 2). Faecal and biliary excretion was minor (4–7%), and no residues were detected in the expired air following single doses. For all routes, excretion was rapid, with 95% of the radioactivity excreted within the first 24–48 hours. Very little flonicamid was retained in the tissues, and repeated dosing of rats did not indicate any potential for accumulation. Maximum plasma concentrations (C_{max}) (Table 3) and area under the plasma concentration–time curve (AUC) values were directly proportional to dose level in both sexes. Time to maximum plasma concentration (T_{max}) was similar in low-dose males and females; however, T_{max} was increased in high-dose males relative to females, probably in relation to the prolonged plateau observed in plasma concentrations in males at this dose. Tissue distribution of radiolabel was similar in all groups following single dosing. Radioactivity was rapidly and widely distributed throughout the tissues at levels similar to plasma concentrations; however, slightly higher concentrations of radiolabel were noted in the liver, kidneys, adrenals, thyroid and ovaries (Table 4). Males also had increased levels of radiolabel in the lungs following repeated dosing.

1.2 Biotransformation

The urinary and faecal samples from Dow (2002) and Neal, Savides & Dow (2002a,b) were subjected to high-performance liquid chromatographic analysis to determine the metabolic fate of

Table 3. Plasma kinetic parameters in rats after administration of [^{14}C]flonicamid

Group	T_{max} (h)	C_{max} (ng eq/g)	$AUC_{0-\infty}$ (h·ng eq/g)	$t_{½}$ (h)
2 mg/kg bw oral M	0.4 ± 0.1	2 074 ± 70	16 447 ± 1 729	5.20 ± 0.64
2 mg/kg bw oral F	0.4 ± 0.2	2 112 ± 97	14 457 ± 1 241	4.48 ± 0.38
400 mg/kg bw oral M	0.9 ± 0.4	249 618 ± 45 370	4 320 416 ± 474 589	11.58 ± 2.19
400 mg/kg bw oral F	0.5 ± 0.1	367 583 ± 29 093	3 831 101 ± 1 515 408	6.79 ± 2.36

AUC: area under plasma concentration–time curve; bw: body weight; C_{max}: maximum plasma ^{14}C concentration; eq: equivalents; F: female; M: male; $t_{½}$: half-life for plasma elimination; T_{max}: time to maximum ^{14}C plasma concentration (using times estimated by WinNonlin® rather than the discrete collection times)
Source: Neal & Savides (2001b)

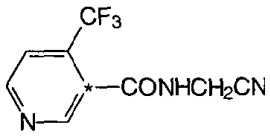
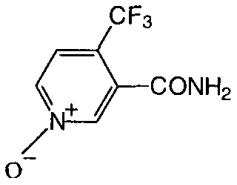
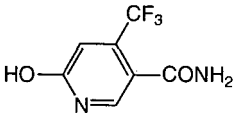
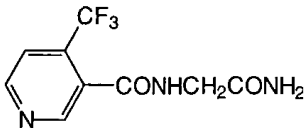
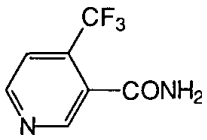
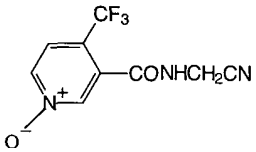
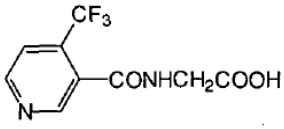
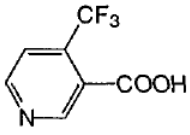
Table 4. Distribution of radioactivity in rat tissues/organs at 168 hours after administration of [^{14}C]flonicamid

	% of radioactive dose administered			
	Single low dose (2 mg/kg bw)		Single high dose (400 mg/kg bw)	
Tissue/organ	**Males**	**Females**	**Males**	**Females**
Blood	0.052	0.050	0.073	0.299
Bone marrow	ND	ND	ND	ND
Brain	ND	0.002	0.004	0.005
Heart	0.010	0.010	0.007	0.003
Lungs	0.008	0.008	0.002	0.003
Liver	0.154	0.106	0.042	0.028
Adrenals	ND	ND	< 0.001	< 0.001
Kidneys	0.020	0.020	0.009	0.009
Gastrointestinal tract + contents	0.002	0.010	0.018	0.028
Bone	ND	ND	0.011	0.010
Muscle	0.598	0.484	0.380	0.337
Fat	0.022	0.012	ND	ND
Testes/ovaries	0.010	ND	0.008	ND
Uterus	N/A	ND	N/A	< 0.001
Spleen	ND	ND	< 0.001	< 0.001
Pancreas	ND	ND	ND	< 0.001
Thyroid	ND	ND	< 0.001	< 0.001
Thymus	ND	ND	< 0.001	ND
Carcass	1.834	1.364	1.350	0.882
Total	2.094	1.570	1.515	1.260

bw: body weight; N/A: not applicable; ND: below the limit of detection
Source: Neal, Savides & Dow (2002a)

flonicamid (Table 5; Fig. 2). In urine, the major residue was unchanged parent, followed by the metabolite 4-trifluoromethylnicotinamide (TFNA-AM), and was generally similar between the sexes after single and repeated dosing. TFNA-AM was also the predominant metabolite in the faeces and bile. In the faeces, 4-trifluoronicotinic acid (TFNA) was found only in low-dose animals, whereas

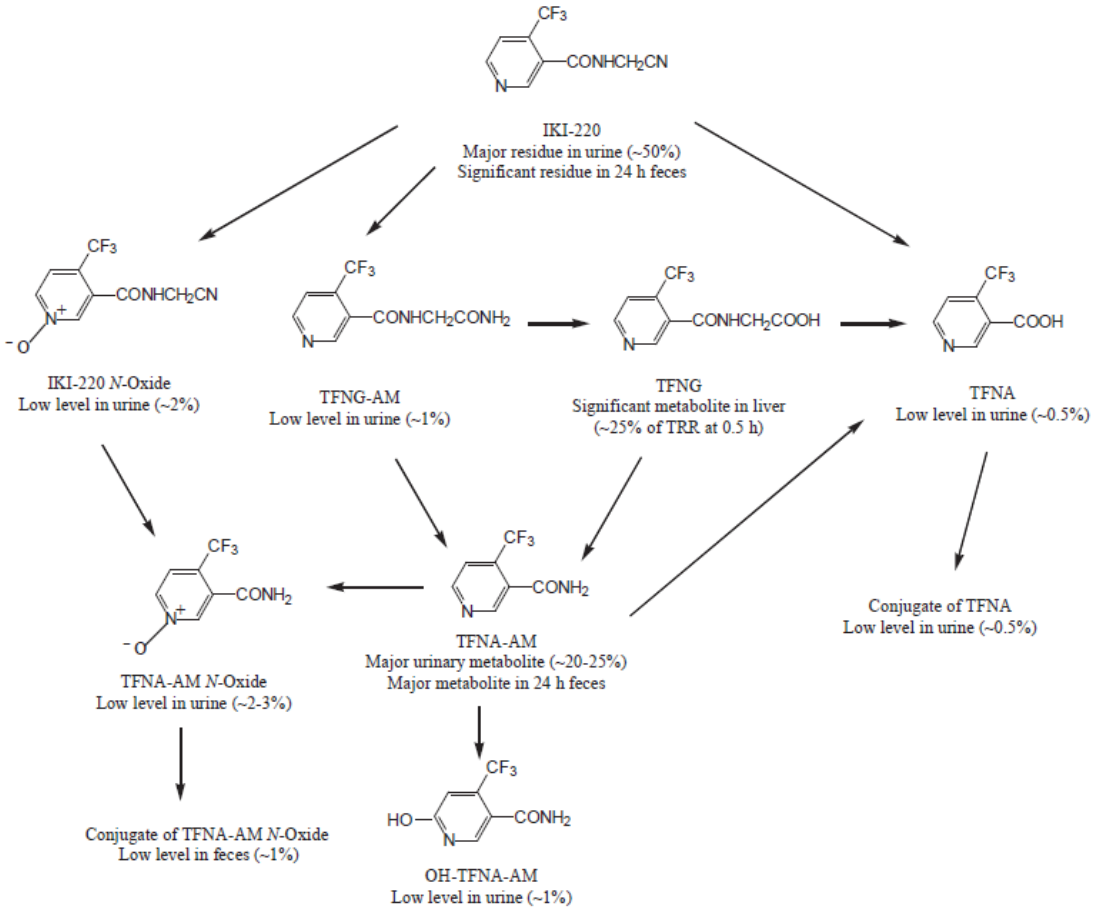
Table 5. Identified metabolites

Metabolite designation	Structure	Urine	Faeces	Bile
Flonicamid		+	+	+
TFNA-AM *N*-oxide conjugate		+	+	–
TFNA conjugate	NG	+	+	–
TFNA-AM *N*-oxide	NG	+	+	–
OH-TFNA-AM		+	+	–
TFNG-AM		+	–	+
TFNA-AM		+	+	+
Flonicamid *N*-oxide		+	+	–
TFNG		–	–	–
TFNA		+	+	–

NG: structure not given
Source: Gupta, Shah & McClanahan (2002)

TFNA-AM *N*-oxide conjugate was found only in high-dose animals. Unchanged parent was the predominant residue in the bile, and *N*-(4-trifluoromethylnicotinoyl)glycinamide (TFNG-AM) was

Fig. 2. Proposed metabolic pathway of flonicamid in the rat



Source: Gupta, Shah & McClanahan (2002)

unique to the bile of high-dose animals. In the liver, the predominant residue was unchanged parent, with *N*-(4-trifluoronicotinoyl)glycine (TFNG) and TFNA-AM noted as minor metabolites.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Flonicamid is of moderate acute oral toxicity in rats. It is of low acute dermal and inhalation toxicity (Table 6).

In the acute oral toxicity study, animals were given a dose of flonicamid (purity 98.7%) at 625, 1250, 2500 or 5000 mg/kg body weight (bw), with mortalities noted at 1250 mg/kg bw and above. Clinical signs of toxicity consisted of few faeces, loss of mobility, decreased activity, hunched posture, hypothermia, laboured breathing, tremors, convulsions, ataxia and prostration. Surviving animals were free of clinical signs by day 3. All surviving animals gained weight in the first week following treatment; however, one male and one female in the 625 mg/kg bw group and one female in the 2500 mg/kg bw group lost weight from day 7 to day 14. There were no gross changes at necropsy in surviving animals; however, some decedent animals exhibited dark red-black spots on the serosal surface of the stomach, and one female exhibited anogenital staining (Ridder, Yoshida & Watson, 2001a).

Table 6. Acute toxicity of flonicamid

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Crl:CD (SD)BR(IGS)	Male and female	Oral	98.7	LD_{50} = 884 mg/kg bw (M) LD_{50} = 1 768 mg/kg bw (F)	Ridder, Yoshida & Watson (2001a)
Rat	Crl:CD (SD)BR(IGS)	Male and female	Dermal	98.7	LD_{50} > 5 000 mg/kg bw	Ridder, Yoshida & Watson (2000a)
Rat	Sprague-Dawley	Male and female	Inhalation	98.7	LC_{50} > 4.90 mg/L	Paul (2000)

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

In the acute dermal toxicity study, animals were given a limit dose of flonicamid (purity 98.7%) at 5000 mg/kg bw. There were no mortalities. Clinical signs consisting of coloured material around the nose and eyes of most animals and anogenital staining were noted from day 1 to day 4. All animals gained weight throughout the study, and there were no gross changes at necropsy (Ridder, Yoshida & Watson, 2000a).

In the acute inhalation toxicity study, male and female Sprague-Dawley-derived rats were exposed to 4.90 mg/L flonicamid (purity 98.7%) via nose-only exposure for 4 hours. Clinical signs of toxicity consisted of exaggerated breathing up until day 2 and brown staining around the snout and/or jaws during exposure and up to day 2. All animals gained weight throughout the study. At gross necropsy, minimal congestion was noted in all lung lobes, dark foci were noted in the lungs of one male and severe congestion was noted in the right posterior lobe of another male (Paul, 2000).

(b) Dermal irritation

In a dermal irritation study, 0.5 g of flonicamid (purity 98.7%) was moistened with approximately 1 mL of distilled water and applied to the right dorsal skin site on each of six male New Zealand White rabbits. There were no dermal or abnormal clinical findings observed in the test animals. All scores at the 1-, 24-, 48- and 72-hour time points for erythema and oedema were zero, for both the control animals and the test animals. Flonicamid is considered non-irritating to the skin of the rabbit (Ridder, Yoshida & Watson, 2000b).

(c) Ocular irritation

In an eye irritation study, 0.1 mL of flonicamid (purity 98.7%) was instilled into one eye of each of six young male albino New Zealand White rabbits. There were no effects observed in the cornea or iris at any time. Redness (grade 2), chemosis (grades 1–2) and discharge (grades 1–3) were observed at the 1-hour time point. There was no evidence of conjunctivitis at 72 hours post-instillation. Flonicamid is considered slightly irritating to the eye of the rabbit (Ridder, Yoshida & Watson, 2000c).

(d) Dermal sensitization

In a dermal sensitization study using a maximization protocol, 20 4- to 7-week-old male Hartley albino guinea-pigs were each injected on day 1 with three pairs of injections, consisting of 1) 0.1 mL 1:1 volume per volume (v/v) Freund's Complete Adjuvant (FCA)/sterile water; 2) 0.1 mL 10% weight per volume (w/v) flonicamid (purity 98.7%) in mineral oil; and 3) 0.1 mL 10% w/v flonicamid in 1:1 FCA/distilled water. One week later, a topical induction dose of 0.4 mL of flonicamid (10% w/v) in mineral oil was applied to a filter paper, which was placed on the application site for 48 hours. Challenge was 14 days after the topical application, with 0.4 mL 10% w/v applied to

the test site with 24-hour exposure. An additional group of 20 guinea-pigs was treated in the same manner, except that they were exposed to the test material during the challenge phase only. The concentrations of flonicamid used were based on preliminary irritation studies.

At challenge, 2/20 of the previously induced guinea-pigs and 2/20 of the control animals scored 1 (mild erythema) at 48 hours. All other scores were zero. It is therefore concluded that there was no evidence of a dermal sensitization response (Ridder & Watson, 2000).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In an oral 13-week toxicity study, groups of 10 Crl:CD-1 (ICR) BR mice of each sex received flonicamid (purity 98.7%) in the diet at a dose level of 0, 100, 1000 or 7000 parts per million (ppm) (equal to 0, 15, 154 and 1069 mg/kg bw per day for males and 0, 20, 192 and 1248 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity, and a detailed clinical examination was performed weekly. Body weight and feed consumption were measured weekly. At study termination, select haematology and clinical chemistry parameters were evaluated; in addition, all animals were subject to gross necropsy, and the liver with gallbladder, kidneys and spleen were weighed. The bone marrow (sternum), liver and spleen were examined histopathologically in all groups, along with all macroscopic abnormalities and the kidneys for control and high-dose animals.

At 7000 ppm, body weight and body weight gain were decreased in the first 30 days of treatment in males and females. Males lost body weight in this period, and females gained 23.7% of the weight gained by control females. Feed consumption was decreased in high-dose females from weeks 3 to 7.

Erythrocyte, haemoglobin and haematocrit values were decreased compared with control values in males and females at 7000 ppm. Reticulocyte counts were increased in males and females at the same dose. Mean corpuscular volume and mean corpuscular haemoglobin were increased in males and females at 7000 ppm. In clinical chemistry examination, total bilirubin level was increased in males at 7000 ppm, and glucose level was increased in non-fasted males and females at 7000 ppm (statistically significant in females only).

Absolute weight of liver with gallbladder was increased in males at 7000 ppm, and relative weight of liver with gallbladder was increased in males and females at 7000 ppm. Minimal to moderate centrilobular hepatocellular hypertrophy was observed in males and females at 7000 ppm, and minimal centrilobular hepatocellular hypertrophy was observed in males at 1000 ppm. Absolute and relative spleen weights were increased in both sexes at 7000 ppm. Gross and histopathological changes consisted of minimally to moderately severe extramedullary haematopoiesis in the spleen at doses of 1000 ppm and above and minimal to moderate increased pigment deposition in the spleen in all animals at 7000 ppm. In the bone marrow, there was minimal to mild hypocellularity and increased pigment deposition at 7000 ppm in males and females.

As there was only histopathological examination of the bone marrow, spleen, liver and kidneys and, specifically, no examination of the lung tissue, no end-points were selected for this study (Ridder, Yoshida & Watson, 2001b).

Rats

In an oral 28-day toxicity study, groups of six Wistar (Jcl:Wistar) rats of each sex received flonicamid (purity 98.7%) in the diet at a dose level of 0, 50, 100, 500, 1000 or 5000 ppm in males (equal to 0, 3.61, 7.47, 36.5, 73.8 and 353 mg/kg bw per day, respectively) and 0, 100, 500, 1000, 5000 or 10 000 ppm in females (equal to 0, 8.36, 41.2, 81.9, 373 and 642 mg/kg bw per day, respectively). Additionally, there was a satellite group of two males per dose given 0 or 5000 ppm (equal to 0 and 353.4 mg/kg bw per day, respectively) for 28 days that were subjected to

immunostaining of the kidney for α_{2u}-globulin. Animals were observed daily for morbidity, moribundity and overt clinical signs. Body weight and feed consumption measurements and a detailed examination for clinical signs were performed once per week. An ophthalmoscopic examination was performed on high-dose males and females prior to treatment and during week 4. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, the weights of the liver, kidney and spleen were recorded, assessed by gross examination and examined histopathologically. There were no effects on mortality, clinical signs of toxicity or ophthalmoscopic examination. Body weights and feed consumption were decreased compared with controls in 5000 ppm males and 10 000 ppm females. Feed efficiency was also decreased in 10 000 ppm females. Clinical chemistry changes occurred at 5000 ppm in males and females, with increased cholesterol and decreased calcium levels in males and decreased alanine aminotransferase activity and increased protein and albumin levels in females. At 10 000 ppm in females, gamma-glutamyltranspeptidase (GGT) activity and globulin and cholesterol levels were increased, and albumin/globulin ratios and triglyceride levels were decreased. Changes to haematological parameters consisted of decreased haematocrit and red blood cells at 5000 ppm in males and increased platelet counts in females at 10 000 ppm. Kidney changes were limited to males and were noted at doses at and above 100 ppm, with hyaline droplet depositions in proximal tubular cells of the kidney. At 5000 ppm, there were increased incidences of pale kidneys and increased kidney weights in main group males and hyaline droplets and granular casts in proximal tubular cells of kidneys that were positive for α_{2u}-globulin antibodies in the satellite group. As a result, the hyaline droplets of the kidneys were considered to be specific to male rats and not applicable to the human risk assessment. Gross and histopathological changes in the liver were noted at 5000 ppm in males and females, with hepatocellular hypertrophy and increased incidence of liver enlargement. In males, there were increased incidences of dark liver. In females at 10 000 ppm, liver weights were increased, and there was an increased incidence of centrilobular hepatocellular hypertrophy. Relative spleen weights were increased in females at 10 000 ppm (Kuwahara, 2002a).

In a 90-day study, groups of 12 Wistar (Jcl:Wistar) rats of each sex received flonicamid (purity 98.7%) in the diet at a dose level of 0, 50, 200, 1000 or 2000 ppm in males (equal to 0, 3.08, 12.1, 60.0 and 119 mg/kg bw per day, respectively) and 0, 200, 1000 or 5000 ppm in females (equal to 0, 14.5, 72.3 and 340 mg/kg bw per day, respectively). Animals were observed daily for morbidity, moribundity and overt clinical signs. Body weight and feed consumption measurements and a detailed examination for clinical signs were performed once per week. An ophthalmoscopic examination was performed on high-dose males and females prior to treatment and during week 13. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, the weights of selected organs were recorded, assessed by gross examination and examined histopathologically.

There were no effects on mortality, clinical signs, body weight, feed efficiency or urine analysis parameters. Feed consumption was decreased in high-dose females; however, in the absence of effects on body weight, the change was not considered to be adverse. High-dose males and females showed no changes under ophthalmoscopic examination. There were no effects on the functional observational battery and no consistent, treatment-related effects on motor activity. Changes to the kidneys were noted at doses at and above 200 ppm in males, with increased kidney weights, hyaline droplets and granular casts in the tubules and tubular basophilic changes. Effects at 200 ppm were considered to be related to the rat-specific α_{2u}-globulin-positive hyaline droplets seen in the 28-day study. However, as there were kidney changes at higher doses in female rats and at lower doses in female dogs (see below), changes that were not specifically linked to positive α_{2u}-globulin staining – increased kidney weights, granular casts and increased basophilic changes in the renal tubules – were considered relevant to the human risk assessment. At 2000 ppm, males exhibited pale kidneys. At 5000 ppm, kidney weights were increased in females, along with increased cytoplasmic vacuolation of the proximal tubules. In the liver, centrilobular hepatocellular hypertrophy was noted at the high dose in males (2000 ppm) and females (5000 ppm). In the 5000 ppm females, liver weights were also increased. Haematocrit was decreased in 5000 ppm females.

The no-observed-adverse-effect level (NOAEL) was 200 ppm (equal to 12.11 mg/kg bw per day) in males and 1000 ppm (equal to 72.3 mg/kg bw per day) in females. The lowest-observed-adverse-effect level (LOAEL) was 1000 ppm (equal to 60.0 mg/kg bw per day) in males, based on increased kidney weights, granular casts in the tubules and increased basophilic changes in the renal tubules, and 5000 ppm (equal to 340.1 mg/kg bw per day) in females, based on decreased haematocrit, increased liver and kidney weights, and increased cytoplasmic vacuolation of the proximal tubules of the kidneys (Kuwahara, 2002b).

Dogs

In a 90-day oral toxicity study in dogs, groups of four Beagle dogs of each sex received flonicamid (purity 98.7%) in capsules at a dose level of 0, 3, 8 or 20 mg/kg bw per day for males and females and 50 mg/kg bw per day for females only. Animals were observed for mortality twice daily and for clinical signs of toxicity, moribundity and feed consumption daily, and detailed physical examinations and body weight measurements were performed weekly. Clinical chemistry and haematological parameters were measured prior to the initiation of dosing, at weeks 6–7 and prior to termination. Select organs were weighed at necropsy, assessed by gross observations and examined histopathologically.

One female in the 50 mg/kg bw per day group was sacrificed moribund by week 3. Clinical signs of toxicity consisted of vomiting, ataxia, decreased activity, laboured breathing, prostration and diarrhoea in both males and females at 20 mg/kg bw per day and in the female-only 50 mg/kg bw per day dose group. In the 50 mg/kg bw per day group, severity of symptoms increased and included additional observations of excessive salivation, tremors, convulsions, few/no/soft faeces, partially closed eyelids and circling behaviour. Body weights and total body weight gain were decreased in males and females at 20 mg/kg bw per day and above when compared with controls. Decreased feed consumption was noted in the 20 and 50 mg/kg bw per day female dose groups during various weeks throughout the study period. In the female high-dose group, this decrease was directly related to feed rejection. Blood analysis at week 7 and study termination revealed a decrease in red blood cells and an increased reticulocyte count in females at 50 mg/kg bw per day. Spleen weights were decreased and lung weights were increased in high-dose females. Thymus weights were decreased in high-dose males; however, the weights were still within the historical control range for Beagle dogs between 8 and 10 months of age (Nomura, 2015a). Minimal to mild tubular vacuolation of the inner cortex of the kidneys was noted in 2/4 females at 50 mg/kg bw per day.

The NOAEL was 8 mg/kg bw per day in males and females, based on vomiting, ataxia, decreased activity, laboured breathing, prostration, and decreased body weight and body weight gain in both sexes as well as decreased feed consumption in females observed at 20 mg/kg bw per day (Ridder & Watson, 2001).

In a 1-year oral toxicity study in dogs, groups of six Beagle dogs of each sex received flonicamid (purity 98.7%) in capsules at a dose level of 0, 3, 8 or 20 mg/kg bw per day. Animals were observed for mortality twice daily and clinical signs of toxicity, moribundity and feed consumption daily, and detailed physical examinations and body weight measurements were performed weekly. Clinical chemistry and haematological parameters were measured prior to the initiation of dosing, every 12 weeks and prior to termination. Select organs were weighed at necropsy, assessed by gross observations and examined histopathologically.

There was no mortality, and no gross or histopathological changes were noted. Treatment-related changes were limited to the top dose. Vomiting was noted in males and females in all groups; however, an increase in incidence indicating adversity occurred at 20 mg/kg bw per day. Increased percentages of reticulocytes were noted at the 12-month time point in males and females at the high dose. Body weight gains were decreased in females at 20 mg/kg bw per day.

The NOAEL was 8 mg/kg bw per day, based on vomiting and increased reticulocytes in males and females and decreased body weight gain in females at 20 mg/kg bw per day (Ridder & Watson, 2003a).

(b) Dermal application

No dermal toxicity studies were submitted.

(c) Exposure by inhalation

No repeated-dose inhalation toxicity studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a carcinogenicity study in mice, flonicamid (purity 98.7%) was administered in the diet to 60 CD-1 mice of each sex per dose at 0, 250, 750 or 2250 ppm (equal to 0, 29, 88 and 261 mg/kg bw per day for males and 0, 38, 112 and 334 mg/kg bw per day for females, respectively) for up to 78 weeks. Additionally, satellite groups of 10 mice of each sex from the control and 2250 ppm dose groups were killed at week 26 (interim sacrifice 1) and week 52 (interim sacrifice 2). The animals were observed twice daily on weekdays and once daily on weekends and holidays for viability, clinical signs were recorded daily and a detailed physical examination was performed weekly. Haematology and clinical chemistry samples were collected from the satellite groups at interim kill and from surviving animals at 12 months and terminal kill; however, haematological parameters were evaluated only in the control and 2250 ppm dose groups, and evaluation of the clinical chemistry samples was not performed. Liver, kidney and spleen weights were taken for all animals killed at 26, 52 and 78 weeks, although not from animals found dead or killed in extremis. Additionally, in animals killed at 78 weeks, organ weights were taken from 10 animals of each sex per dose for the adrenals, brain, heart, testes, epididymides, ovaries and uterus. All animals were necropsied, and tissues from all control and high-dose animals and from all animals found dead or moribund were examined microscopically. The liver, spleen and bone marrow from control and high-dose animals were examined in the 26- and 52-week satellite groups. Gross lesions, liver, spleen, bone marrow and lungs were examined microscopically, regardless of dose group, in the terminal kill groups.

There were no treatment-related clinical signs of toxicity or effects on body weight, body weight gain or feed consumption. In females at all doses, there was a decrease in cellularity of the femoral bone marrow, whereas in males, there was an increase in extramedullary haematopoiesis in the spleen, an increase in pigment deposition in the femoral and sternal bone marrow, increased centrilobular hepatocellular hypertrophy and an increase in masses/nodules in the lung. In both sexes, there was an increase in incidences of hyperplasia/hypertrophy of the epithelial cells of the terminal bronchioles at all doses (Table 7). Hypertrophy of lung epithelial cells is considered a marker of lung injury and is seen as round to oval or cuboidal often/mostly hypertrophic cells with abundant eosinophilic cytoplasm prominently outlining alveolar walls (Renne et al., 2009). Epithelial cell hypertrophy was correlated with an increase in alveolar/bronchiolar adenomas in all treated dose groups, and the statistically significant increase in lung epithelial cell hyperplasia was correlated with alveolar/bronchiolar carcinomas at 750 and 2250 ppm in both males and females. At 750 ppm and above, there was a decrease in cellularity of the bone marrow in the femur of males and the sternum of both sexes. Females exhibited an increase in the incidence of extramedullary haematopoiesis of the spleen and an increase in pigment deposition in the sternal bone marrow. At 2250 ppm, there were increased liver weights of both sexes and increased centrilobular hepatocellular hypertrophy in females. Additionally, there was increased pigment deposition in the spleen of both sexes and increased pigment deposition in the femur of females. At the interim kills, similar effects were noted in the bone marrow, spleen and liver. In all treated groups, there was an increase in alveolar/bronchiolar adenomas and combined alveolar/bronchiolar adenomas and/or carcinomas. At

Table 7. Non-neoplastic and neoplastic lesions in mice after 78 weeks of treatment

	Incidence of lesion							
	Males (n = 60)				Females (n = 60)			
Histopathological lesion	**0 ppm**	**250 ppm**	**750 ppm**	**2 250 ppm**	**0 ppm**	**250 ppm**	**750 ppm**	**2 250 ppm**
Bone marrow (sternum)								
Hypocellularity[a]	0	1	5*	22*	1	3	6	12**
Deposition, brown pigment[a]	0	7**	15**	31**	5	6	10	24**
Bone marrow (femur)								
Hypocellularity[a]	0	1	7*	24**	4	2	7	25**
Deposition, brown pigment[a]	0	4	12**	32**	4	2	7	25**
Lungs								
Epithelial cells, hyperplasia[a]	2	5	11**	13**	5	3	6	11
Epithelial cells, hypertrophy[a]	2	22**	46**	46**	4	20**	41**	42**
Alveolar/bronchiolar adenoma	9	26**	24**	32**	9	21*	30**	25**
Alveolar/bronchiolar carcinoma	2	4	9*	10*	0	3	4	5*
Total mice with primary lung tumours	10	27	29	36	9	22	32	26

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test)
[a] Values and statistical analysis clarified in Nomura (2015b).
Source: Ridder & Watson (2003b)

750 ppm and above in males and at 2250 ppm in females, there was an increase in alveolar/bronchiolar carcinomas.

A NOAEL was not identified. The LOAEL was 250 ppm (equal to 29 and 38 mg/kg bw per day for males and females, respectively), the lowest dose tested, based on hyperplasia/hypertrophy of the epithelial cells of the terminal bronchioles, increased incidence of tissue masses/nodules in the lungs and increased alveolar/bronchiolar adenomas in both sexes, as well as centrilobular hepatocellular hypertrophy, extramedullary haematopoiesis of the spleen and pigment deposition in the femoral and sternal bone marrow in males and decreased cellularity in the femoral bone marrow in females (Ridder & Watson, 2003b).

In a second carcinogenicity study in mice, flonicamid (purity 98.7%) was administered in the diet to 50 CD-1 mice of each sex per dose at 0, 10, 25, 80 or 250 ppm (equal to 0, 1.2, 3.1, 10.0 and 30.3 mg/kg bw per day for males and 0, 1.4, 3.7, 11.8 and 36.3 mg/kg bw per day for females, respectively) for up to 78 weeks. The animals were observed twice daily on weekdays and once daily on weekends and holidays for viability, clinical signs were recorded daily and a detailed physical examination including palpations was performed weekly starting at week 41. Haematology and clinical chemistry samples were collected from surviving animals at terminal kill, but were not evaluated. Liver, kidney, spleen and brain weights were measured for all animals killed at 78 weeks, although not for animals found dead or killed in extremis. All animals were necropsied, and tissues from all control and high-dose animals and from all animals found dead or moribund were examined

Table 8. Non-neoplastic and neoplastic lesions in mice after 78 weeks of treatment

	Incidence of lesion									
	Males (n = 50)					Females (n = 50)				
Histopathological lesion	**0 ppm**	**10 ppm**	**25 ppm**	**80 ppm**	**250 ppm**	**0 ppm**	**10 ppm**	**25 ppm**	**80 ppm**	**250 ppm**
Bone marrow (sternum)										
Deposition, brown pigment	0	0	0	0	1	2	5	1	2	5
Bone marrow (femur)										
Deposition, brown pigment	0	0	0	0	2	3	5	3	3	8
Lungs										
Hyperplasia/ hypertrophy	5	5	5	3	14**	3	5	2	4	11*
Alveolar/bronchiolar adenoma	8	11	12	11	21**	10	8	11	14	13
Alveolar/bronchiolar carcinoma	3	6	3	4	9	1	4	2	3	3
Total mice with primary lung tumours	11	16	15	14	27**	10†	12	12	16	16

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test); †: $P < 0.05$ (trend test)
Source: Nagaoka (2004)

microscopically. Gross lesions, liver, spleen, bone marrow and lungs were examined microscopically, regardless of dose group.

There were no treatment-related clinical signs of toxicity or effects on body weight, body weight gain, feed consumption or organ weights. At 250 ppm, there was an increase in lung masses and an increase in hyperplasia/hypertrophy in the terminal bronchiole epithelial cells in both sexes. There was a slight, non-statistically significant, increase in brown pigment deposition in the bone marrow in 250 ppm females (Table 8). Although this finding is consistent with those seen in the previous study, there is little evidence of treatment-related change in this study. There were no treatment-related changes in any other organ system.

The incidences of alveolar/bronchiolar adenomas and of combined alveolar/bronchiolar adenomas and/or carcinomas were increased in high-dose males, and there was a trend of increased incidence of combined alveolar/bronchiolar adenomas and/or carcinomas in mid-high- and high-dose females. Combined adenomas and/or carcinomas were above the historical control range in males and just under the upper range of historical controls in the top two female dose groups.

The non-neoplastic NOAEL was 80 ppm (equal to 10.0 and 11.8 mg/kg bw per day for males and females, respectively). The non-neoplastic LOAEL was 250 ppm (equal to 30.3 and 36.3 mg/kg bw per day for males and females, respectively), based on an increase in lung adenomas in males, a slight lung hyperplasia/hypertrophy in the terminal bronchiole epithelial cells in males and females and an increased incidence of hyperplasia of alveolar epithelial cells in females (Nagaoka, 2004).

Rats

In a combined chronic toxicity and carcinogenicity study in rats, flonicamid (purity 98.7%) was administered in the diet to 52 Wistar rats of each sex per dose at 0, 50, 100, 200 or 1000 ppm (equal to 0, 1.84, 3.68, 7.32 and 36.5 mg/kg bw per day, respectively) for males and 0, 200, 1000 or

5000 ppm (equal to 0, 8.92, 44.1 and 219 mg/kg bw per day, respectively) for females for up to 24 months. Additionally, satellite groups of 10 or 14 rats of each sex per dose were similarly treated at the same dose levels and sacrificed at 6 and 12 months, respectively. The animals were observed twice daily on weekdays and once daily on weekends and holidays for viability, clinical signs were recorded daily and a detailed physical examination was performed weekly. Functional observations were performed on 10 animals of each sex per dose group from one of the satellite groups at week 49. Body weights and feed consumption were recorded weekly for 13 weeks and monthly from week 16 and thereafter. Feed consumption was measured for each cage of four rats on a weekly basis for 13 weeks, in week 16 and once every 4 weeks thereafter. An ophthalmological examination was conducted on all main study animals in all groups prior to study initiation and at week 104. Urine analysis, haematological examination and clinical chemistry analysis were performed during study weeks 13/14, 26, 52, 77/78 and 103/104. Animals were selected for analysis (10 of each sex per dose) from one of the satellite groups at weeks 13 and 26. Thereafter, 10 animals of each sex per dose were selected from the main group, with animals that were showing clinical signs unrelated to treatment that could interfere with testing excluded. Organ weight analysis was also performed on 10 animals of each sex from each dose group after 6, 12 and 24 months. All animals except those in the satellite group that were not selected for scheduled kill after 52 weeks of treatment were subjected to necropsy, and their tissues were examined histopathologically.

There was no treatment-related mortality in this study, and there were no effects on urine analysis. Clinical signs of toxicity consisted of a decrease in rearing and an increase in keratitis in males at 1000 ppm. At 5000 ppm, there was an increase in rhinitis and opacity, cataracts and retinal atrophy in the eyes in females. Effects on body weight were limited to the second half of the study, with decreases in body weight and body weight gain in males at doses of 1000 ppm and in females at 5000 ppm. Decreases in feed consumption were limited to females in the 5000 ppm dose group. Haematological changes consisted of decreased haematocrit, red blood cells and haemoglobin in females at 5000 ppm. Clinical chemistry changes consisted of decreased triglyceride levels in females at 1000 ppm and above and increased cholesterol level and GGT activity in females at 5000 ppm. Other liver changes occurred in females at 5000 ppm, including increased liver weights, increased dark coloration of the liver and livers with accentuated lobular patterns, increased centrilobular hepatocellular hypertrophy and increased foci of cellular alteration (eosinophilic type). In the kidneys, there was an increase in slight to severe hyaline droplet deposition in renal proximal tubular cells and an increase in kidney pelvic dilatation in males at 1000 ppm. In females at 5000 ppm, there was an increase in kidney weights, an increase in cytoplasmic vacuolation of the renal proximal tubular cells and an increase in chronic nephropathy. There was an increased incidence of striated muscle atrophy in females at 1000 ppm. At 5000 ppm, there was also an increase in pituitary anterior cell hyperplasia in females.

The NOAEL was 200 ppm (equal to 7.32 and 8.92 mg/kg bw per day for males and females, respectively). The LOAEL was 1000 ppm (equal to 36.5 and 44.1 mg/kg bw per day for males and females, respectively), based on decreased body weight and body weight gain, decreased rearing, increased incidences of keratitis and pelvic dilatation in the kidneys in males and decreased triglyceride levels and increased striated muscle atrophy in females (Kuwahara, 2002c).

2.4 Genotoxicity

(a) In vitro studies

A range of GLP-compliant in vitro studies of the genotoxicity of flonicamid was conducted to assess its potential for inducing chromosomal aberration, gene mutation and reverse gene mutation (summarized in Table 9). There was no evidence for genotoxicity or mutagenicity in the presence or absence of metabolic activation.

Table 9. Genotoxicity studies with flonicamid

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 61.7, 185, 313, 556, 625, 1 250, 1 667, 2 500 and 5 000 µg/plate (±S9)	98.7	Negative	Matsumoto (2002a)
Chromosomal aberrations	Chinese hamster lung cells	573, 1 145 and 2 290 µg/mL (±S9) Exposure: 6, 24 and 48 h	98.7	Negative	Matsumoto (2002b)
Mammalian cell gene mutation	L5178Y mouse lymphoma cells, *TK* locus	28.3, 84.8, 143, 254, 286, 573, 763, 1 145 and 2 290 µg/mL	98.7	Negative	Matsumoto (2002c)
In vivo					
Mouse micronucleus	CD-1 mouse bone marrow, males and females	125 (F), 250, 500 and 1 000 (M) mg/kg bw Harvest time: 24, 48 and 72 h	98.7	Negative	Matsumoto (2002d)
Unscheduled DNA synthesis	SD rats primary hepatocytes, males	0, 600 and 2 000 mg/kg bw Scored: 2 and 14 h	98.7	Negative	Mehmood (2003)

bw: body weight; F: females; M: males; S9: 9000 × *g* supernatant fraction from liver homogenate from Aroclor-treated rats; SD: Sprague-Dawley; *TK*: thymidine kinase

(b) In vivo studies

GLP-compliant unscheduled DNA synthesis and micronucleus assays were conducted to assess the potential of flonicamid to damage DNA and impede repair in vivo (summarized in Table 9). There was no evidence of genotoxicity.

Overall, flonicamid did not demonstrate any genotoxic potential.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a range-finding multigenerational reproductive toxicity study, flonicamid (purity 98.7%) was administered continuously in the diet to Jcl:Wistar rats (eight of each sex per dose) at a dose level of 0, 50, 200, 1000 or 2000 ppm (equal to 0, 3.3, 13.1, 65.9 and 131 mg/kg bw per day for males and 0, 3.8, 14.9, 76.5 and 155 mg/kg bw per day for females, respectively). The parental animals were given test article diet formulations for 3 weeks prior to mating to produce the F_1 litters. After weaning, the study was terminated. Animals were examined daily for clinical signs and mortality, weekly for body weight and daily for feed consumption parameters. Reproductive performance was examined, with estrous cycles, mating, fertility and gestation indices, duration of gestation and number of implantation sites reported. Gross pathological examination was performed on all parental animals. Further, liver and kidney weights were measured, and a histopathological examination was performed for all parental animals. Offspring were examined for clinical signs and mortality during the lactation period, body weights, number of pups, sex ratio and viability index. A gross pathological examination was performed on culled, found dead and terminal kill pups.

In parental animals, there were no treatment-related effects on survival, body weight, body weight gain or feed consumption. There were no effects on reproductive performance. Pale kidneys were noted in parental males at doses of 1000 ppm and above, and kidney weights were increased in males at 2000 ppm. In males, histopathological changes were noted at 200 ppm and above. At 200

ppm and above, there was an increase in hyaline droplet deposition in the proximal tubular cells. At 1000 ppm and above, there was an increased incidence of tubular basophilic change. At 2000 ppm, there was an increase in granular casts in the dilated tubules. In females, there were no histopathological changes.

In the offspring, there were no clinical signs of toxicity and no effects on mortality. There were no effects on numbers of pups delivered, sex ratio, viability index or pup body weights. There were no treatment-related gross changes noted in weanlings or pups culled at day 4 (Takahashi, 2002a).

In a multigenerational reproductive toxicity study, flonicamid (purity 98.7%) was administered continuously in the diet to Jcl:Wistar rats (24 of each sex per dose) at a dose level of 0, 50, 300 or 1800 ppm (equal to 0, 3.7, 22.3 and 133 mg/kg bw per day for males and 0, 4.4, 26.5 and 153 mg/kg bw per day for females, respectively). The parental (P) animals were given test article diet formulations for 10 weeks prior to mating to produce the F_1 litters. After weaning, F_1 animals (24 of each sex per dose) were selected to become the parents of the F_2 generation and were given the same concentration of test formulation as was administered to their parents, starting at 10 weeks prior to mating to produce the F_2 litters. In addition to the typical parameters examined in a reproductive toxicity study, serum concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured in males and females, as well as testosterone in males and 17β-estradiol and progesterone in female parents in the F_1 generation, using a radioimmunoassay method. In a supplemental in vitro study (reported in Takahashi, 2002b), flonicamid technical solutions were serially diluted (ranging from 10^{-2} to 10^{-9} mol/L), and estrogen receptor (ER) binding assays were conducted in triplicate analyses per concentration.

In parental animals, there were no treatment-related effects on survival, clinical signs, body weight, body weight gain or feed consumption. At doses of 300 ppm and above, relative kidney weights were increased in F_1 males, and there was an increased incidence of hyaline droplet deposition in the proximal tubule cells in males of both generations. These changes were considered specific to the rat and not relevant to the risk assessment. At 1800 ppm, kidney weights were increased in males of both generations, as were pale kidneys. In P and F_1 males, there were increased incidences of tubular basophilic change and granular casts in dilated tubules in kidneys; in P and F_1 females, there were increased incidences of vacuolation of the proximal tubule cells.

In the ER binding assay, results demonstrated that the test substance had no clear binding affinity for the ERs α and β. Because blood serum concentrations of the test substance were not determined in the main study, it was not possible to compare the concentration of the test substance that resulted in ER binding with levels of the test substance actually in the blood in animals fed up to 153 mg/kg bw per day.

In the offspring, there were no treatment-related effects on body weight, anogenital distance, gross pathology or birth, live birth, viability or lactation indices and no macroscopic findings in the F_1 or F_2 pups or weanlings that could be attributed to treatment. Microscopic examinations were not performed. At 1800 ppm, there was a 4% increase in days to vaginal opening in F_1 pups, which coincided with a 4% increase in body weight at time of vaginal opening; however, this finding was not seen in the F_2 pups. Absolute and relative (to body weight) uterine weights were significantly decreased by 19% in the F_1 weanlings sacrificed at 25–27 days of age.

For the reproductive parameters, there were no effects of treatment on precoital or gestation intervals; mating, fertility or gestation indices; estrous cycle duration or cyclicity; sperm enumeration, motility or morphology; or number of primordial ovarian follicles. In the F_1 females, blood serum levels of LH were significantly increased at 300 ppm and higher. FSH was significantly increased and 17β-estradiol was decreased by 27% (not significantly) for the females in the 1800 ppm dose group. However, owing to the variable and cyclic nature of hormone levels and the lack of adverse effects on the reproductive parameters, these changes were not considered to be adverse.

The NOAEL for parental toxicity was 300 ppm (equal to 22.3 mg/kg bw per day). The LOAEL for parental toxicity was 1800 ppm (equal to 133 mg/kg bw per day), based on increased kidney weights, tubular basophilic change and granular casts observed in males and increased proximal tubule cell vacuolation of the kidney observed in females.

The NOAEL for offspring toxicity in females was 300 ppm (equal to 26.5 mg/kg bw per day). The LOAEL was 1800 ppm (equal to 153 mg/kg bw per day), based on delayed sexual maturation and decreased uterine weight in F_1 females. The NOAEL for offspring toxicity in males was 1800 ppm (equal to 133 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 1800 ppm (equal to 133 mg/kg bw per day), the highest dose tested (Takahashi, 2002b).

(b) Developmental toxicity

Rats

In a range-finding developmental toxicity study, flonicamid (purity 98.7%) was administered to mated Jcl:Wistar rats (eight per dose) via gavage from gestation day (GD) 6 to GD 19 at a dose level of 0, 30, 100, 300 or 1000 mg/kg bw per day at a dosing volume of 1 mL/kg bw in 1% aqueous sodium carboxymethyl cellulose. The animals were terminated on day 20 after mating for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. The ovaries and uterine contents of adult females were examined macroscopically at necropsy on day 20 after mating, and all fetuses were examined macroscopically at maternal necropsy.

Toxicity was limited to the 1000 mg/kg bw per day group. Six of the eight dams died between GD 9 and GD 13. Clinical signs of toxicity consisted of eye discharge, forelimb wounding, loss of abdominal fur, soiled fur around the external genital region, vaginal haemorrhage and white discharge on the tray. Body weight gains were decreased from GD 6 to GD 9. Feed consumption decreased from GD 6 to GD 9 and from GD 18 to GD 20. There were no external abnormalities in fetuses from the two surviving dams at 1000 mg/kg bw per day and no external abnormalities in fetuses from the lower-dose groups (Hojo, 2002a).

In a developmental toxicity study, flonicamid (purity 98.7%) was administered to mated Jcl:Wistar rats (24 per dose) via gavage from GD 6 to GD 19 at a dose of 0, 20, 100 or 500 mg/kg bw per day at a dosing volume of 1 mL/kg bw in 1% aqueous sodium carboxymethyl cellulose. The animals were killed on day 20 after mating for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. Adult females were examined macroscopically at necropsy on day 20 after mating, and all fetuses were examined macroscopically at maternal necropsy and subsequently by detailed internal visceral or skeletal examination. Kidney and liver weights were measured, and histopathological examination of both organs were performed for the 0 and 500 mg/kg bw per day dose groups.

There were no maternal deaths. In dams, there was an increase in liver weights, centrilobular hepatocyte hypertrophy and increased cytoplasmic vacuolation of the proximal tubules of the kidney at 500 mg/kg bw per day. At the same dose in the fetuses, there was an increase in cervical rib skeletal variations as a function of both fetuses and litters.

The NOAEL for maternal toxicity was 100 mg/kg bw per day. The LOAEL for maternal toxicity was 500 mg/kg bw per day, based on increased liver weights and histopathological changes in the liver and kidneys.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day. The LOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, based on an increase in cervical rib skeletal variations (Hojo, 2002b).

Rabbits

In a range-finding developmental toxicity study, flonicamid (purity 98.7%) was administered to artificially inseminated Japanese White (Kbl:JW) rabbits (six per dose) via gavage from GD 6 to GD 27 at a dose of 0, 30, 100, 300 or 1000 mg/kg bw per day. Following mortalities in all groups at and above 100 mg/kg bw per day, flonicamid (purity 98.7%) was administered to artificially inseminated Japanese White (Kbl:JW) rabbits (six per dose) via gavage from GD 6 to GD 27 at a dose of 0, 3, 10 or 30 mg/kg bw per day. The animals were killed on day 20 after insemination for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. The ovaries and uterine contents of adult females were examined macroscopically at necropsy on day 28 after insemination, and all fetuses were examined macroscopically at maternal necropsy.

In the 30 mg/kg bw per day group, clinical signs of toxicity included reddish urine in one animal and red discharge in another animal, observed during the dosing period. Two maternal rabbits in that dose group aborted (day 22 and day 25) and were euthanized. One of these animals showed posterior paralysis and soiled fur in the lower abdominal region before abortion. The other animal showed reddish urine on GDs 23 and 24. Gross pathological examination of these two animals revealed soiled fur on the external genital region, hair bolus in the stomach, urinary bladder distended with urine, fracture of the lumbar vertebra and haemorrhagic pelvic cavity in one dam and black stomach contents and yellow liver colour in the other dam. The study author was unable to elucidate whether the abortions were related to treatment, but it was thought that for at least one animal, the accidental bone fracture of the lumbar vertebra may have been the cause. No other clinical signs of toxicity were observed except for one female from the control group, which had reddish urine on GD 17.

During the dosing period, group mean body weights for maternal animals at the highest dose tested, as well as adjusted weights, were slightly, but consistently, lower than those of the control group. Body weight gains were consistently negative and were statistically significantly different on GD 24 when compared with the control group. Feed consumption was slightly lower when compared with the control group during the latter half of the dosing period.

For this same dose group, the caesarean sections revealed a significant decrease in the gravid uterine weight (54% of control), reduced number of implantations (5 versus 9.7) and reduced number of live fetuses (4.8 versus 8.8). The placental weight was slightly increased over that of the control group for both the 10 and the 30 mg/kg bw per day dose groups. Mean fetal weights were decreased both for males (−11%, not significant) and females (−6%, not significant). The percentage of male fetuses was decreased at this dose (36.8%) compared with controls (56.6%) (Takahashi, 2002c).

In a developmental toxicity study, flonicamid (purity 98.7%) was administered to artificially inseminated Japanese White (Kbl:JW) rabbits (25 per dose) via gavage from GD 6 to GD 27 at a dose of 0, 2.5, 7.5 or 25 mg/kg bw per day. Clinical signs, body weight and feed consumption were recorded. Adult females were examined macroscopically at necropsy on day 30 after insemination, and all fetuses were examined macroscopically at maternal necropsy and subsequently by detailed examination for external and visceral variations and abnormalities and skeletal variations and abnormalities.

There were no effects on maternal mortality or on embryo/fetal caesarean section parameters. Maternal body weight was decreased by 83%, feed consumption was decreased by 15–44% and gravid uterine weights were decreased by 8% at 25 mg/kg bw per day. Fetal weights were decreased by 10% at 25 mg/kg bw per day. There was no evidence of teratogenicity or sensitivity of the young.

The NOAEL for maternal toxicity was 7.5 mg/kg bw per day. The LOAEL for maternal toxicity was 25 mg/kg bw per day, based on decreased body weight, feed consumption and gravid uterine weight.

The NOAEL for embryo and fetal toxicity was 7.5 mg/kg bw per day. The LOAEL for embryo and fetal toxicity was 25 mg/kg bw per day, based on decreased fetal weights (Takahashi, 2002d).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, flonicamid (purity 98.7%) was administered by gavage in 0.5% (w/v) aqueous methyl cellulose to fasted young adult Sprague-Dawley rats (10 of each sex per dose) at a dose of 0, 100, 300, 600 (males only) or 1000 mg/kg bw (females only). A satellite group of five males was also treated at 1000 mg/kg bw. All animals were observed for up to 14 days post-dosing. Functional observational battery and motor activity were evaluated pretreatment and on days 0 (approximately 30–60 minutes post-dosing), 7 and 14. At termination, six rats of each sex per dose (four males in the 1000 mg/kg bw satellite group) were perfused in situ for neurohistological examination. Positive control data conducted at the same laboratory were provided.

There were no effects at doses at or below 600 mg/kg bw. There was one mortality in the five males given 1000 mg/kg bw, on the day following dosing. Clinical signs in this male consisted of flattened posture, impaired respiration, little or no response to external stimuli, tremors and impaired locomotion during the evaluation at 30–60 minutes post-dosing. In other males in that dose group, total session locomotor activity (distance) was decreased by 63% and mean session resting time was increased by 27%, with both these parameters attaining statistical significance when compared with controls during the evaluations 30–60 minutes post-dosing. These changes were considered part of the overt toxicity of the test substance, as the acute median lethal dose (LD_{50}) was 884 mg/kg bw. In animals given 1000 mg/kg bw, there were significant increases in resting times in males, forelimb grip strength in females and landing foot splay in both males and females.

The NOAEL was 300 mg/kg bw for females and 600 mg/kg bw for males. The LOAEL was 1000 mg/kg bw for males and females, based on increased resting time and increased landing foot splay observed in both sexes, systemic toxicity and decreased total locomotor activity in males, and increased forelimb grip strength observed in females at the high dose (Ridder & Watson, 2002).

In a range-finding subchronic neurotoxicity study, flonicamid (purity 98.7%) was administered in the diet to Sprague-Dawley rats (five of each sex per dose) at a dose of 0, 200, 500, 1000, 5000 or 10 000 ppm (equal to 0, 17, 41, 84, 388 and 712 mg/kg bw per day for males and 0, 18, 46, 84, 429 and 807 mg/kg bw per day for females, respectively) and to a group of five females at a dose of 20 000 ppm (equal to 1012 mg/kg bw per day) for 28 days. All animals were monitored twice daily for mortality and moribundity and daily for clinical observations, and body weight and feed consumption were measured weekly. A gross necropsy was performed on all animals.

At 5000 ppm, feed consumption was decreased in males. At 10 000 ppm, body weight, body weight gain and feed consumption were decreased in males and females. In the female-only 20 000 ppm dose group, there was one mortality, with hunched and/or pale appearance, decreased defecation, decreased body weight, body weight gain and feed consumption, thin appearance at necropsy and pale pancreas (Schaefer, 2003a).

In a subchronic neurotoxicity study, flonicamid (purity 98.7%) was administered in the diet to Sprague-Dawley rats (10 of each sex per dose) at a dose of 0, 200, 1000 or 10 000 ppm (equal to 0, 13, 67 and 625 mg/kg bw per day for males and 0, 16, 81 and 722 mg/kg bw per day for females, respectively) for 13 weeks. All animals were monitored twice daily for mortality and moribundity and daily for clinical observations, and body weight and feed consumption were measured weekly. Functional observational battery and motor activity were evaluated prior to initiation of treatment and at weeks 3, 7 and 12. At termination, five rats of each sex per group were perfused in situ, and tissues

from the control and 10 000 ppm groups were examined microscopically. Positive control data conducted at the same laboratory were provided.

There were no treatment-related effects on mortality, clinical signs, and gross pathology or neuropathology parameters. Changes at 1000 ppm were limited to a decreased incidence of rearing in open-field observations in males throughout the treatment period. Body weights were decreased in 10 000 ppm males throughout the treatment period and in females at the same dose in weeks 1, 2, 9 and 11–13. Body weight gains were decreased throughout the study in males and females at 10 000 ppm, as was feed consumption. Total motor activity was decreased by 48% at week 7 in 10 000 ppm males, and locomotor activity was decreased by 55% at week 7 in the same males and by 37% in 10 000 ppm females. Landing foot splay was increased in males at 10 000 ppm.

The NOAEL was 1000 ppm (67 and 81 mg/kg bw per day for males and females, respectively). The LOAEL was 10 000 ppm (625 and 722 mg/kg bw per day for males and females, respectively), based on decreased body weights, body weight gain and feed consumption in males and females, decreased rearing and total motor activity in males, decreased locomotor activity in males and females, and increased landing foot splay in males (Schaefer, 2003b).

(b) Immunotoxicity

In a 28-day immunotoxicity study, flonicamid (purity 98.7%) was administered in the diet to groups of 10 female Crl:CD-1 (ICR) mice at a dose of 0, 100, 600 or 6000 ppm (equal to 0, 23.2, 142 and 1540 mg/kg bw per day, respectively). A positive control group of 10 females was administered control diet and cyclophosphamide monohydrate in phosphate-buffered saline via gavage at 50 mg/kg bw per day.

There were no effects on mortality. Body weight and body weight gain were decreased compared with controls in 6000 ppm mice throughout the treatment period, and feed consumption was increased on days 0–3 and 14–17, but decreased on days 24–28. Clinical signs of toxicity in the 6000 ppm dose group consisted of unkempt appearance, cool-to-touch extremities, decreased defecation, small faeces and thin body condition. Spleen weights were increased and thymus weights were decreased at 6000 ppm; however, there was no statistical significance to either change. The number of spleen cells per sample was increased in the 6000 ppm dose group. A splenic antibody-forming cell assay indicated an increase in spleen activity in all groups treated with flonicamid; however, there was no dose–response relationship, and the change was of unknown significance. In animals treated with cyclophosphamide monohydrate, spleen and thymus weights were decreased and spleen activity was reduced to zero, validating the test.

The NOAEL was 600 ppm (equal to 142 mg/kg bw per day). The LOAEL was 6000 ppm (equal to 1540 mg/kg bw per day), based on clinical signs of toxicity and decreased body weight and body weight gain (Setser, 2012).

(c) Studies on metabolites

Acute toxicity

Acute toxicity studies on the metabolites of flonicamid are summarized in Table 10.

Short-term studies of toxicity

In a 90-day study, groups of 12 Wistar (Jcl:Wistar) rats of each sex received TFNA (purity > 99%) in the diet at a dose level of 0, 50 or 2000 ppm in males (equal to 0, 3.42 and 136 mg/kg bw per day, respectively) and of 0, 200 or 5000 ppm in females (equal to 0, 15.9 and 409 mg/kg bw per day, respectively). Animals were observed daily for morbidity, moribundity and overt clinical signs. Body weight and feed consumption measurements were performed once per week. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, select organs were weighed, assessed by gross examination and examined histopathologically.

Table 10. Acute toxicity of flonicamid metabolites

Test substance	Species	Strain	Sex	Route	Purity (%)	Result	Reference
TFNA	Rat	HanBrl: Wist (SPF)	Male and female	Oral	99.4	LD_{50} > 2 000 mg/kg bw	Damme (2002a)
TFNG	Rat	HanBrl: Wist (SPF)	Male and female	Oral	99.4	LD_{50} > 2 000 mg/kg bw	Damme (2002b)
TFNA-AM	Rat	HanBrl: Wist (SPF)	Male and female	Oral	100	LD_{50} > 2 000 mg/kg bw	Damme (2002c)
TFNA-OH[1]	Rat	HanBrl: Wist (SPF)	Male and female	Oral	100	LD_{50} > 2 000 mg/kg bw	Damme (2002d)
TFNG-AM	Rat	HanBrl: Wist (SPF)	Male and female	Oral	99.5	LD_{50} > 2 000 mg/kg bw	Damme (2002e)

bw: body weight; LD_{50}: median lethal dose; TFNA: 4-trifluoronicotinic acid; TFNA-AM: 4-trifluoromethylnicotinamide; TFNA-OH: 6-hydroxy-4-trifluoromethylnicotinic acid; TFNG: *N*-(4-trifluoronicotinoyl)glycine; TFNG-AM: *N*-(4-trifluoromethylnicotinoyl)glycinamide

There was no effect on mortality or clinical signs of toxicity. Body weights in males at 2000 ppm were decreased to a slight, but not adverse, extent. Feed consumption was likewise decreased, but in the absence of adverse effects on body weight, the change was not considered to be adverse. Blood glucose level was decreased in 5000 ppm females; however, the modest decreases were not considered toxicologically relevant in light of the rest of the database.

The NOAEL was 2000 ppm (equal to 136 mg/kg bw per day) for males and 5000 ppm (equal to 409 mg/kg bw per day) for females, the highest doses tested (Nagaike, 2003a).

In a 90-day study, groups of 12 Wistar (Jcl:Wistar) rats of each sex received TFNG (purity > 98%) in the diet at a dose level of 0, 50 or 2000 ppm in males (equal to 0, 3.56 and 135 mg/kg bw per day, respectively) and 0, 200 or 5000 ppm in females (equal to 0, 16.5 and 411 mg/kg bw per day, respectively). Animals were observed daily for morbidity, moribundity and overt clinical signs. Body weight and feed consumption measurements were performed once per week. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, select organs were weighed, assessed by gross examination and examined histopathologically.

There were no treatment-related mortalities or clinical signs. There were no effects on body weight, feed consumption, feed efficiency, haematology, clinical chemistry, macropathology or micropathology.

The NOAEL was 2000 ppm (equal to 135 mg/kg bw per day) for males and 5000 ppm (equal to 411 mg/kg bw per day) for females, the highest doses tested (Nagaike, 2003b).

Genotoxicity studies

Genotoxicity studies on flonicamid metabolites are summarized in Table 11.

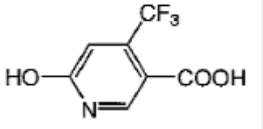


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Table 11. Genotoxicity studies with flonicamid metabolites

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro						
TFNA	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate (±S9)	99.4	Negative	Wollny (2002a)
TFNG	Reverse mutation	*S. typhimurium* and *E. coli*	0, 50, 150, 500, 1 500 and 5 000 µg/plate (±S9)	99.4	Negative	May (2002)
TFNA-AM	Reverse mutation	*S. typhimurium* and *E. coli*	0, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate (±S9)	99	Negative	Wollny (2002b)
TFNA-OH	Reverse mutation	*S. typhimurium* and *E. coli*	0, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate (±S9)	99	Negative	Wollny (2002c)
TFNG-AM	Reverse mutation	*S. typhimurium* and *E. coli*	0, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate (±S9)	99.5	Negative	Wollny (2002d)

S9: 9000 × *g* supernatant fraction from liver homogenate from Aroclor-treated rats; TFNA: 4-trifluoronicotinic acid; TFNA-AM: 4-trifluoromethylnicotinamide; TFNA-OH: 6-hydroxy-4-trifluoromethylnicotinic acid; TFNG: *N*-(4-trifluoronicotinoyl)glycine; TFNG-AM: *N*-(4-trifluoromethylnicotinoyl)glycinamide

(d) Mode of action studies

A number of studies have been conducted with flonicamid to investigate the lung tumours in mice, the differences in lung toxicity between rats and mice and the differences in lung toxicity between mouse strains. Appendix 1 contains further summaries of the mode of action studies within the International Programme on Chemical Safety (IPCS) framework.

In a 3-day dietary toxicity study, groups of five male Crj:CD-1(ICR) strain mice received flonicamid (purity 98.7%) in the diet at a dose level of 0, 80, 250, 750 or 2250 ppm (equal to 0, 12.3, 40.9, 129.6 and 339.3 mg/kg bw per day, respectively) for 72 hours. Morbidity/mortality checks were performed at least twice daily, feed consumption was monitored and body weights were recorded pre-dosing and at necropsy. 5-Bromo-2′-deoxyuridine (BrdU) in saline was administered by intraperitoneal injection at a dose of 100 mg/kg bw to all mice 2 hours prior to the termination of treatment. All mice were killed and subjected to necropsy 72 hours after the start of treatment. The lungs and trachea and the small intestine were removed and infused directly with 4% paraformaldehyde to fix the organs in toto. The preserved organs were subjected to immunohistopathological examination according to the streptavidin-biotin method using an antibody against BrdU as the primary antibody, with the immunohistochemical technique confirmed by a positive reaction in the G zone (cell renewal area) of the small intestine.

There were no mortalities. Three of five mice in the 2250 ppm dose group lost body weight over the course of the 3 days. There were no effects on feed consumption. The BrdU indices were increased to a statistically significant extent in dose groups above 750 ppm and increased to a non-statistically significant extent in the 250 ppm dose group (Table 12), indicating a possible threshold for mitogenic effects between 80 and 250 ppm (equal to 12.3 and 40.9 mg/kg bw per day, respectively) in males (Nomura, 2003a).

Table 12. BrdU indices in the lung in a 3-day dietary toxicity study in mice

Dietary concentration (ppm)	Positive cells/1 000 counted cells (mean ± SD)
0	7.8 ± 2.4
80	7.8 ± 2.2
250	16.0 ± 8.9 (↑105)
750	25.2 ± 6.9** (↑223)
2 250	28.4 ± 5.3** (↑264)

ppm: parts per million; SD: standard deviation; **: $P < 0.01$ (Dunnett's multiple comparison test)
Source: Nomura (2003a)

In a short-term dietary toxicity study, groups of five female Crj:CD-1(ICR) mice and five female Jcl:Wistar rats were given flonicamid (purity 98.7%) in the diet for 3 or 7 days. Mice were given 0 or 2250 ppm (equal to 0 mg/kg bw per day in the control group and 386 and 374 mg/kg bw per day in the 3- and 7-day test groups, respectively), and rats were given 0 or 5000 ppm (equal to 0 mg/kg bw per day in the control group and 403 and 392 mg/kg bw per day in the 3- and 7-day test groups, respectively). Morbidity/mortality checks were performed at least twice daily, feed consumption was monitored and body weights were recorded pre-dosing and at necropsy. BrdU in saline was administered by intraperitoneal injection at a dose of 100 mg/kg bw to all animals after 3 or 7 days of exposure. Animals were killed and subjected to necropsy after 3 or 7 days, depending on group assignment. The lungs and trachea and the small intestine were removed and infused directly with 4% paraformaldehyde to fix the organs in toto. The preserved organs were subjected to immunohistopathological examination according to the streptavidin-biotin method using an antibody against BrdU as the primary antibody, with the immunohistochemical technique confirmed by a positive reaction in the G zone (cell renewal area) of the small intestine.

There were no mortalities, no clinical signs of toxicity and no effects on body weight. After 3 days of treatment, the BrdU index was increased by 280% in mice and unaffected in rats (Table 13). After 7 days of treatment, the BrdU index was increased by 560% in mice and unaffected in rats (Table 13; Nomura, 2003b).

Table 13. BrdU indices in the lung in a 3- and 7-day dietary toxicity study in mice and rats

Dietary concentration (ppm)	Positive cells/1 000 counted cells (mean ± SD)
3-day mice	
0	3.6 ± 1.1
2 250	13.6 ± 3.6** (↑277)
3-day rats	
0	3.8 ± 0.8
5 000	3.6 ± 1.1
7-day mice	
0	3.8 ± 1.3
2 250	10.4 ± 2.1** (↑560)
7-day rats	
0	3.2 ± 1.5
5 000	3.8 ± 1.3

ppm: parts per million; SD: standard deviation; **: $P < 0.01$ (binomial test)
Source: Nomura (2003b)

In a short-term dietary toxicity study, groups of five male Crj:CD-1(ICR) mice were given flonicamid (purity 98.7%) in the diet for 28 days followed by a recovery period of 1, 2 or 4 weeks. Four groups were given 0 ppm (0 mg/kg bw per day), and four groups were given 2250 ppm (295–303 mg/kg bw per day). Morbidity/mortality checks were performed at least twice daily, feed consumption was monitored and body weights were recorded pre-dosing and at necropsy. BrdU in saline was administered by intraperitoneal injection at a dose of 100 mg/kg bw to all animals 2 hours before the terminal sacrifice of each group. All mice were killed and subjected to necropsy at 28 days after the start of treatment or following the 1-, 2- and 4-week recovery periods, depending on group assignment. The lungs and trachea and the small intestine were removed and infused directly with 4% paraformaldehyde to fix the organs in toto. The preserved organs were subjected to immunohistopathological examination according to the streptavidin-biotin method using an antibody against BrdU as the primary antibody, with the immunohistochemical technique confirmed by a positive reaction in the G zone (cell renewal area) of the small intestine. Additionally, CC-10 staining was performed with polyclonal antibody against the Clara cells at the terminal bronchioles.

There was no mortality noted in the study, and there were no clinical signs of toxicity. There were no effects on body weight, body weight gain or feed consumption. At necropsy, livers of the group without a recovery period exhibited trace enlargement, whereas control and recovery animals showed no significant lesions. At the end of the treatment period, all mice in the 2250 ppm dose group without a recovery period exhibited elongation and hyperplasia/hypertrophy of the Clara cells. These lesions were not seen in any of the control animals or in any of the recovery animals. The BrdU index was increased by 188% above controls in animals killed immediately at the end of the treatment period and was unchanged from controls in the three recovery groups (Nomura, 2003c).

In a short-term dietary toxicity study, groups of five male Crj:CD-1(ICR) mice were given flonicamid (purity 98.7%) or metabolites of flonicamid – TFNG (purity 99.4%), TFNA (purity 99.4%) or TFNA-AM (purity 100%) – in the diet for either 3 or 7 days. Animals were given control diet or diet containing flonicamid at 2250 ppm (equal to 389 mg/kg bw per day over 3 days and 330 mg/kg bw per day over 7 days), TFNG at 2250 ppm (equal to 402 mg/kg bw per day over 3 days and 318 mg/kg bw per day over 7 days), TFNA at 2250 ppm (equal to 364 mg/kg bw per day over 3 days and 336 mg/kg bw per day over 7 days) or TFNA-AM at 2250 ppm (equal to 385 mg/kg bw per day over 3 days and 332 mg/kg bw per day over 7 days). Morbidity/mortality checks were performed at least twice daily, feed consumption was monitored and body weights were recorded pre-dosing and at necropsy. BrdU in saline was administered by intraperitoneal injection at a dose of 100 mg/kg bw to all animals after 3 or 7 days of exposure. Animals were killed and subjected to necropsy after 3 or 7 days, depending on group assignment. The lungs and trachea and the small intestine were removed and infused directly with 4% paraformaldehyde to fix the organs in toto. The preserved organs were subjected to immunohistopathological examination according to the streptavidin-biotin method using an antibody against BrdU as the primary antibody, with the immunohistochemical technique confirmed by a positive reaction in the G zone (cell renewal area) of the small intestine.

There were no mortalities, no clinical signs of toxicity and no gross changes at necropsy. Body weight and feed consumption were unaffected by treatment. After 3 days of treatment, animals given flonicamid had a 250% increase in the BrdU index, whereas there was no effect in animals treated with metabolites. After 7 days of treatment, the BrdU index was increased by 194% in animals given flonicamid and was likewise unaffected in animals given the metabolites.

In conclusion, it was determined that the increase in cell division is a result of exposure to flonicamid and not a result of exposure to the most common metabolites (Nomura, 2003d).

In a short-term dietary toxicity study, groups of five male CD-1, B6C3F1 or C57 mice were given flonicamid (purity 98.7%) in the diet for 3 days. Each strain had a control group, a group given 2250 ppm flonicamid (equal to 299, 316 and 306 mg/kg bw per day in CD-1, B6C3F1 and C57 strains, respectively) and a group given 2250 ppm isoniazid (equal to 298, 325 and 290 mg/kg bw per

day in CD-1, B6C3F1 and C57 strains, respectively) as a positive control. Morbidity/mortality checks were performed at least twice daily, feed consumption was monitored and body weights were recorded pre-dosing and at necropsy. BrdU in saline was administered by intraperitoneal injection at a dose of 100 mg/kg bw to all mice 2 hours prior to the termination of treatment. All mice were killed and subjected to necropsy 72 hours after the start of treatment. The lungs and trachea and the small intestine were removed and infused directly with 4% paraformaldehyde to fix the organs in toto. The preserved organs were subjected to immunohistopathological examination according to the streptavidin-biotin method using an antibody against BrdU as the primary antibody, with the immunohistochemical technique confirmed by a positive reaction in the G zone (cell renewal area) of the small intestine. Additionally, CC-10 staining was performed with polyclonal antibody against the Clara cells at the terminal bronchioles.

There was no mortality, and there were no clinical signs of toxicity. There were no effects on body weight or feed consumption. In the CD-1 strain mice, both flonicamid and isoniazid induced an increase in the BrdU index, although the effect of isoniazid was greater than that of flonicamid. In the B6C3F1 and C57 strains, the BrdU index was increased after treatment with isoniazid, but not with flonicamid (Table 14).

In conclusion, CD-1 mice were more susceptible than the B6C3F1 and C57 strains to either flonicamid or isoniazid. Additionally, flonicamid caused less of an increase in the BrdU index in the CD-1 mice than did isoniazid and did not result in a significant increase in the BrdU indices in either the B6C3F1 or C57 strain (Nomura, 2003e).

3. Observations in humans

A report published in *Human and Experimental Toxicology* (Su et al., 2011) described a poisoning as a result of ingestion of 80 mL of Conserve containing spinosad and 2–3 g of flonicamid. Clinical signs of toxicity consisted of consciousness disturbance, shock, respiratory failure, pneumonitis and urinary retention, along with grade 2a oesophageal injury. The investigators attributed the toxicity to the presence of spinosad, and this case is inconclusive as to the toxic properties of flonicamid in humans.

Table 14. BrdU indices in the lung in a 3-day dietary toxicity study in three strains of mice

Dietary concentration (ppm)	Positive cells/1 000 counted cells (mean ± SD)
CD-1	
0	3.4 ± 1.3
Flonicamid	10.6 ± 3.6** (↑212)
Isoniazid	23.6 ± 2.7** (↑594)
B6C3F1	
0	3.0 ± 0.5
Flonicamid	3.0 ± 1.4
Isoniazid	12.0 ± 3.3** (↑300)
C57	
0	2.0 ± 0.8
Flonicamid	2.2 ± 0.7 (↑10)
Isoniazid	4.0 ± 1.6* (↑100)

ppm: parts per million; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$ (binomial test)
Source: Nomura (2003e)

No information was provided on the health of workers involved in the manufacture or use of flonicamid.

Comments

Biochemical aspects

In metabolism studies conducted in rats using flonicamid labelled with ^{14}C at the 3-nicotinamide position, flonicamid was rapidly absorbed. T_{max} values were under 1 hour at the low and high doses (2 and 400 mg/kg bw, respectively), and half-lives were between 4.5 hours at the low dose and 11.6 hours at the high dose. Radiolabel concentrations in the plasma in all dose groups decreased in a manner consistent with first-order kinetics. The majority of administered radioactivity was excreted in the urine within the first 24 hours. There was no evidence of bioaccumulation following repeated dosing. Distribution to the tissues was extensive, with levels similar to concentrations in plasma; however, slightly higher concentrations were seen in the liver, kidneys, adrenals, thyroid and ovaries following single or repeated dosing in both sexes and in the lungs following repeated dosing in males.

The main urinary residue was unchanged parent, followed by 4-trifluoromethylnicotinamide (TFNA-AM), which was also the predominant metabolite in the faeces and bile. Other metabolites were 4-trifluoromethylnicotinic acid (TFNA), TFNA-AM *N*-oxide conjugate (not specified), *N*-(4-trifluoromethylnicotinoyl)glycinamide (TFNG-AM) and *N*-(4-trifluoromethylnicotinoyl)glycine (TFNG) (Neal & Savides, 2001a,b; Dow, 2002; Gupta, Shah & McClanahan, 2002; Neal, Savides & Dow, 2002a,b).

Toxicological data

In rats, flonicamid is of moderate acute oral toxicity (LD_{50} = 884 mg/kg bw), low acute dermal toxicity (LD_{50} > 5000 mg/kg bw) and low acute inhalation toxicity (LC_{50} > 4.90 mg/L). Flonicamid was slightly irritating to the eyes and non-irritating to the skin of rabbits. It was not a dermal sensitizer in guinea-pigs (Paul, 2000; Ridder & Watson, 2000; Ridder, Yoshida & Watson, 2000a,b,c, 2001a).

The main target organs are the liver, haematopoietic system and lungs in mice and the liver and kidneys in rats. In long-term studies in rats, the skeletal muscles, eyes and stomach are also targets. In dogs, effects on general condition and clinical signs are the main signs of toxicity.

In a 90-day range-finding study in mice, which tested dietary flonicamid concentrations of 0, 100, 1000 and 7000 ppm (equal to 0, 15, 154 and 1069 mg/kg bw per day for males and 0, 20, 192 and 1248 mg/kg bw per day for females, respectively), increased incidences of minimal centrilobular hypertrophy in the liver in males and increased incidences of minimal to moderately severe extramedullary haematopoiesis in the spleen were seen in both sexes at 1000 ppm (equal to 154 mg/kg bw per day). The lungs were not evaluated in this study (Ridder, Yoshida & Watson, 2001b).

In a 28-day range-finding study in rats, dietary flonicamid concentrations of 0, 50, 100, 500, 1000 and 5000 ppm (equal to 0, 3.61, 7.47, 36.5, 73.8 and 353 mg/kg bw per day, respectively) for males and 0, 100, 500, 1000, 5000 and 10 000 ppm (equal to 0, 8.36, 41.2, 81.9, 373 and 642 mg/kg bw per day, respectively) for females were tested. The kidneys of two males per dose at 0 and 5000 ppm were immunostained for α_{2u}-globulin. In addition to effects on clinical chemistry and haematology parameters and on the liver (e.g. dark coloration, hepatocellular hypertrophy and increased incidence of liver enlargement) at 5000 ppm in males and females, males also exhibited increased incidence of pale kidneys, increased kidney weights, increased incidence of hyaline droplet depositions in proximal tubular cells and granular casts of the kidneys. Hyaline droplets were positive for α_{2u}-globulin (Kuwahara, 2002a). As a result, the Meeting considered hyaline droplets of the kidneys to be specific to male rats and not applicable to the human risk assessment.

In a 90-day study in the rat, which tested dietary flonicamid concentrations of 0, 50, 200, 1000 and 2000 ppm (equal to 0, 3.08, 12.1, 60.0 and 119 mg/kg bw per day, respectively) for males and 0, 200, 1000 and 5000 ppm (equal to 0, 14.5, 72.3 and 340 mg/kg bw per day, respectively) for females, the NOAEL was 200 ppm (equal to 12.1 mg/kg bw per day), based on increased kidney weights, granular casts and increased basophilic changes in the renal tubules in males at 1000 ppm (equal to 60.0 mg/kg bw per day) (Kuwahara, 2002b). The Meeting was unable to dismiss the possible human relevance of the kidney findings in the male rats because of the observation of kidney effects in female rats at higher doses in this study and in female dogs (see below).

In a 90-day study in dogs, which tested capsule flonicamid doses of 0, 3, 8, 20 and 50 (females only) mg/kg bw per day, the NOAEL was 8 mg/kg bw per day, based on vomiting, ataxia, decreased activity, laboured breathing, prostration, and decreased body weight and body weight gain in both sexes, as well as decreased feed consumption in females, observed at 20 mg/kg bw per day. Tubular vacuolation of the inner cortex of the kidney was noted in 2/4 females at 50 mg/kg bw per day (Ridder & Watson, 2001).

In a 1-year study in dogs, which tested capsule flonicamid doses of 0, 3, 8 and 20 mg/kg bw per day, the NOAEL was 8 mg/kg bw per day, based on vomiting and increased reticulocytes in males and females and decreased body weight gain in females at 20 mg/kg bw per day (Ridder & Watson, 2003a).

The Meeting concluded that the overall NOAEL for oral toxicity in dogs was 8 mg/kg bw per day, and the overall LOAEL was 20 mg/kg bw per day.

In an 18-month study in CD-1 mice, which tested dietary flonicamid concentrations of 0, 250, 750 and 2250 ppm (equal to 0, 29, 88 and 261 mg/kg bw per day for males and 0, 38, 112 and 334 mg/kg bw per day for females, respectively), a NOAEL was not identified, as an increase in the combined incidence of alveolar/bronchiolar adenomas in both sexes, an increase in extramedullary haematopoiesis of the spleen, increased pigment deposition in the femoral and sternal bone marrow, increased centrilobular hepatocellular hypertrophy, increased incidence of hyperplasia/hypertrophy of the epithelial cells of the terminal bronchioles and masses/nodules in lung of males and decreased cellularity of the femoral bone marrow in females were observed at 250 ppm (equal to 29 mg/kg bw per day), the lowest dose tested (Ridder & Watson, 2003b).

In a second 18-month study in CD-1 mice, which tested dietary flonicamid concentrations of 0, 10, 25, 80 and 250 ppm (equal to 0, 1.2, 3.1, 10.0 and 30.3 mg/kg bw per day for males and 0, 1.4, 3.7, 11.8 and 36.3 mg/kg bw per day for females, respectively), the NOAEL was 80 ppm (equal to 10.0 mg/kg bw per day), based on an increase in lung adenomas in males, a slight lung hyperplasia/hypertrophy in the terminal bronchiole epithelial cells in males and females and an increased incidence of hyperplasia of alveolar epithelial cells in females at 250 ppm (equal to 30.3 mg/kg bw per day) (Nagaoka, 2004).

The overall NOAEL for the two long-term mouse studies was 80 ppm (equal to 10.0 mg/kg bw per day), and the overall LOAEL was 250 ppm (equal to 29 mg/kg bw per day).

In a 2-year study in rats, which tested dietary flonicamid concentrations of 0, 50, 100, 200 and 1000 ppm (equal to 0, 1.84, 3.68, 7.32 and 36.5 mg/kg bw per day, respectively) for males and 0, 200, 1000 and 5000 ppm (equal to 0, 8.92, 44.1 and 219 mg/kg bw per day, respectively) for females, the NOAEL was 200 ppm (equal to 7.32 mg/kg bw per day), based on decreased body weight and body weight gain, decreased rearing, and increased incidences of keratitis and pelvic dilatation in the kidneys in males and decreased triglyceride levels and increased striated muscle atrophy in females at 1000 ppm (equal to 36.5 mg/kg bw per day). No treatment-related tumours were observed in this study (Kuwahara, 2002c).

The Meeting concluded that flonicamid causes lung tumours in CD-1 mice, but is not carcinogenic in rats.

Flonicamid was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found (Matsumoto, 2002a,b,c,d; Mehmood, 2003).

The Meeting concluded that flonicamid is unlikely to be genotoxic.

Mechanistic studies were performed in support of the hypothesis that lung tumours caused by flonicamid were due to a non-genotoxic proliferative process specific to the Clara cells of CD-1 mice. The investigators identified a possible threshold for mitogenic effects between 80 and 250 ppm (equal to 12.3 and 40.9 mg/kg bw per day, respectively) in a 3-day dietary toxicity study in male mice. Flonicamid caused a transient increase in elongation and hyperplasia/hypertrophy of the Clara cells in the lungs of male mice. In a short-term dietary study of flonicamid and its metabolites in CD-1 mice, proliferation of the respiratory bronchiolar epithelial cells was specific to the parent compound, as male mice exposed to TFNG, TFNA and TFNA-AM did not exhibit this finding. Flonicamid did not cause such proliferation in female rats or in B6C3F1 and C57 mice. Overall, the mechanistic studies support the plausibility of the proposed mode of action (Nomura, 2003a,b,c,d,e).

In view of the lack of genotoxicity, the absence of carcinogenicity in rats and the fact that lung tumours were observed only in CD-1 mice with a plausible mode of action, the Meeting concluded that flonicamid is unlikely to pose a carcinogenic risk to humans from the diet.

In a reproductive toxicity study in rats, which tested dietary concentrations of 0, 50, 300 and 1800 ppm (equal to 0, 3.7, 22.3 and 133 mg/kg bw per day for males and 0, 4.4, 26.5 and 153 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 300 ppm (equal to 22.3 mg/kg bw per day), based on increased proximal tubule cell vacuolation of the kidney observed in females and increased kidney weights, tubular basophilic change and granular casts observed in males at 1800 ppm (equal to 133 mg/kg bw per day). The NOAEL for offspring toxicity was 300 ppm (equal to 26.5 mg/kg bw per day), based on delayed sexual maturation and decreased uterine weight in F_1 females at 1800 ppm (equal to 153 mg/kg bw per day). The NOAEL for reproductive toxicity was 1800 ppm (equal to 133 mg/kg bw per day), the highest dose tested. Minor changes in levels of reproductive hormones observed at 300 ppm and above in the absence of any adverse effects on reproduction were not considered toxicologically relevant (Takahashi, 2002b).

In a range-finding developmental toxicity study in rats, which tested gavage flonicamid doses of 0, 30, 100, 300 and 1000 mg/kg bw per day, the first signs of toxicity in the dams were clinical signs of eye discharge, forelimb wounding, loss of abdominal fur, soiled fur around the external genital region, vaginal haemorrhage, white discharge on the tray, and decreased body weight gain and feed consumption at 1000 mg/kg bw per day. There were no external abnormalities at the highest dose tested (Hojo, 2002a).

In a developmental toxicity study in rats, which tested gavage flonicamid doses of 0, 20, 100 and 500 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased liver weights and histopathological changes in the liver and kidneys observed at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on an increase in cervical rib skeletal variations observed at 500 mg/kg bw per day (Hojo, 2002b).

In a range-finding developmental toxicity study in rabbits, which tested gavage flonicamid doses of 0, 3, 10 and 30 mg/kg bw per day, decreased body weight gain, feed consumption and gravid uterine weight were observed in the dams at 30 mg/kg bw per day. In the offspring, a decreased number of live fetuses, decreased fetal weights and decreased percentage of male fetuses were observed at 30 mg/kg bw per day (Takahashi, 2002c).

In a developmental toxicity study in rabbits, which tested gavage flonicamid doses of 0, 2.5, 7.5 and 25 mg/kg bw per day, the NOAEL for maternal toxicity was 7.5 mg/kg bw per day, with decreased body weight, feed consumption and gravid uterine weight being observed at 25 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 7.5 mg/kg bw per day, with decreased fetal weights being observed at 25 mg/kg bw per day (Takahashi, 2002d).

In an acute neurotoxicity study in rats, which tested flonicamid at gavage doses of 0, 100, 300, 600 (males only) and 1000 (females only) mg/kg bw per day, increased resting time and increased landing foot splay in both sexes, systemic toxicity and decreased total locomotor activity in males, and increased forelimb grip strength in females were observed at the high dose. The effects

were not considered specific to neurotoxicity, but rather indicative of systemic toxicity (Ridder & Watson, 2002).

In a 13-week neurotoxicity study in rats, which tested dietary concentrations of 0, 200, 1000 and 10 000 ppm (equal to 0, 13, 67 and 625 mg/kg bw per day for males and 0, 16, 81 and 722 mg/kg bw per day for females, respectively), effects at the high dose included decreased body weights, body weight gain and feed consumption in males and females, decreased rearing and total motor activity in males, decreased locomotor activity in males and females, and increased landing foot splay in males. The effects were not considered specific to neurotoxicity, but rather indicative of systemic toxicity (Schaefer, 2003b).

The Meeting concluded that flonicamid is not neurotoxic.

In a 28-day immunotoxicity study in female mice, which tested dietary flonicamid concentrations of 0, 100, 600 and 6000 ppm (equal to 0, 23.2, 142 and 1540 mg/kg bw per day, respectively), clinical signs of toxicity and decreased body weight and body weight gain were observed at the high dose. No specific immunotoxic effects were observed (Setser, 2012).

The Meeting concluded that flonicamid is not immunotoxic.

Toxicological data on metabolites and/or degradates

Acute toxicity and genotoxicity studies were performed on five metabolites: TFNA (plants, also in rat), TFNA-AM (all livestock commodities, also in rat), TFNG (plants, also in rat), TFNG-AM (rat) and 6-hydroxy-4-trifluoromethylnicotinic acid (TFNA-OH) (secondary crops). Additionally, short-term dietary studies were conducted with TFNA and TFNG.

TFNA was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and did not show evidence of genotoxicity in an Ames test. In a 90-day toxicity study in rats, TFNA was given at a dietary concentration of 0, 50 or 2000 ppm for males (equal to 0, 3.42 and 136 mg/kg bw per day, respectively) and 0, 200 or 5000 ppm for females (equal to 0, 15.9 and 409 mg/kg bw per day, respectively). The modest increase in blood glucose levels in females at 5000 ppm was not considered toxicologically significant, and no other changes were observed. The NOAEL was 2000 ppm (equal to 136 mg/kg bw per day) for males and 5000 ppm (equal to 409 mg/kg bw per day) for females, the highest doses tested (Damme, 2002a; Wollny, 2002a; Nagaike, 2003a). The Meeting concluded that TFNA is markedly less toxic than the parent compound.

TFNA-AM was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and did not show evidence of genotoxicity in an Ames test (Damme, 2002b; Wollny, 2002b). As TFNA-AM is a major rat metabolite, the Meeting concluded that TFNA-AM would be no more toxic than the parent compound.

TFNG, a minor metabolite found in the rat liver, but a major plant metabolite, was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and did not show evidence of genotoxicity in an Ames test. In a 90-day toxicity study in rats, TFNG was given at a dietary concentration of 0, 50 or 2000 ppm for males (equal to 0, 3.56 and 135 mg/kg bw per day, respectively) and 0, 200 or 5000 ppm for females (equal to 0, 16.5 and 411 mg/kg bw per day, respectively). The NOAEL was 2000 ppm (equal to 135 mg/kg bw per day) for males and 5000 ppm (equal to 411 mg/kg bw per day) for females, the highest doses tested (Damme, 2002c; May, 2002; Nagaike, 2003b). The Meeting concluded that TFNG is markedly less potent than the parent compound.

TFNA-OH was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and did not show evidence of genotoxicity in an Ames test (Damme, 2002d; Wollny, 2002c). The Meeting concluded that TFNA-OH would likely be less potent than the parent compound, taking into consideration the limited data available and the structural similarity to TFNA.

TFNG-AM was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and did not show evidence of genotoxicity in an Ames test (Damme, 2002e; Wollny, 2002d). The Meeting concluded that TFNG-AM would be no more toxic than the parent compound.

Human data

No information was provided on the health of workers involved in the manufacture or use of flonicamid.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.07 mg/kg bw on the basis of a NOAEL of 7.32 mg/kg bw per day in the 2-year rat study, based on decreased body weight, decreased rearing, effects on clinical chemistry and effects on kidney and muscle observed at 36.5 mg/kg bw per day. This ADI is supported by the overall NOAEL of 8 mg/kg bw per day in dogs and the NOAELs of 7.5 mg/kg bw per day for maternal and embryo/fetal toxicity in the developmental toxicity study in rabbits. A safety factor of 100 was applied. The margin between the upper bound of the ADI and the LOAEL of 29 mg/kg bw per day for lung adenomas in male mice is about 415.

The Meeting concluded that the ADI would apply to the sum of flonicamid and the metabolites TFNA-AM and TFNG-AM, expressed as flonicamid.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for flonicamid in view of its low acute toxicity and the 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 flonicamid

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month studies of toxicity and carcinogenicity[a,b]	Toxicity	80 ppm, equal to 10.0 mg/kg bw per day	250 ppm, equal to 29 mg/kg bw per day
		Carcinogenicity	80 ppm, equal to 10.0 mg/kg bw per day	250 ppm, equal to 29 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	200 ppm, equal to 7.32 mg/kg bw per day	1 000 ppm, equal to 36.5 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 36.5 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 800 ppm, equal to 133 mg/kg bw per day[c]	–
		Parental toxicity	300 ppm, equal to 22.3 mg/kg bw per day	1 800 ppm, equal to 133 mg/kg bw per day
		Offspring toxicity	300 ppm, equal to 26.5 mg/kg bw per day	1 800 ppm, equal to 153 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	100 mg/kg bw per day	500 mg/kg bw per day
		Embryo and fetal	100 mg/kg bw per	500 mg/kg bw per

Species	Study	Effect	NOAEL	LOAEL
		toxicity	day	day
	Acute neurotoxicity study[d]	Neurotoxicity	600 mg/kg bw per day[c]	–
	Subchronic neurotoxicity study[a]	Neurotoxicity	625 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	7.5 mg/kg bw per day	25 mg/kg bw per day
		Embryo and fetal toxicity	7.5 mg/kg bw per day	25 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity[b,e]	Toxicity	8 mg/kg bw per day	20 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI) (for sum of flonicamid and metabolites TFNA-AM and TFNG-AM, expressed as flonicamid)

0–0.07 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 flonicamid

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, almost complete at low and high doses
Dermal absorption	Not given
Distribution	Widely distributed at levels similar to plasma, higher concentrations in liver, kidneys, adrenals, thyroid and ovaries and in lungs in males following repeated dosing
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	93–98% excreted within 7 days; predominantly in urine within the first 24 h
Metabolism in animals	Multiple metabolites, approximately 50% excreted unchanged
Toxicologically significant compounds in animals and plants	Flonicamid, TFNA-AM, TFNG-AM

Acute toxicity	
Rat, LD_{50}, oral	884 mg/kg bw
Rat, LD_{50}, dermal	> 5 000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.90 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Slightly irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson and Kligman maximization test or Buehler method)
Short-term studies of toxicity	
Target/critical effect	Clinical signs of toxicity and decreased body weight (dog)
Lowest relevant oral NOAEL	8 mg/kg bw per day (dog)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decreased body weight, decreased rearing, effects on clinical chemistry, effects on kidney and muscle
Lowest relevant NOAEL	7.32 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice, but not in rats[a]
Genotoxicity	
	Not genotoxic[a]
Reproductive toxicity	
Target/critical effect	Kidney effects in parents; delayed sexual maturation and decreased uterine weight in female offspring
Lowest relevant parental NOAEL	22.3 mg/kg bw per day
Lowest relevant offspring NOAEL	26.5 mg/kg bw per day
Lowest relevant reproductive NOAEL	133 mg/kg bw per day (highest dose tested)
Developmental toxicity	
Target/critical effect	Decreased maternal body weight, gravid uterine weight and fetal weight
Lowest relevant maternal NOAEL	7.5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	7.5 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	600 mg/kg bw (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	625 mg/kg bw per day (highest dose tested; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 540 mg/kg bw per day (highest dose tested; mouse)
Studies on toxicologically relevant metabolites	*TFNA-AM* Oral LD_{50} > 2 000 mg/kg bw (rat) No evidence of genotoxicity *TFNG-AM* Oral LD_{50} > 2 000 mg/kg bw (rat) No evidence of genotoxicity

Mechanistic/mode of action studies	Causes increased cell division in CD-1 mouse lungs after 3 days at a threshold between 12.3 and 40.9 mg/kg bw per day Does not cause similar increases in rats, B6C3F1 mice or C57 mice TFNG, TFNA and TFNA-AM do not cause an increase in cell division Following a recovery period of 1–3 weeks, there is no evidence of increased cell division
Medical data	
	No information was provided

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

Summary

	Value	Study	Safety factor
ADI	0–0.07 mg/kg bw	Two-year toxicity study (rat)	100
ARfD	Unnecessary	–	–

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Appendix 1: Mode of action

Rationale for the proposed mode of action (MOA)

Exposure to flonicamid over a period of 18 months produced increased incidences of preneoplastic and neoplastic lesions in the lungs of CD-1 mice. Incidences of alveolar/bronchiolar

adenoma and/or carcinoma were increased in both male and female mice. These tumours were observed at all doses in the initial (high-dose) study and at 250 ppm (30.3 and 36.3 mg/kg bw per day for males and females, respectively) in the second mouse oncogenicity study performed at lower doses.

Postulated MOA

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])

In the working document provided by the sponsor (Ishihara Sangya Kaisha, Ltd, 2014) to support mitogenesis as a non-linear, non-genotoxic MOA responsible for the development of mouse lung tumours, treatment of CD-1 mice with flonicamid was said to result in increased proliferation of lung tissue, progressing to hyperplasia, adenoma and, eventually, carcinoma. A description of the requirements or relevant information for each subsection of the weight of evidence framework is listed under each heading below. Five additional supplemental studies were conducted using 5-bromo-2′-deoxyuridine (BrdU) to assess rates of cell division in lung tissue of animals treated with flonicamid. These studies included a 3-day dietary study to determine a no-observed-adverse-effect level (NOAEL) for increases in BrdU index, a 3- and 7-day dietary comparative study between the rat and the mouse, a 28-day dietary/28-day recovery study including examination of Clara cells using electron microscopy, a 3- and 7-day dietary study comparing the technical material with metabolites and a 3-day dietary study comparing effects of the technical material with isoniazid in three different mouse strains. In addition to the two oncogenicity studies, a 90-day dietary study was conducted in the CD-1 mouse, although many of the tissues, including the lung, were not subjected to a histopathological examination in that study. The United States Environmental Protection Agency's (USEPA) Cancer Assessment Review Committee (CARC) (USEPA, 2005) report was available in addition to the data package. This report was factored into the assessment and interpretation of carcinogenicity.

Key events

Clear description of each of the key events (measurable parameters) that are thought to underlie the MOA. (ILSI/RSI HRF)

Key events in the development of lung tumours in CD-1 mice treated with flonicamid were proposed by the sponsor as follows:

1. Ingestion of the chemical (oral)
2. Distribution to the lung
3. Mitogenic stimulation of Clara cells
4. Increased proliferation, leading to hyperplasia, adenomas and, ultimately, carcinomas.

The sponsor provided data to support some aspects of the key events; however, there were several parameters that are considered critical to support the proposed MOA that were not measured or supported with data. Metabolism data were provided for the rat only. Hence, there are no toxicokinetic data for flonicamid, including information on metabolic profile, in the mouse or in species other than the rat. In addition, although the Clara cell has been demonstrated to be responsive to treatment, the involvement of other cell types has not been ruled out. Hyperplasia was observed at terminal sacrifice in the oncogenicity study and in animals found dead or killed in extremis during the latter phase of the study. However, there are no data for earlier time points with which to clearly establish a temporal relationship between the development of hyperplasia and adenomas and/or carcinomas. As a result, it is difficult to demonstrate a clear progression through the proposed sequence of events leading to tumour formation.

Table A-1. Incidence of lung neoplasms in second (low-dose) CD-1 mouse oncogenicity study[a]

		Incidence of neoplasms					
Tumour		**0 ppm**	**10 ppm**	**25 ppm**	**80 ppm**	**250 ppm**	**Historical controls[b]**
Alveolar/bronchiolar adenoma	M	8/46** (17%)	11/48 (23%)	12/45 (27%)	11/46 (24%)	21/48** (44%)	19.9% (9.6–32%)
	F	10/49 (20%)	8/48 (17%)	11/41 (27%)	14/42 (33%)	13/45 (29%)	13.7% (3.8–26.9%)
Alveolar/bronchiolar carcinoma	M	3/46 (7%)	6/48 (13%)	3/45 (7%)	4/46 (9%)	9/48 (19%)	12.1% (2–30.8%)
	F	1/49 (2%)	4/48 (8%)	2/41 (5%)	3/42 (7%)	3/45 (7%)	8% (2–15.7%)
Alveolar/bronchiolar adenoma and/or carcinoma combined	M	11/46** (24%)	16/48 (33%)	15/45 (33%)	14/46 (30%)	27/48** (56%)	29.3 (18–42.3%)
	F	10/49* (20%)	12/48 (25%)	12/41 (29%)	16/42 (38%)	16/45 (36%)	20.2 (10–38.5%)

F: female; M: male; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (statistical significance at controls designates trend across the data set, whereas statistical significance at treatment is indicative of pair-wise comparison with controls)

[a] Dietary concentrations of flonicamid in diet equal to doses of 0, 1.2, 3.1,10.0 and 30.3 mg/kg bw per day for males and 0, 1.4, 3.7, 11.8 and 36.3 mg/kg bw per day for females, respectively.

[b] Historical control data are from 17 oncogenicity studies conducted by IET laboratories between 1990 and 2003.

Source: Nagaoka (2004)

Concordance of dose–response relationship

Dose–response relationships identified for each key event, and comparisons presented of dose–response relationships among key events with cancer. (ILSI/RSI HRF)

The discussion document provided by the sponsor made reference to a concordance between the effect level for neoplasms in the mouse oncogenicity studies and the BrdU labelling index (cell proliferation) studies. The sponsor proposes a threshold for tumour response and increase in BrdU labelling index between 80 and 250 ppm. Tables A-1 and A-2 present the tumour data from the two oncogenicity studies. Table A-3 presents the incidence of hyperplasia, as recorded at terminal sacrifice or time of death for animals killed in extremis/found dead from the low-dose oncogenicity study. Results from a preliminary study to determine a threshold for increased cell division and proliferation are indicated in Table A-4.

An increased incidence of hyperplasia in the epithelial cells of the terminal bronchioles was evident in both sexes at the highest dose of 250 ppm following long-term dosing in the low-dose oncogenicity study (Table A-3) and at all doses in the high-dose study. The 80 ppm dose level was considered to be the NOAEL for this response. The sponsor notes in its discussion document (Cohen et al., 2004) that "Although complete concordance between the doses for early and late events is not essential, it is essential that the NOEL [no-observed-effect level] in the short-term, precursor-related study (BrdU labeling index) is the same or lower than that for the carcinogenicity endpoint."

Temporal association

Sequence of key events over time that lead to tumour formation. (ILSI/RSI HRF)

The sponsor has provided some evidence to demonstrate a temporal association between the key events and development of tumours. An increase in BrdU index, indicative of increased cell proliferation, was evident in multiple special studies with durations of dosing ranging from 3 to 28 days. There did not appear to be a durational effect for the BrdU studies, with similar results observed regardless of duration of dosing. Morphological changes in Clara cells suggesting increased cell size

Table A-2. Incidence of lung neoplasms in first (high-dose) CD-1 mouse oncogenicity study[a]

Tumour		Incidence of neoplasms				
		0 ppm	**250 ppm**	**750 ppm**	**2 250 ppm**	**Historical controls[b]**
Alveolar/bronchiolar adenoma	M	7/55* (13%)	25/59** (42%)	25/58** (43%)	33/56** (59%)	14.3% (2–42%)
	F	9/57* (16%)	20/58* (34%)	30/59** (51%)	24/58** (41%)	8.51% (1.7–26.7%)
Alveolar/bronchiolar carcinoma	M	4/55* (7%)	6/59 (10%)	12/58* (21%)	12/56* (21%)	6.9% (1.4–26%)
	F	0/57**	3/58 (5%)	3/59 (5%)	7/58** (12%)	4.1% (0.8–18.4%)
Alveolar/bronchiolar adenoma and/or carcinoma combined	M	10/55** (18%)	27/59** (46%)	29/58** (50%)	36/56** (64%)	Not provided
	F	9/57* (16%)	22/58** (38%)	31/59** (53%)	25/58** (43%)	

F: female; M: male; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (statistical significance at controls designates trend across the data set, whereas statistical significance at treatment is indicative of pair-wise comparison with controls)

[a] Dietary concentrations of flonicamid in diet equal to doses of 0, 29, 88 and 261 mg/kg bw per day for males and 0, 38, 112 and 334 mg/kg bw per day for females, respectively.

[b] Historical control data are from Charles River (2000).

Source: Ridder & Watson (2003b)

Table A-3. Incidence of hyperplasia in CD-1 mice from the low-dose oncogenicity study, as recorded at terminal sacrifice or time of death for animals killed in extremis/found dead

	Incidence of hyperplasia				
	0 ppm	**10 ppm**	**25 ppm**	**80 ppm**	**250 ppm**
Males					
Hyperplasia/hypertrophy, epithelial cell, terminal bronchiole	0/50	5/50	5/50	3/50	14/50*
Females					
Hyperplasia/hypertrophy, epithelial cell, terminal bronchiole	3/50	5/50	2/50	4/50	11/50*

ppm: parts per million; *: $P < 0.05$

[a] Dietary concentrations of flonicamid in diet equal to doses of 0, 1.2, 3.1, 10.0 and 30.3 mg/kg bw per day for males and 0, 1.4, 3.7, 11.8 and 36.3 mg/kg bw per day for females, respectively.

Source: Nagaoka (2004)

Table A-4. BrdU index (number of positive stained cells/1000) for groups of five male CD-1 mice treated with flonicamid[a] for 3 days

	0 ppm	**80 ppm**	**250 ppm**	**750 ppm**	**2 250 ppm**
BrdU index	7.8 ± 2.4	7.8 ± 2.2	16.0 ± 8.9	25.2 ± 6.9**	28.4 ± 5.3**

BrdU: 5-bromo-2′-deoxyuridine; ppm: parts per million; **: $P < 0.01$

[a] Dietary concentrations of flonicamid in diet are equal to doses of 0, 12.3, 40.9, 129.6 and 339.3 mg/kg bw per day, respectively.

Source: Nomura (2003a)

and metabolic activity were also observed in these studies. These studies demonstrated evidence of the reversibility of these changes after 28 days of dosing. The sponsor (Cohen et al., 2004) stated that "The question could be raised that this [cell proliferation rate] has not been followed to subsequent time points; however, clear evidence of increased proliferation was present based on the histopathology observance of increased incidences of hyperplasia." As stated previously in this monograph, histopathology of lung tissue was available only for animals treated long term. There are no short-term studies or interim sacrifice data that examined lung tissue at earlier time periods, and therefore there is uncertainty as to how early hyperplasia is occurring or to confirm that it is evident prior to tumour development. In the absence of this information, the Meeting is of the opinion that a temporal relationship has not been adequately demonstrated.

Strength, consistency and specificity of association of tumour response with key events

Complete assessment and presentation of the relationships among the key events, precursor lesions and tumours. (ILSI/RSI HRF)

The sponsor stated that there are considerable data supporting the sequence of events for lung tumours in this strain of mice, from mitogenesis to hyperplasia to adenoma to carcinoma, not only for flonicamid, but also based on numerous studies with other chemicals. Although the literature acknowledges that progression through this sequence of events (mitogenesis to hyperplasia to adenoma to carcinoma) can be responsible for tumour formation, the Meeting is of the opinion that this has not been clearly demonstrated for flonicamid. The Meeting acknowledges that the sponsor has provided some evidence to support the relationships among the key events, precursor lesions and tumours. Consistency of effects was noted between the two mouse oncogenicity studies, with similar tumour response observed in both studies at the overlapping dose of 250 ppm. Consistency was also noted within the BrdU labelling cell proliferation studies, and results appear to be similar across all studies in the 2250 ppm dose group (the response level in one study was higher in all test groups owing to a second administration of BrdU; the other studies were single-dose administration). The Meeting would concur that, as stated by the sponsor, there did not appear to be any evidence of cell necrosis in lung tissue, indicating that cytotoxicity and subsequent regeneration were not likely explanations for these tumours. In addition, the Meeting would agree that the non-genotoxic profile of flonicamid renders increased mitogenesis as a plausible MOA. However, the key events in the proposed MOA, as mentioned previously, are not adequately supported by measurable parameters. This lowers the overall strength of the MOA argument.

Biological plausibility and coherence

Determination of whether key events and the sequence of events are consistent with current biological thinking, both regarding carcinogenesis in general and for the specific chemical under review. (ILSI/RSI HRF)

The sponsor provided discussion to address the issues of biological plausibility and coherence. This includes discussion regarding issues of the strain specificity of the development of lung tumours in the CD-1 mouse, proper classification of mouse lung tumours and sequence of events for development of lung tumours (from Clara cell proliferation through to carcinoma). The Meeting has considered the sponsor's arguments and acknowledges that the CD-1 mouse model has been recognized to have a high rate of spontaneous lung tumour development. Historical control data are supplied to account for this. The historical control data supplied for these tumours in studies conducted in both IET and Charles River laboratories are consistent with those reported in the literature (22–37%) (Manenti et al., 2003). As mentioned previously, the statistically significantly increased incidence of lung tumours in treated animals at 250 ppm and above relative to concurrent controls was either above or at the high end of the range of these historical control values.

Classification of lung tumours is acknowledged to have improved over time. The definition and classification of cancerous and precancerous lesions are now more precise; however, the labelling

of mouse lung neoplasms as bronchoalveolar has been questioned. In humans, bronchoalveolar tumours are rare and can be distinguished from other neoplasms by a characteristic lepidic growth pattern (Nikitin et al., 2004). This morphology is rarely mirrored in the mouse. The Nikitin et al. (2004) paper notes that an expert panel of pathologists recommended that it would be more appropriate to classify these tumours by tumour type, adenocarcinoma, rather than by location. In humans, adenocarcinoma is the most common type of lung cancer and is estimated to originate from Clara cells in approximately 50–90% of the incidences, as indicated by immunostaining and electron microscopy (Broers et al., 1992; Albertine et al., 1998). The Nikitin et al. (2004) paper goes on to state that this would indicate that the lesions observed in mouse lungs, which are said to originate from the Clara cells, may in fact be of greater concern when evaluating human risk than originally expected.

It is acknowledged that the CD-1 mouse strain does have a higher background incidence for adenoma and/or carcinoma relative to other mouse strains. In addition, the CD-1 mouse appeared to be more sensitive to flonicamid in a comparison BrdU labelling cell proliferation study with B6C3F1 and C57BL mice. As already stated, however, historical control data specific to the CD-1 mouse have been utilized in the interpretation of tumour data. It is acknowledged that lung tumours were not observed in the rat oncogenicity study, nor was there any alteration in BrdU index in rats treated with flonicamid for 7 days. Limited further comparison can be made of the differences in tumour development between the two species, however, as metabolism data are available for the rat only. Hence, it is not known what amounts of flonicamid reach the mouse lung or whether the metabolic profile in the mouse is the same as that in the rat.

Isoniazid is proposed by the sponsor to follow a similar MOA for lung tumours. Isoniazid is an anti-tuberculosis drug that causes bronchoalveolar tumours in mice, but not in rats. The sponsor cites this chemical as producing identical lesions to those produced by flonicamid in the mouse through the same MOA, and for which human epidemiological data (no increased lung tumour rates) are available. Both chemicals produce lung tumours in mice; however, isoniazid is mutagenic, whereas flonicamid is non-mutagenic. Although a comparative cell proliferation study was conducted in CD-1 mice with flonicamid and isoniazid, which demonstrated increased cell proliferation in alveolar/bronchiolar tissue, no further investigation has been conducted at a molecular level. There are no studies exploring various enzyme pathways to confirm that the changes observed in BrdU index are representative of all preneoplastic lung lesions in the mouse. The USEPA's (2005) CARC report notes that it is not clear if the tumour type and cell type are the same for the isoniazid- and flonicamid-induced mouse neoplasms. There are no data to confirm that flonicamid affects the same target and cascade of molecular events that are affected by isoniazid; therefore, the Meeting concurs with the CARC findings that there is not enough information to make extrapolations from isoniazid to flonicamid for the human situation.

There is evidence in the literature that mouse lung tissue can undergo a sequence of events, beginning with increased proliferation, which progresses through to hyperplasia, adenoma and, eventually, carcinoma. However, there seems to be a lack of consensus regarding the cell type from which these lesions originate. There is speculation that these tumours originate in Clara cells; however, it has been acknowledged that type II alveolar cells may also be involved. The USEPA's (2005) CARC report alludes to the fact that there does not appear to be a consensus on the origins of such tumours. According to research by Kauffman (1981), mouse lung adenomas have two characteristic histological patterns, alveolar or bronchiolar, depending on the cell of origin. Bronchiolar tumours originate from Clara cells, whereas alveolar tumours originate from type II alveolar epithelium. There is evidence in the literature from ultrastructural studies that papillary tumours, which are said to grow faster and become larger and possibly malignant, originate exclusively from Clara cell lines. Within the USEPA's (2005) CARC report, a consulting pathologist for the USEPA references *Pathology of the Mouse* (Maronpot, Boorman & Gaul, 1999), which states that the majority of mouse lung tumours arise from type II pneumocytes and that primary hyperplasia of the alveolar epithelium is thought to be a precursor to alveolar/bronchiolar adenomas and carcinomas and derived from type II cells. In the section on histogenesis of lung tumours in the mouse, it reports that tumours can originate from either Clara or type II cells; in some cases, both cell

characteristics are present in the tumour cells. However, the majority of lung tumours arise from type II cells. Another consulting pathologist (USEPA, 2005) comments that alveolar/bronchiolar tumours in mammals are thought to arise from alveolar epithelial cells (type II pneumocytes) rather than bronchiolar epithelial cells; however, cells resembling Clara cells (dense granules in apical cytoplasm) have been found in some alveolar/bronchiolar neoplasms. This pathologist concluded that the origin of the alveolar/bronchiolar tumours is still subject to debate, as what is recognized and diagnosed as alveolar/bronchiolar adenomas or carcinomas may have arisen from either cell type: type II pneumocytes or Clara cells. It is indicated in the CARC report (USEPA, 2005) that the input received from the consulting pathologists supported a weight of evidence approach for the assessment of the lung tumour MOA. The CARC report (USEPA, 2005) concludes that the sponsor's proposed MOA was considered to be plausible and that the data supported the proposed MOA. However, the report (USEPA, 2005) noted that human relevance could not be dismissed, stating that

> Although the sponsor has shown that Clara cells are involved in the toxicological response to treatment with the test compound, they have not ruled out if other cell types are involved in the tumorigenic response to treatment. Further, although Clara cells are more numerous in the mouse than in humans, Clara cells are present in the human lung and have been shown to be responsive to the metabolic activity of xenobiotics.

The Meeting concurs that the proposed MOA is plausible.

Alternative modes of action

Alternative MOAs that may be applicable for the chemical under review. Comparison of their likelihood vis-à-vis the proposed MOA. (ILSI/RSI HRF)

The sponsor examined a short list of alternative MOAs, but these considerations were limited. It is acknowledged that flonicamid is non-DNA reactive, as indicated by the negative results from the genotoxicity battery. In addition, as mentioned previously, there did not appear to be any evidence of cell necrosis, indicating that cytotoxicity and subsequent regeneration were not likely explanations for these tumours. The discussion document also describes changes in apoptosis as an alternative MOA; however, there is no specific mention in the database that apoptosis was assessed in lung tissue.

Uncertainties, inconsistencies and data gaps

Identification of information deficiencies in the case; description of inconsistent findings in the data at large; evaluation of uncertainties; proposal of pointed research that could significantly inform the case. (ILSI/RSI HRF)

The discussion document and supporting studies provide insight into a plausible MOA for these lung tumours, but lacks information or measurements to confirm and quantify the proposed key events in tumour formation.

The sponsor acknowledges that there are limitations with the proposed MOA, and the following uncertainties/data gaps were identified:

- Studies could be performed with long-term administration of flonicamid in the B6C3F1 or C57BL mouse, extending the lack of changes in proliferation at the earlier times.
- Specific molecular changes and mechanism of action have not been defined. The specific cellular target of flonicamid leading to the mitogenic response is unknown.
- It has not been definitively determined if it is the parent compound or a specific metabolite that produces the mitogenic response in the mouse. The sponsor goes on to state that administration of the major metabolites of flonicamid to the CD-1 mouse did not produce an increase in cell proliferation. It is important to note, however, that these metabolites are those identified in the rat. This concern notwithstanding, the sponsor also indicates that "there may have been metabolic and kinetic differences producing the metabolites at the purported target

site (bronchiolar Clara cell) after flonicamid administration compared to direct oral administration of that metabolite."

The following uncertainties were noted during evaluation of the MOA framework:

- The "no effect level" for the tumours is below the proposed threshold for increased cell proliferation, as indicated by the BrdU index; there does not appear to be a clear point of departure for these tumours, as would be expected with the proposed MOA.
- Lung tissue was not examined earlier than at the 78-week terminal sacrifice in mice; there is no information on hyperplasia in the 90-day dietary study or at interim sacrifice in the first oncogenicity study. Without this information, it is not possible to clearly establish or confirm a temporal relationship for the progression from cell proliferation to hyperplasia and, eventually, adenoma and/or carcinoma.
- In the BrdU labelling cell proliferation studies, Clara cells were identified through immunostaining with the CC-10 protein to be reactive to flonicamid; however, other cell types have not been eliminated. As discussed previously, there is a lack of consensus over the cell of origin involved with lung tumours in the mouse. In the histology report for the oncogenicity studies, there is speculation in the discussion that Clara cells were involved with hyperplasia; however, no further examination or testing was conducted to confirm this.
- Human relevance cannot be ruled out based on cell of origin. Human lung tissue does contain Clara cells, although at lower concentrations, and these cells have been shown to be responsive to the metabolic activity of xenobiotics. Furthermore, there is evidence in the literature of alveolar/bronchiolar tumours originating from Clara cells in humans.
- The mouse model has been extensively used for the modelling of human lung adenocarcinomas in research, and this should be taken into account when examining and evaluating all available data.
- There is not enough information to make extrapolations from isoniazid to flonicamid for the human situation.
- The sponsor made reference to literature on species-specific effects following isoniazid and styrene administration in the discussion of the MOA. Both styrene and isoniazid have been flagged by the International Agency for Research on Cancer as either possibly carcinogenic or non-classifiable, and both have "inadequate evidence of carcinogenicity in humans", indicating that there is a lack of information available.

Overall assessment of the MOA

The Meeting is of the opinion that the weight of evidence suggests a plausible mitogenic MOA for the mouse lung tumours; however, key events were not adequately supported by measurable parameters. No data or information is available on mouse metabolism of flonicamid. A clear point of departure for tumour development has not been adequately demonstrated for alveolar/bronchiolar neoplasms. Lung tumours were observed at doses at which the precursor events were not clearly demonstrated. The absence of examination of lung tissue at earlier time periods (i.e. following less than chronic dosing) results in uncertainty as to how early hyperplasia is occurring. Consequently, it is not possible to confirm that it is evident prior to tumour development. Although the scientific literature supports the possibility that tumours may develop in accordance with the proposed MOA, cell types other than Clara cells have not been investigated and/or ruled out as being involved in tumour development. There was no clear demonstration of progression through the proposed sequence of events leading to tumour formation.

The absence of supporting data for the key events lowers the overall strength of the MOA argument. There is speculation among pathologists regarding the origin of lung alveolar/bronchiolar tumours, in that what is recognized and diagnosed as alveolar/bronchiolar adenomas or carcinomas

may have arisen from either cell type: type II pneumocytes or Clara cells. A limited number of alternative MOAs were explored and ruled out; however, effects on apoptosis do not appear to have been examined, according to the study report. Many of the uncertainties flagged by the Meeting have also been identified by the USEPA. Although the USEPA accepted the proposed MOA, it did not dismiss human relevancy, citing an absence of information ruling out other (than Clara cell) cell types. Only limited speculation can be made between lung metabolism in laboratory animals and how this reflects the situation in the human lung. In addition to differences in classification of tumours for the species, there are many uncertainties with the current understanding of quantitative lung metabolism pathways in the mouse and human.

Conclusion

Overall indication of the level of confidence in the postulated MOA. (ILSI/RSI HRF)

Although the proposed MOA was considered to be plausible, there are residual concerns regarding the weight of evidence supporting this MOA in animals. However, in light of the information presented and the threshold nature of the lung tumours in mice, there is evidence that the risk to human health can be characterized.

Additional data that would augment the MOA argument include:

- a mouse metabolism study to provide information on the distribution and metabolic profile for flonicamid in the lung of CD-1 mice;
- comparative (mouse, rat and human) in vitro assays examining flonicamid activity on cell type (Clara cell versus type II pneumocytes) and enzymatic pathways in lung tissue;
- short-term dietary study in the CD-1 mouse to establish and confirm key events, in particular early changes in cell morphology and hyperplasia, to establish a temporal relationship for these effects.

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FLUMIOXAZIN

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Explanation

Flumioxazin (code S-53482) is the International Organization for Standardization (ISO)–approved common name for *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboxamide (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 103361-09-7. It acts as a herbicide by inhibition of protoporphyrinogen oxidase (PPO), resulting in an accumulation of porphyrins. It is used against weeds and mosses.

Flumioxazin 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.

This evaluation is based mainly on the study reports submitted by the sponsor. All critical studies contained statements of compliance with good laboratory practice (GLP), unless otherwise specified. 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.

Most toxicity studies were conducted according to internationally recognized guidelines (generally United States Environmental Protection Agency [USEPA]/Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA]), except where indicated.

The authors conducted a literature search on 7 May 2015 in the databases PubMed, PubMedCentral and Scopus (keyword: flumioxazin, no restriction for publication date). Thirty-six, 25 and 224 references were retrieved, respectively. Articles appearing to be obviously non-relevant for a toxicological or human health evaluation were excluded from the results list based on their titles and/or abstracts. For the remaining seven references, the full articles were retrieved. These articles were included in the evaluation and are described in the appropriate sections.

Evaluation for acceptable intake

1. Biochemical aspects

Two ^{14}C-labelled flumioxazin molecules were used for all absorption, distribution, metabolism and excretion (ADME) studies: [phenyl-^{14}C]flumioxazin and [tetrahydro-phthaloyl-1,2-^{14}C]flumioxazin (also indicated as [phthalimide-^{14}C]flumioxazin) (for the chemical structures and the positions of the label, see Fig. 1 and Fig. 2 below). According to the study reports cited below, some parts of the studies were not conducted according to GLP principles (e.g. no audit by a quality assurance unit).

1.1 Absorption, distribution and excretion

In the first study, groups of five male and five female rats were given, by gavage, 1) a single oral dose of [phenyl-^{14}C]flumioxazin at 1 mg/kg body weight (bw) (low dose), 2) a single oral dose of [phenyl-^{14}C]flumioxazin at 100 mg/kg bw (high dose) or 3) a dose of non-radiolabelled flumioxazin at 1 mg/kg bw per day for 14 days followed by a single oral dose of [phenyl-^{14}C]flumioxazin at 1 mg/kg bw (repeated-dose experiment). Excreta were collected for 7 days after the administration of the radiolabelled compound. Afterwards, rats were sacrificed, and specimens such as blood and selected organs were collected for further analysis.

Most (97.9–102.3%) of the radioactivity, expressed as a percentage of the administered dose, was eliminated within 7 days post-dosing, the majority within 2 days post-administration. Within 24 hours post-dosing, excretion via urine and faeces was 86% for both males and females in the low-dose group, 82% and 72% for males and females, respectively, in the high-dose group and 87% and 83% for males and females, respectively, in the repeated-dose group. Total recovery of radioactivity was nearly quantitative. In the low- and repeated-dose groups, slightly more radioactivity was excreted in faeces (56.4–71.5%) than in urine (28.6–42.8%). In the high-dose group, more of the radioactivity was excreted in faeces (78.1–85.2%) than in urine (13.0–23.4%). Urinary radiocarbon excretion was greater in females than in males in all dose groups.

Tissue ^{14}C residue levels, expressed as nanogram flumioxazin equivalents (eq) per gram tissue or parts per billion (ppb), were generally low on the seventh day post-dosing. Radiocarbon residue was higher in blood cells than in tissues. Tissue ^{14}C residue levels, including those for fat,

were lower than blood levels, which suggested little potential for bioaccumulation. Tissue ^{14}C residue levels were highest in the metabolic organs: liver (12.2–24.3 ppb in the low- and repeated-dose groups; 523–713 ppb in the high-dose group) and kidney (11.2–16.0 ppb in the low- and repeated-dose groups; 397 ppb in the high-dose group). Relative to the dose, tissue levels in the high-dose animals were lower than those in animals of the low- or repeated-dose groups (Matsunaga, 1993).

In the second study, groups of five male and five female rats were given, by gavage, 1) a single oral dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw (low dose) or 2) a single oral dose of [phthalimide-^{14}C]flumioxazin at 100 mg/kg bw (high dose). Further groups of eight males and eight females were given, via gavage, a dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw after pretreatment with unlabelled flumioxazin at 1 mg/kg bw per day for 14 consecutive days (repeated-dose experiment; however, samples of only five animals of each sex were analysed). Excreta were collected for 7 days after the administration of the radiolabelled compound. Afterwards, rats were sacrificed, and specimens such as blood and selected organs were collected for further analysis.

The radioactivity was almost completely eliminated from the body in all groups within 7 days after administration, the majority within 2 days post-administration. Within 24 hours post-dosing, excretion via urine and faeces was 76% and 71% for males and females, respectively, in the low-dose group, 84% and 69% for males and females, respectively, in the high-dose group and 86% and 87% for males and females, respectively, in the repeated-dose group. Nearly 100% of the dose was excreted via faeces and urine. Recoveries of ^{14}C (expressed as a percentage relative to the dosed ^{14}C) from faeces and urine were 59.6–65.8% and 30.7–36.8%, respectively, in the low-dose group, 83.4–87.5% and 11.8–14.1%, respectively, in the high-dose group and 62.5–65.7% and 31.7–35.3%, respectively, in the repeated-dose group. The proportion of urinary ^{14}C excretion in the high-dose group was significantly smaller than in the low- and repeated-dose groups in both sexes ($P < 0.001$). Urinary ^{14}C excretion in females of the low- and high-dose groups was significantly greater than that in males ($P < 0.005$ for the low-dose group, $P < 0.01$ for the high-dose group).

Tissue and blood ^{14}C residues on the seventh day after administration were generally low. In all groups, blood cells showed the highest ^{14}C residue levels, which were 41.2–46.6 ppb flumioxazin equivalents in the low- and repeated-dose groups and 2175–2268 ppb flumioxazin equivalents in the high-dose group. However, ^{14}C levels in the blood cells in the high-dose group did not increase in proportion to the dose. In addition, ^{14}C residue levels in blood, heart, kidney, liver and skin were relatively higher than those in any other examined tissues in both sexes for all groups. In all groups, tissue and blood contained less than 1.0% of the administered dose. Total residue in blood amounted to no more than 0.2% of the dose for all groups (Shiba, 1994).

The biliary excretion of flumioxazin was investigated in bile duct–cannulated male and female rats. Animals received a single oral dose of [phthalimide-^{14}C]flumioxazin at 1 mg/kg bw, and bile, urine and faeces were collected up to 72 hours post-dosing. The gastrointestinal tract (with contents) from each animal was also collected at terminal sacrifice.

Most of the radioactivity was excreted in the urine (37% in both males and females) or bile (41% and 37% in males and females, respectively) within 24 hours. At 72 hours after administration, mean radioactivity concentrations in the urine accounted for 43% and 41% of the dose in males and females, respectively, and those in bile, for 43% and 39% of the dose in males and females, respectively. Mean radioactivity concentrations in faeces accounted for 6% and 9%, and residues remaining in the gastrointestinal tract accounted for only 1% and 2%, in males and females, respectively. Total recoveries were above 90%. Flumioxazin was almost completely bioavailable in the rat after oral administration. Only a minor amount of the parent compound was detected in the faeces, amounting to approximately 1% of the dose (Gibson et al., 1997).

1.2 Biotransformation

In the samples of urine and faeces collected in the studies by Matsunaga (1993) and Shiba (1994), metabolites were separated by thin-layer chromatography using several different mobile phases, their retention times were compared with synthetic reference standards and spots were detected using autoradiography. The metabolites were quantified by liquid scintillation counting. Taking into account the results of all three reports, flumioxazin was extensively metabolized (Table 1). Thirty-five metabolites were detected and quantified after administration of [phenyl-^{14}C]flumioxazin, and seven were identified. Twenty-nine metabolites were detected and quantified after administration of [phthalimide-^{14}C]flumioxazin, and 10 were identified.

The main metabolic reactions were hydroxylation of the cyclohexene ring of the tetrahydrophthalimide moiety, cleavage of the imide linkage, cleavage of the amide linkage in the benzoxazine ring, reduction of the double bond in the tetrahydrophthalimide ring, acetylation of the amino group of the aniline derivative and incorporation of a sulfonic acid group in the tetrahydrophthalimide ring.

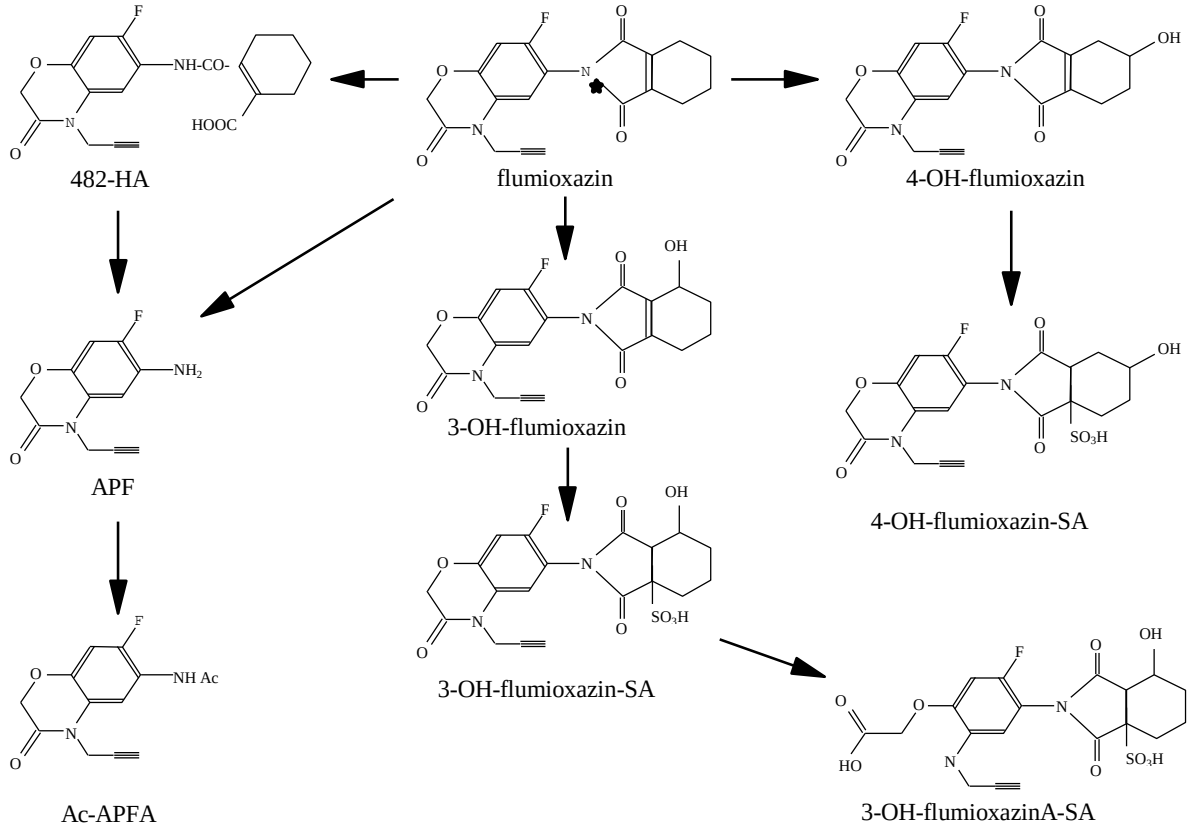
The proposed flumioxazin metabolic pathways in rats, based on the studies with [phenyl-^{14}C]flumioxazin and [phthalimide-^{14}C]flumioxazin, are summarized in Fig. 1 and Fig. 2, respectively.

Table 1. Amount of radioactive metabolites in urine and faecal extracts

	% of total administered radioactivity (male/female)					
	[phenyl-^{14}C]Flumioxazin			**[phthalimide-^{14}C]Flumioxazin**		
Metabolite[a]	**Low dose**	**High dose**	**Repeated dose**	**Low dose**	**High dose**	**Repeated dose**
Total excreted ^{14}C	102.3/99.2	98.2/101.5	97.9/98.9	96.5/96.4	99.4/97.5	97.5/97.8
Flumioxazin	0.3 / 0.4	51.0/46.6	0.3/5.2	2.8/1.9	66.1/63.0	1.2/1.3
3-OH-flumioxazin	3.2/3.9	2.3/3.1	3.1/4.5	6.2/5.3	2.5/2.5	6.4/6.3
4-OH-flumioxazin	2.0/3.0	1.8/3.2	2.3/3.7	5.8/6.1	2.1/2.7	6.5/7.0
APF	2.5/6.8	1.8/4.0	3.8/4.4	–	–	–
Ac-APFA	5.9/4.0	2.2/2.0	5.3/4.5	–	–	–
SAT-482	–	–	–	0.6/0.6	0.2/0.3	0.6/0.5
3-OH-SAT-482	–	–	–	2.2/2.0	0.7/0.7	1.9/1.6
4-OH-SAT-482	–	–	–	2.3/2.0	0.5/0.5	1.5/1.5
THPA	–	–	–	0.9/1.3	0.4/0.5	1.5/1.5
1-OH-HPA-1	–	–	–	1.3/1.4	0.7/0.8	1.8/2.0
3-OH-flumioxazin-SA	14.4/18.6	6.9/8.1	13.4/11.6	16.1/17.6	5.7/6.1	16.4/16.8
4-OH-flumioxazin-SA	5.2/7.0	2.9/3.1	6.0/4.7	7.4/7.8	2.3/2.4	8.3/8.3
3-OH-flumioxazin-A-SA	4.0/2.4	1.8/1.4	3.8/1.9	3.2/2.4	0.8/0.7	3.4/2.6
Identified metabolites	37.5/46.1	70.7/71.5	38.0/40.5	48.8/48.3	82.1/80.2	49.5/49.3
Sum of unknown metabolites	54.3/48.6	22.9/24.6	50.7/49.8	39.8/40.1	13.0/12.3	40.4/41.1
Unextractable ^{14}C	8.9/2.8	3.9/3.6	7.8/6.9	5.7/4.8	3.6/3.7	6.1/5.5

[a] See Figs 1 and 2 for chemical structures of metabolites.
Source: Matsunaga (1993); Shiba (1994)

Fig. 1. Proposed metabolic pathway of flumioxazin in the rat, based on [phenyl-^{14}C]flumioxazin



*: position of the label

Source: Matsunaga (1993)

Apparently, the results of the studies by Matsunaga (1993) and Shiba (1994) were also published in the open literature (Tomigahara et al., 1999a,b).

2. Toxicological studies

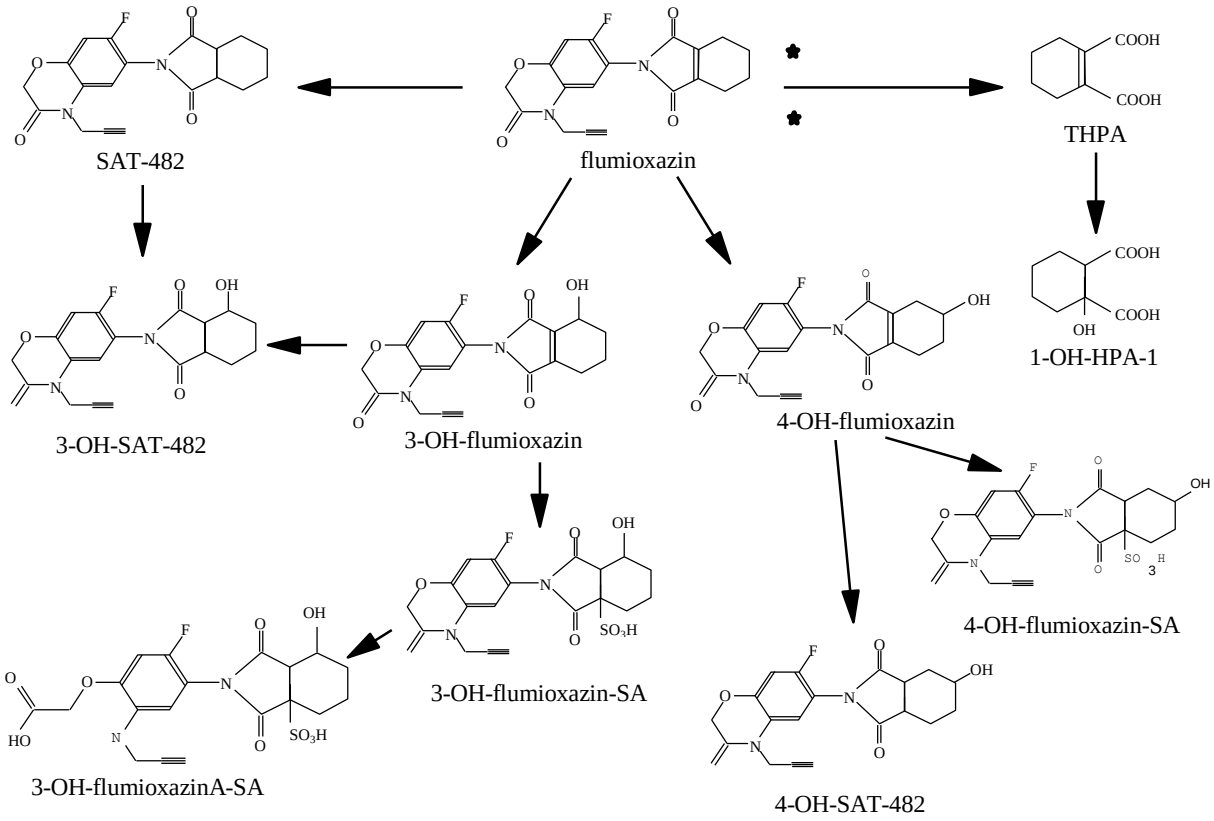
2.1 Acute toxicity

The results of acute toxicity studies with flumioxazin 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 2.

(a) Lethal doses

Flumioxazin technical (purity 94.8%) was administered by gavage (suspension in 1% weight per volume [w/v] aqueous methyl cellulose, dosing volume of 20 mL/kg bw) to five male and five female Sprague-Dawley rats at a dose level of 0 or 5000 mg/kg bw. Mortality and clinical signs were recorded on five occasions on the day of dosing and daily thereafter for the remainder of the 14-day observation period. Individual body weights were measured on days 0, 7 and 14. At the end of the study, all animals were sacrificed, and any gross pathological changes were recorded. No mortality was observed, and there were no overt signs of reaction to treatment. No abnormalities were detected at necropsy. The acute oral median lethal dose (LD_{50}) of flumioxazin in rats was greater than 5000 mg/kg bw for both sexes under the conditions of the study (Hiromori, 1990a).

Fig. 2. Proposed metabolic pathway of flumioxazin in the rat, based on [phthalimide-^{14}C]flumioxazin



*: position of the labels

Source: Shiba (1994)

Table 2. Acute toxicity of flumioxazin

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Sprague-Dawley	Male + female	Oral	94.8	LD_{50} > 5 000 mg/kg bw	Hiromori (1990a)
Rat	Sprague-Dawley	Male + female	Dermal	94.8	LD_{50} > 2 000 mg/kg bw	Hiromori (1990b)
Rat	Sprague-Dawley	Male + female	Inhalation	98.3	LC_{50} > 3.93 mg/L (maximum attainable concentration)	Kawaguchi (1990)
Rabbit	New Zealand White	Male + female	Skin irritation	94.8	Non-irritating	Nakanishi (1989)
Rabbit	New Zealand White	Male + female	Eye irritation	94.8	Minimally irritating	Nakanishi (1989)
Guinea-pig	Hartley	Male	Skin sensitization	94.8	Non-sensitizing (Magnusson & Kligman)	Nakanishi (1990)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

Flumioxazin technical (purity 94.8%) suspended in 1% w/v aqueous methyl cellulose was applied to the clipped skin of five male and five female Sprague-Dawley rats at a dose level of 0 or 2000 mg/kg bw. The test material was covered for 24 hours with surgical tape, after which test material and the tape were removed. Mortality and clinical signs were recorded on five occasions on the day of dosing and daily thereafter for the remainder of the 14-day observation period. Individual body weights were measured on days 0, 7 and 14. At the end of the study, all animals were sacrificed, and any gross pathological changes were recorded. No mortality was observed, and there were no overt signs of reaction to treatment. No abnormalities were detected at necropsy. The acute dermal LD_{50} of flumioxazin in rats was greater than 2000 mg/kg bw for both sexes under the conditions of the study (Hiromori, 1990b).

Two groups of five male and five female Sprague-Dawley albino rats were subjected to a 4-hour nose-only exposure to a dust of flumioxazin (purity 98.3%) at an analytically determined concentration of 0 (control), 1550 or 3930 mg/m^3, the higher concentration being the maximum attainable. The dust had a mass median aerodynamic diameter of 4.99–6.18 µm. Mortality and clinical signs were recorded on five occasions on the day of exposure and at least daily during 14 days after dosing. Individual body weights were measured pre-dosing on day 0 and on days 3, 7 and 14. No mortalities occurred at any dose level. There were no clinical signs in the control group. In both treated groups, the following clinical signs were observed in both sexes: irregular respiration or bradypnoea from 0.5 hour after commencement of exposure, lasting up to 2 hours after cessation of exposure; and a decrease in spontaneous activity during exposure only. Body weight was unaffected by treatment. There were no gross necropsy findings that were attributable to treatment. Therefore, the acute inhalation median lethal concentration (LC_{50}) of flumioxazin in rats was greater than 3930 mg/m^3 air for both sexes under the conditions of the study (Kawaguchi, 1990).

(b) Dermal irritation

Flumioxazin technical (purity 94.8%) was applied at a dose of 0.5 g to the clipped dorsal region of three male and three female New Zealand White rabbits under a gauze patch moistened with corn oil 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 4.5, 24, 48 and 72 hours after application. No cutaneous irritation was observed at any test site during the study (Nakanishi, 1989).

(c) Ocular irritation

Flumioxazin technical (purity 94.8%) was instilled at a dose of 100 mg into the conjunctival sac of one eye of each of three male and three female New Zealand White rabbits. Observations of ocular lesions were conducted at 1, 24, 48 and 72 hours after instillation. The test material induced slight redness and very slight chemosis of the conjunctiva in all rabbits and slight congestion in the iris in two rabbits 1 hour after application. Twenty-four hours after application, slight redness of the conjunctiva was observed in four rabbits. These reactions disappeared 48 hours after application. The test material caused slight, transient irritation to the eyes of rabbits (Nakanishi, 1989).

(d) Dermal sensitization

In a dermal sensitization study conducted according to the Magnusson & Kligman method, flumioxazin technical (purity 94.8%) was injected intradermally (for each animal: two injections of Freund's Complete Adjuvant [FCA] in water, two injections of flumioxazin as a 1% solution in corn oil, and two injections of flumioxazin in FCA and water) into 20 male Hartley guinea-pigs; 6 days later, a 10% sodium lauryl sulfate solution in petrolatum was applied, which was followed on the next day by an epidermal application of 25% test substance in petrolatum under occlusive dressing pads for 48 hours. Fourteen days after the epidermal application, the challenge treatment was performed. Flumioxazin at 25% in petrolatum was applied to the intact right flank of each animal for 24 hours. Observations were made 24 and 48 hours after removal of the patches. The control group (20 animals)

was treated in a similar fashion, but with no test item. 2,4-Dinitrochlorobenzene was used as the positive control (five animals). Intradermal injection concentrations were limited by precipitation. Topical application did not produce any skin reactions. No sensitization reactions were produced at the challenge site. The positive control produced satisfactory results. Under the conditions of the study, flumioxazin did not show skin sensitizing potential (Nakanishi, 1990).

2.2 Short-term studies of toxicity

(a) Oral administration

The short-term toxicity of flumioxazin was evaluated in mice, rats and dogs.

Mice

A 4-week dietary toxicity study in mice with flumioxazin technical (purity 94.8%) was conducted as a dose range–finding study to determine the dose levels for an oncogenicity study. Three groups of nine male and nine female Crj:CD-1 (ICR) mice received flumioxazin in the diet at a concentration of 1000, 3000 or 10 000 parts per million (ppm) for 4 weeks (equal to 152, 420 and 1367 mg/kg bw per day for males and 165, 482 and 1698 mg/kg bw per day for females, respectively). A similarly constituted control group received untreated diet.

No deaths or treatment-related clinical signs were noted during the study. No treatment-related changes in body weight, feed consumption, haematology, blood biochemistry or gross pathology were observed. Increases in both absolute and relative liver weights were observed in males at 10 000 ppm and in females at 3000 ppm, and relative liver weights of females were increased at 10 000 ppm (always less than a 15% increase). No treatment-related histopathological alterations were noted in the liver (other organs were apparently not evaluated). Flumioxazin (technical grade) affected liver weights in mice, but the toxicological significance of these changes was considered low, even at 10 000 ppm.

In conclusion, 7000 ppm was selected as the highest dose level for the oncogenicity study in mice, based on 7000 ppm (approximately 1000 mg/kg bw per day) being the highest USEPA guideline dose level for mouse oncogenicity studies. Considering the limited parameters evaluated, no no-observed-adverse-effect level (NOAEL) was identified for this study (Seki, 1990).

In a further study, four groups of 10 male and 10 female Crj:CD-1 (ICR) mice received flumioxazin (purity 98.4%) in the diet at a concentration of 100, 1000, 3000 or 10 000 ppm for 13 weeks (equal to 16.3, 164, 459 and 1062 mg/kg bw per day for males and 18.6, 202, 595 and 2163 mg/kg bw per day for females, respectively). A similarly constituted control group received untreated diet.

No deaths or treatment-related clinical signs were noted during the study. No treatment-related changes in body weight, feed and water consumption, urine analysis, haematology, blood biochemistry, ophthalmology or gross pathology were observed. Low reticulocyte counts and decreased calcium values in males at 10 000 ppm and increased creatinine values in females at 10 000 ppm were considered to be of little biological significance. Bone marrow smears were not examined. Increases in both absolute and relative liver weights were observed in males and females at 10 000 ppm (always less than a 15% increase). Ovary weights were reduced (by approximately 35%) in females at 10 000 ppm, No treatment-related histopathological alterations were noted.

The NOAEL was 3000 ppm (equal to 459 and 595 mg/kg bw per day for males and females, respectively), based on increased liver weights and decreased ovary weights at 10 000 ppm (equal to 1062 and 2163 mg/kg bw per day for males and females, respectively) (Hagiwara, 1990).

Rats

Flumioxazin (purity 98.4%) was offered to five groups of 12 male and 12 female Sprague-Dawley rats (aged 5 weeks) at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 2.28, 20.7, 69.7 and 244 mg/kg bw per day for males and 0, 2.21, 21.7, 71.5 and 230 mg/kg bw per day for females, respectively) for 90 days. Achieved concentrations and homogeneity were verified by analysis. The animals were observed at least once daily for clinical signs, behaviour and mortalities. Ophthalmological examination was performed on all animals during the quarantine period and on all animals from the control and highest-dose groups after 90 days. Body weights, feed consumption and water consumption were determined at weekly intervals for all animals. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine analyses were conducted on all animals at termination. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (brain, heart, liver, spleen, thymus, kidneys, pituitary, adrenals, thyroid with parathyroid, ovaries or testes) were recorded. In the control and 3000 ppm groups, histological evaluation was performed on heart, spleen, lymph nodes (mandibular and mesenteric), thymus, tongue, salivary gland, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (caecum, colon, rectum), liver, pancreas, trachea, lungs, kidneys, urinary bladder, testes, ovaries, prostate, seminal vesicle, epididymides, uterus, mammary gland (female), vagina, pituitary, thyroid (with parathyroids), adrenals, brain (cerebrum and cerebellum), spinal cord (cervical, mid-thoracic and lumbar), sciatic nerve, eyes, Harderian glands, skin, skeletal muscle, bone and bone marrow (femur and sternum), aorta and gross lesions. In the remaining dose groups, histopathological examination was restricted to the lung, liver, kidneys, spleen, bone marrow and gross lesions.

No treatment-related effects on survival, clinical signs, feed consumption, water consumption or ophthalmological findings were observed throughout the study. Urine analysis parameters at week 13 were similar to control values. Body weights of rats receiving 3000 ppm were significantly reduced; feed consumption efficiency tended to be decreased. Haematological effects were observed at both 1000 and 3000 ppm. At 1000 ppm, these effects included significantly reduced mean corpuscular volume (males and females), significantly reduced mean corpuscular haemoglobin (females) and significantly increased platelet count (females). For animals at 3000 ppm, the haematological effects included significantly lower haemoglobin level, haematocrit value, mean corpuscular haemoglobin, mean corpuscular volume and myeloid/erythroid ratios for both sexes and significantly reduced red blood cell counts for females. Haematological parameters, which were significantly increased, included platelet count, reticulocyte count and erythroblast count for both sexes. Total bilirubin was increased in males at and above 1000 ppm. Liver and kidney weights relative to body weight were significantly higher in 1000 ppm males. In the 3000 ppm group, significant increases were observed in absolute spleen weights and relative liver and spleen weights in both sexes; absolute thyroid weights and relative brain, heart and thyroid weights in females; and relative kidney weights in males. Histopathological examination of tissues from the 3000 ppm group revealed increased incidences of extramedullary haematopoiesis in the spleen of both males and females. Haematopoiesis (erythroid hyperplasia) in the bone marrow and extramedullary haematopoiesis in the liver were seen in one female.

The NOAEL was 300 ppm (equal to 20.7 and 21.7 mg/kg bw per day for males and females, respectively), based on changes in haematological and clinical chemistry parameters at 1000 ppm (equal to 69.7 and 71.5 mg/kg bw per day for males and females, respectively) (Hagiwara, 1989).

Flumioxazin technical (purity 94.8%) was offered to five groups of 16 male and 16 female Sprague-Dawley rats (aged 5 weeks) at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 1.9, 19.3, 65.0 and 197 mg/kg bw per day for males and 0, 2.2, 22.4, 72.9 and 218 mg/kg bw per day for females, respectively) for 90 days. After 5 weeks of dosing, six rats of each sex per dose group were used for an interim sacrifice. Achieved concentrations, stability and homogeneity were verified by analysis. The animals were observed at least twice daily for clinical signs, behaviour and mortalities, except on weekends, when the frequency was once per day. Ophthalmological

examination was performed on all animals of both sexes in the control group, on females at 1000 ppm and on both sexes at 3000 ppm in week 13 after application of a mydriatic agent. Body weights, feed consumption and water consumption were determined at weekly intervals for all animals. For assessment of blood biochemical and haematological parameters, blood samples were taken after overnight fasting at sacrifice of both the interim and main study groups. Urine analyses were conducted on samples collected in the week before sacrifice for all animals. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (liver, kidneys, spleen, heart, brain, testes, prostate, adrenals, ovaries, thymus, thyroid including parathyroid, and pituitary) were recorded. Histological evaluation was performed on abdominal skin, adrenals, aorta (thoracic), brain, epididymides, oesophagus, eyeballs (fixed in Davidson's fixative at scheduled sacrifice), femur (bone marrow), Harderian glands, heart, kidneys, large intestine (caecum, colon, rectum), liver, lungs, mammary glands (female only), mesenteric lymph nodes, ovaries, pancreas, parathyroids, pituitary, prostate, salivary gland (submandibular), sciatic nerve, seminal vesicles, skeletal muscle, small intestine (duodenum, jejunum, ileum), spinal cord (mid-thoracic area), spleen, sternum (bone marrow), stomach, submandibular lymph nodes, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus, vagina and macroscopically abnormal tissue.

One female from the 3000 ppm group was found dead in week 12. This death was considered related to treatment. Pallor of the auricles, eyes and/or all limbs was observed in this animal and in some additional females in this same group beginning at week 8. Blood vessels in fundus oculi were not clearly observed in two females in the 3000 ppm dose group. No treatment-related changes in body weights, water consumption or urine analysis were observed. The primary toxicological findings were related to changes in the haematopoietic system, including decreases in haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and/or haematocrit values for males in the 1000 and 3000 ppm groups and for females at 300 ppm and above (Table 3). Signs of anaemia (decreased erythrocyte count) were also observed in females at 3000 ppm. Increases in reticulocyte and erythroblast ratios for females at 1000 ppm and above and for males at 3000 ppm and increases in leukocyte count or neutrophil count for females at 3000 ppm were also noted. Bone marrow differentiation revealed a decrease in myeloid/erythroid ratios for males at 3000 ppm and for females at 1000 ppm and above. Other indications of toxicity related to the haematopoietic system included dark and enlarged spleens and increased levels of plasma total bilirubin in animals exposed to 3000 ppm and extramedullary haematopoiesis in the spleen of males at 1000 ppm and above and in females at 300 ppm and above. Hypercellularity in the femoral bone marrow and extramedullary haematopoiesis in the liver were also noted in females at and above 1000 ppm. Increases in relative weights of liver, heart, kidney and thyroid were observed for males at and above 1000 ppm. For females, increases in liver weights were observed at 1000 and 3000 ppm, and increases in spleen, heart and kidney weights were observed at 3000 ppm. Liver sinusoidal cells containing brown pigments were reported for one male at the top dose and for females at 1000 ppm and above. In several females at 3000 ppm, centrilobular ballooning degeneration and necrosis of hepatocytes and brown pigment in hepatocytes and canaliculi in the liver, myelofibrosis and osteosis in the femoral bone marrow, brown pigment in tubular epithelial cells and tubular vacuolation in the kidney, cortical cytoplasmic vacuolation in the adrenal, atrophic changes with foam cells in the thymus, sinus histiocytosis in the lymph nodes, thrombosis in the lung and/or erosion in the glandular stomach were seen.

The lowest NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day), based on haematological changes (including anaemia and extramedullary haematopoiesis) in females at 300 ppm (equal to 22.4 mg/kg bw per day), although the effects were marginal. For males, the NOAEL was 300 ppm (equal to 19.3 mg/kg bw per day), based on increases in liver, heart, kidney and thyroid weights at 1000 ppm (equal to 65.0 mg/kg bw per day) (Adachi, 1991).

Table 3. Selected findings in 90-day rat study

	Males					Females				
	0 ppm	**30 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**	**0 ppm**	**30 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**
Haemoglobin (g/dL)	14.6	14.8	14.8	13.9**	13.6**	13.7	14.1	13.8	12.4	8.8**
MCV (fL)	47.6	47.4	47.3	44.6**	42.2**	49.6	49.0	47.9*	42.9**	40.7**
MCH (pg)	17.5	17.6	17.3	16.2**	15.1**	17.9	17.7	17.3*	14.9**	13.4**
MCHC (g/dL)	36.8	37.2	36.5	36.3*	35.9**	36.2	36.1	36.0	34.6**	32.8**
Liver, relative weight (g%)	2.54	2.54	2.62	2.71*	2.88**	2.45	2.54	2.53	2.63*	2.95**
Heart, relative weight (g%)	0.32	0.31	0.34	0.34*	0.34*	0.35	0.34	0.33	0.36	0.49**
Kidney, relative weight (g%)	0.66	0.65	0.67	0.70*	0.71**	0.69	0.71	0.71	0.70	0.83**
Thyroid, relative weight (mg%)	5.0	5.8*	5.7	6.0*	7.0**	6.7	6.9	6.8	7.8	7.5
Spleen, increased extramedullary haematopoiesis	0	0	0	1	6	0	0	1	8	10

MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean cell volume; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (significantly different from the vehicle control group; least significant difference test)
Source: Adachi (1991)

Dogs

Flumioxazin technical (purity 94.8%) was administered in gelatine capsules to three groups of four male and four female Beagle dogs (aged 6 months) at a dose of 10, 100 or 1000 mg/kg bw per day for 13 weeks. A concurrent control group of four males and four females received empty capsules. The animals were observed for clinical signs before administration (which took place at approximately 09:00), every 2–3 hours after administration until about 17:00 on working weekdays and at least 3 times per day on other days. Feed consumption was monitored daily, and body weight measurements were taken at weekly intervals from 2 weeks before commencement of dosing until study termination. Ophthalmological examination was performed on weeks 0, 5 and 11 of treatment. Electrocardiograms were recorded via standard limb lead II on weeks 0, 7 and 13 of administration. Haematology and clinical chemistry were undertaken at weeks 0, 4, 8 and 12. Bone marrow smear and myelocrit measurements were made using bone marrow samples obtained from sternal puncture prior to necropsy. Urine analysis was performed for all animals on weeks 0, 6 and 12. Faeces were collected during a 24-hour period on weeks 0, 6 and 12 and examined for faecal occult blood. To examine liver function, a bromosulfophthalein (BSP) retention test was performed at weeks 0, 5 and 11. To examine kidney function, a *para*-aminohippurate retention test was performed at weeks 0, 7 and 13. At necropsy, all animals were subjected to thorough gross examination and organ weight analysis: brain, pituitary, mandibular glands, thyroids (including parathyroids), thymus, lungs, heart, liver (including gallbladder), pancreas, spleen, kidneys, adrenals, testes, prostate, ovaries and uterus. Histopathology was also performed on these organs, as well as on eyeballs (including optic nerve), parotid glands, tongue, submandibular lymph nodes, larynx, trachea, aorta, urinary bladder (including ureters), oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (caecum, colon, rectum), mesenteric lymph nodes, spinal cord (mid-thoracic), sciatic nerve, femoral skeletal

muscle, abdominal skin, mammary glands, sternal bone marrow, femoral bone marrow, epididymides and vagina and on gross lesions (the only one being the skin of the left forelimb of a high-dose animal). In addition to light microscopy, electron microscopy was also performed on suitably prepared samples of liver and kidney of all surviving animals at termination.

There were no deaths throughout the treatment period. As for clinical signs, a slightly higher frequency of loose faeces was noted in the 1000 mg/kg bw per day group. No abnormalities in feed consumption, body weight gain or feed efficiency were noted. No compound-related abnormalities in ophthalmology, electrocardiography, urine analysis, faecal examination, myelocrit measurement or myelogram examination of bone marrow were noted. No remarkable changes in haematology parameters were noted. No effects on kidney or liver function were noted. Treatment-related effects included a prolongation of activated partial thromboplastin time in females of the 1000 mg/kg bw per day group at weeks 4 and 12; increased total cholesterol and phospholipid levels and elevated alkaline phosphatase activity in the 100 and 1000 mg/kg bw per day groups (males and females); and increased absolute and relative liver weights, as well as histological changes in the liver, at 1000 mg/kg bw per day. Treatment-related changes, which were mild in severity (i.e. minimal or slight), were seen in the liver of both sexes from the 1000 mg/kg bw per day group. A minimal to slight proliferation of bile ductules was found in one male and two females from the 1000 mg/kg bw per day group. Two males from the 1000 mg/kg bw per day group exhibited minimal increased fibrous tissue around hepatic centrilobular veins. This change was also considered to be a response to hepatic injury due to this compound. Minimal capillary proliferation was seen in the gross lesion of the heart from one female in the 1000 mg/kg bw per day group. This change may be related to valvular telangiectasia, known as a spontaneous lesion. All other lesions were regarded as agonal and/or minor incidental changes of naturally occurring disease unrelated to the test compound. The incidence and relative severity of these changes were comparable among the control and treatment groups. On electron microscopic examination, proliferation and dilatation of the smooth endoplasmic reticulum were observed in the hepatocytes of all males and 3/4 females that received 1000 mg/kg bw per day. These morphological features, in the absence of damage to other organelles, were considered to represent an intracellular adaptive response to an increased metabolic demand presented by the test material, rather than evidence of overt hepatotoxicity per se.

The NOAEL was 10 mg/kg bw per day, based on increases in total cholesterol and phospholipid levels and elevated alkaline phosphatase activity at 100 mg/kg bw per day (Nakano, 1993).

Flumioxazin technical (purity 94.8%) was administered to three groups of four male and four female Beagle dogs (aged 6 or 7 months) by gelatine capsule at a dose level of 10, 100 or 1000 mg/kg bw per day for 52 consecutive weeks. In a concurrent control group, four males and four females received empty capsules. The animals were observed for clinical signs before administration (which took place at approximately 09:30), every 2–3 hours after administration until about 17:00 on working weekdays and at least 3 times per day on other days. Feed consumption was monitored daily, and body weight measurements were taken at weekly intervals from 2 weeks before commencement of dosing until study termination. Ophthalmological examination was performed at weeks 0, 25 and 51 of treatment. Haematology and clinical chemistry were undertaken at weeks 0, 13, 26, 39 and 52. Bone marrow smear and myelocrit measurements were made using bone marrow samples obtained from sternal puncture prior to necropsy. Urine analysis was performed for all animals at weeks 0, 13, 26, 39 and 52. Faeces were collected during a 24-hour period on weeks 0, 13, 26, 39 and 52 and examined for faecal occult blood. To examine liver function, a BSP retention test was performed on weeks 0, 12, 25, 38 and 51. At necropsy, all animals were subjected to thorough gross examination and organ weight analysis: brain, pituitary, mandibular glands, thyroids (including parathyroids), thymus, lungs, heart, liver (including gallbladder), pancreas, spleen, kidneys, adrenals, testes, prostate, ovaries and uterus. Histopathology was also performed on these organs as well as on eyeballs (including optic nerve), parotid glands, tongue, submandibular lymph nodes, larynx, trachea, aorta, urinary bladder (including ureters), oesophagus, stomach, small intestine (duodenum, jejunum,

ileum), large intestine (caecum, colon, rectum), mesenteric lymph nodes, spinal cord (cervical, mid-thoracic, lumbar), sciatic nerve, femoral skeletal muscle, abdominal skin, mammary glands, sternal bone marrow, femoral bone marrow, epididymides, vagina, lacrimal glands, membrana nictitans and abnormal sites. In addition to light microscopy performed on all the tissues listed above, electron microscopy was also performed on suitably prepared samples of liver of control animals and of the 1000 mg/kg bw per day group.

There were no deaths through the treatment period. As for clinical signs, a slightly higher frequency of loose faeces was noticed in the 1000 mg/kg bw per day group. No consistent abnormalities in feed consumption, body weight gain or feed efficiency were observed. No compound-related abnormalities in ophthalmology, urine analysis, faecal examination, myelocrit measurement or myelogram examination of bone marrow were noted, and no remarkable changes in haematology parameters were observed. No effects on liver function were noted in the BSP retention test. In blood biochemistry, increases in total cholesterol and phospholipid levels and an elevation in α_2-globulin ratio were noted in the 1000 mg/kg bw per day group, and an elevation in alkaline phosphatase activity was noted in the 100 and 1000 mg/kg bw per day groups at all intervals tested (i.e. weeks 13, 26, 39 and 52). The absolute and/or relative weights of the liver were elevated in one female animal in the 100 mg/kg bw per day group and one male and three females in the 1000 mg/kg bw per day group. At necropsy, no gross compound-related changes were observed. Light microscopic examination revealed minimal treatment-related changes in the liver in the 1000 mg/kg bw per day group, which consisted of hyperplasia of connective tissue at Glisson's capsule (adjacent to the gallbladder), accompanied by brown pigment and bile duct proliferation. Ultrastructural analysis showed proliferation and dilatation of smooth endoplasmic reticulum in the hepatocytes, which were presumably related to adaptive changes, in all animals from the 1000 mg/kg bw per day group.

The NOAEL in this study was 10 mg/kg bw per day, based on elevated alkaline phosphatase activity and increased liver weights at 100 mg/kg bw per day (Nakano, 1992).

(b) Dermal application

Rats

Groups of five rats of each sex were treated dermally with flumioxazin at a dose level of 100, 300 or 1000 mg/kg bw per day for a period of 21 days. An additional group of five rats of each sex was dermally exposed to the vehicle (corn oil) and served as a concurrent control group. All animals were observed twice daily for mortality and moribundity and once daily for obvious signs of a toxic response and for indications of dermal irritation. Detailed clinical observations were performed once weekly. Body weight measurements were recorded prior to initiation, and body weight and feed consumption measurements were recorded weekly for weeks 1 through 3 and at termination. At termination, blood was collected for evaluation of haematological effects and for serum chemistry analysis. Animals were subsequently killed and subjected to a complete gross necropsy, organ weight evaluations and a histomorphological examination of protocol-specified tissues.

No mortality, clinical signs of toxicity or signs of dermal irritation were observed. Evaluation of mean body weight and feed consumption revealed no differences between controls and treatment groups. Mean haemoglobin and haematocrit values in 1000 mg/kg bw per day females were reduced. Taking into account the low incidence and lack of dose–response relationship, no adverse findings were detected during gross necropsy examination of treated animals. No adverse changes in organ weight were observed; however, only spleen, kidney, liver and testis/epididymides were weighed. Histomorphological evaluation of gross lesions, spleen, kidneys, treated and untreated skin, and liver revealed no compound-related lesions.

The NOAEL of flumioxazin administered dermally to rats was 1000 mg/kg bw per day, the highest dose tested, for the male rats and 300 mg/kg bw per day for the female rats, based on haematological changes at 1000 mg/kg bw per day (Osherhoff, 1991).

(c) Exposure by inhalation

No short-term toxicity studies involving inhalation exposure were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Flumioxazin technical (purity 94.8%) was administered to groups of 51 male and 51 female Crj:CD-1 (ICR) mice (5 weeks old) at a dietary concentration of 300, 3000 or 7000 ppm (equal to 31, 315 and 754 mg/kg bw per day for males and 37, 346 and 859 mg/kg bw per day for females, respectively) for 78 weeks; a control group received basal diet only. In addition to these animals (designated "main group"), 15 mice of each sex per group were treated with these dietary dose levels for 52 weeks only, for interim sacrifice. Dose levels, homogeneity and stability in the diet were confirmed by analysis. In-life clinical investigations were performed on interim and terminal kill animals. All animals were observed at least once daily for clinical signs and mortality. Detailed physical examinations were performed once per week. Animals in the main groups were weighed on the first day of treatment, then approximately once per week during the first 13 weeks and approximately every 4 weeks thereafter. Animals in the satellite groups were weighed on the first day of treatment and approximately every 4 weeks thereafter. Each animal was weighed prior to necropsy. Feed consumption was determined for each cage (three animals of each sex per cage) approximately once per week in the first 13 weeks and then at approximately 4-week intervals thereafter in the main group and just every 4 weeks in the satellite group. Haematological examination was performed at necropsy, with blood collected from the abdominal vein. Animals were not fasted prior to collection. A detailed gross necropsy was performed at termination, as it was for those found dead or killed in extremis. The following organ weights were measured on the first 12 animals of each sex per dose in the main groups and 10 animals of each sex per dose in the interim sacrifice group: liver, kidneys, brain, testes, adrenals. When compared with current Organisation for Economic Co-operation and Development (OECD) Test Guideline 453, only a limited selection of organs from a limited number of animals were weighed. The following organs and carcasses from all animals (scheduled sacrifice, found dead or killed in extremis) were preserved, and those from all control and 7000 ppm main group animals and the first 10 animals of each sex per dose in the control and 7000 ppm interim sacrifice groups were subjected to histopathological examination: adrenals, aorta (thoracic), brain (medulla, pons, cerebellar and cerebral cortex), epididymides, oesophagus, eyeballs, femur (bone and marrow), gallbladder, Harderian glands, heart, kidneys, large intestine (caecum, colon and rectum), liver, lungs, lymph nodes (submandibular and mesenteric), mammary glands (female only), nerve (sciatic), ovaries, pancreas, parathyroids, pituitary, prostate, salivary glands (submandibular), seminal vesicles, skeletal muscle (femoral), skin (abdominal), small intestine (duodenum, jejunum and ileum), spinal cord (cervical, thoracic and lumbar), spleen, sternum (bone and marrow), stomach, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus and vagina, as well as any macroscopically abnormal tissue. The liver, gallbladder, kidney, lungs, brain and macroscopically abnormal tissues from all animals of the 300 and 3000 ppm main groups were also examined histopathologically. The liver, gallbladder, kidney, lungs, brain, ovaries and macroscopically abnormal tissues from the first 10 survivors of each sex per dose in the satellite group were also examined. When compared with current OECD Test Guideline 453, organs from a limited number of animals were examined. One male and one female died accidentally and were therefore excluded from histopathological evaluation. Tissues and organs showing autolysis were excluded from histopathological evaluation.

There were no changes in clinical signs, body weight, feed consumption, haematology, organ weight measurement or gross pathological examination related to administration of the test compound. Excluding accidental deaths, the mortality rates in the main group for the 0, 300, 3000, and 7000 ppm test groups were 27.5%, 28.0%, 29.4% and 25.5% for males and 35.3%, 31.4%, 29.4% and 30.0% for females, respectively. There were no significant differences in mortality rate between the control group and any of the compound-treated groups. No deaths were considered related to treatment with the test compound. Treatment-related increases in the incidence of hypertrophy of centrilobular hepatocytes were observed for males of the 3000 and 7000 ppm groups; in the absence

of further adverse liver-related effects in these animals, this was considered an adaptive finding. Increases in the incidence of diffuse hypertrophy and single-cell necrosis of hepatocytes were observed for females of the 3000 and 7000 ppm groups. No increase in the incidence of any tumours due to test compound administration was reported.

In this study, the NOAEL was 300 ppm (equal to 37 mg/kg bw per day), based on non-neoplastic changes in the liver in females at 3000 ppm (equal to 346 mg/kg bw per day). For males, the NOAEL was 7000 ppm (equal to 754 mg/kg bw per day), the highest dose tested (Seki, 1993a).

Rats

Flumioxazin technical (purity 94.8%) was administered to groups of 50 male and 50 female Sprague-Dawley rats (6 weeks old) at a dietary concentration of 50, 500 or 1000 ppm (equal to 1.8, 18 and 36.5 mg/kg bw per day for males and 2.2, 21.8 and 43.6 mg/kg bw per day for females, respectively) for 24 months; a control group received basal diet only. In addition to these animals (designated "main group"), 24 rats of each sex per group were treated for up to either 12 months (10 of the 24 animals) or 18 months (remainder of the 24 animals), for interim sacrifice. Dose levels, homogeneity and stability in the diet were confirmed by analysis. In-life clinical investigations were performed on interim and terminal kill animals. All animals were observed at least once daily for clinical signs and mortality. Detailed physical examinations were performed once per week. Animals in the main groups were weighed on the first day of treatment, then approximately once per week during the first 14 weeks and approximately every 4 weeks thereafter. Animals in the satellite groups were weighed on the first day of treatment and approximately every 4 weeks thereafter. Each animal was weighed prior to necropsy (including any decedents), except those necropsied at 18 months. Feed consumption was determined for each cage (animals housed three of each sex per cage) approximately once per week in the first 14 weeks and then at approximately 4-week intervals thereafter in the main groups and every 4 weeks in the satellite groups. Water consumption was measured for animals that underwent urine analysis at weeks 26–27, 50–51, 78–79 or 102–103. Urine analysis was performed from weeks 24 to 26, 51 to 52 and 76 to 77 in animals of the satellite groups and at week 103 in the main group. Ophthalmological examination was performed after application of a mydriatic agent in all animals of the control and 1000 ppm groups in the main group before the start of treatment and at week 53. At week 104, the examination was performed in all males of the control and 1000 ppm groups and all females of each dose group in the main group. Blood samples for haematology and clinical chemistry were taken from alternating sets of 10 animals of each sex per dose of the satellite group from the orbital sinus at weeks 14 and 27 after overnight fasting or from the abdominal aorta at weeks 53 and 79 under anaesthesia prior to necropsy. Blood samples for haematology and clinical chemistry were collected from the abdominal aorta under anaesthesia at necropsy (week 105) of the first 12 animals of each sex per dose of the main group. Serum samples were collected from the remaining animals after overnight fasting. Blood samples were also taken from decedents. Femur bone marrow smears were prepared at weeks 53 and 105. On necropsy, as well as a thorough gross examination, organ weight analyses were performed on the first 10 animals of each sex per dose at week 53 and the first 12 animals of each sex per dose at week 105 for the following organs: liver, kidneys, spleen, heart, brain, testes, adrenals and ovaries. When compared with current OECD Test Guideline 453, only a limited selection of organs in a limited number of animals were weighed in week 53. The organs listed below as well as carcasses collected from the first 10 animals of each sex per dose of the satellite group at week 53, from all survivors at week 105 and from all decedents or animals killed in extremis were prepared for histology: abdominal skin, adrenals, aorta (thoracic), brain (medulla/pons, cerebellar cortex and cerebral cortex), epididymides, oesophagus, eyeball, femur (bone marrow), Harderian glands, heart, kidneys, large intestine (caecum, colon and rectum), liver, lungs, mammary glands (female only), mesenteric lymph nodes, ovaries, pancreas, parathyroids, pituitary, prostate, salivary gland (submandibular), sciatic nerve, seminal vesicles, skeletal muscle, small intestine (duodenum, jejunum and ileum), spinal cord (cervical, thoracic and lumbar), spleen, sternum (bone marrow), stomach, submandibular lymph nodes, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus and vagina, as well as macroscopically abnormal tissues. Slides were examined for all scheduled sacrifices in the control group and 1000

ppm group at weeks 53 and 105 and for all decedents and those killed in extremis (except those decedents or humane kills taking place between weeks 53 and 79). Only the liver, spleen, kidneys, lungs and macroscopically abnormal tissues of animals in the 50 and 500 ppm groups were examined microscopically.

No treatment-related changes were observed in regard to body weight, feed consumption, water consumption, urine analysis, ophthalmology, blood biochemistry, gross pathological examination or organ weight. Mortality was not affected by treatment. In the animals that were killed owing to extreme deterioration of their physical condition, no relationship to treatment was found. Haematological examination revealed a decrease in haemoglobin concentrations in both sexes of the 500 and 1000 ppm groups, decreases in mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in males of the 1000 ppm group and in females of the 500 and 1000 ppm groups, a decrease in haematocrit values in females of the 500 and 1000 ppm groups, an increase in erythrocyte counts in females of the 1000 ppm group, an increase in erythroblast counts in both sexes of the 1000 ppm group and an increase in reticulocyte incidence in females of the 500 and 1000 ppm groups. Bone marrow differentiation revealed a low myeloid to erythroid ratio in females of the 1000 ppm group at week 53. Histopathological examination revealed an increase in the incidence and severity of extramedullary haematopoiesis in the spleen of females in the 500 and 1000 ppm groups at week 53 and of males in the 500 and 1000 ppm groups at week 105 (Table 4). An increase in the incidence and severity of chronic nephropathy in the kidney was observed in males of the 500 and 1000 ppm groups at week 105 (Table 4). No treatment-related increase in tumour incidence during the 2-year treatment was reported.

Under the conditions of this study, the NOAEL was 50 ppm (equal to 1.8 and 2.2 mg/kg bw per day for males and females, respectively), based on anaemia observed at 500 ppm (equal to 18 and 21.8 mg/kg bw per day for males and females, respectively) (Seki, 1993b).

Table 4. Selected findings in 2-year rat study

	Incidence of findings							
	Males				**Females**			
	0 ppm	**50 ppm**	**500 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**1 000 ppm**
Number of animals evaluated for spleen findings (week 53, satellite group)	10	10	10	10	10	10	10	10
Spleen, increased extramedullary haematopoiesis (week 53) Slight-mild-moderate	2-1-0	4-0-0	5-1-0	1-1-0	5-0-1	6-0-0	3-4-0	0*-5-3
Number of animals evaluated for spleen findings (week 105, main group)	50	48	49	49	50	50	50	49
Spleen, increased extramedullary haematopoiesis (week 105) Slight-mild-moderate-severe	4-0-0-0	5-0-0-0	11*-0-2-0	12*-1-0-0	9-8-0-2	14-2-3-0	16-8-2-0	14-4-5-0
Number of animals evaluated for kidney findings (week 105, main group)	50	50	50	50	50	50	50	50
Kidney, chronic nephropathy (week 105) Slight-mild-moderate-severe	8-13-1-2	7-8-8-0	12-15-11-1	17-11-5-0	3-3-0-0	3-4-0-0	6-6-2-0	6-2-0-0

ppm: parts per million; *: $P \leq 0.05$ (Mann-Whitney's *U*-test)
Source: Seki (1993b)

Table 5. Results of genotoxicity studies performed with flumioxazin

Type of study	Organism/cells	Dose range tested	Batch purity (%)	Result	Reference
In vitro gene mutation	Ames: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538, and *Escherichia coli* WP2 uvrA	±S9: 0, 50, 100, 200, 500, 1 000, 2 000 µg/plate	94.8	±S9: Negative	Kogiso (1989)
	Chinese hamster V79 *hprt* forward mutation	±S9: 0, 14.1, 28.1, 56.3, 112.5, 225 µg/mL[a] −S9: 0, 14.1, 28.1, 56.3, 112.5, 225 µg/mL[b] +S9: 0, 28.1, 56.3, 112.5, 337.5, 450 µg/mL[b]	99.6	±S9: Negative	Wollny (2011)
In vitro chromosomal aberration assay	Chinese hamster ovary K1	±S9: 0, 10.6, 35.4, 70.9 µg/mL[c]	98.2	−S9: Negative +S9: Positive	Kogiso (1988)
In vivo micronucleus assay	Mouse bone marrow micronucleus (intraperitoneal injection)	0, 300, 1 000, 5 000 mg/kg bw	98.4	Negative	Hara & Kogiso (1988)
In vivo chromosomal aberration assay	Rat bone marrow chromosomal aberration (oral administration)	Male: 0, 1 250, 2 500, 5 000 mg/kg bw Female: 0, 1 250, 2 500, 4 400, 5 000 mg/kg bw	94.8	Negative	Hara (1990)
In vivo unscheduled DNA synthesis assay	Rat hepatocytes (oral administration)	1 250, 2 500, 5 000 mg/kg bw	94.8	Negative	Kogiso (1990)

bw: body weight; DNA: deoxyribonucleic acid; hprt: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] First mutation assay.

[b] Second mutation assay.

[c] Concentrations equivalent to 0, 0.03, 0.1 and 0.2 mmol/L, respectively.

2.4 Genotoxicity

Flumioxazin was negative in a bacterial mutation assay and a mammalian cell mutation assay in both the presence and absence of metabolic activation and did not induce unscheduled DNA synthesis in rat hepatocytes in an in vivo assay (Table 5).

Treatment of Chinese hamster ovary cells with flumioxazin resulted in an increase in chromosomal aberrations in the presence, but not in the absence, of metabolic activation. However, there was no evidence of clastogenic activity in either an in vivo chromosomal aberration test in rat bone marrow cells or an in vivo mouse micronucleus test in bone marrow cells, which are the appropriate follow-up studies.

(a) In vitro

Flumioxazin technical (purity 94.8%) was tested for its mutagenic potential on *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 and *Escherichia coli* strain WP2 uvrA in a preincubation assay conducted according to the Ames test procedure at concentrations

up to 2000 µg/plate (where precipitation of the test article was observed) in the absence or presence of S9 mix. Two experiments were performed, although concentrations were plated only in duplicate in each experiment. Hence, the study design was less powerful than a study conducted according to current test guidelines (triplicate plating is recommended). The top dose was limited by the presence of precipitate above 2000 µg/plate, which made it difficult to count the colonies. Flumioxazin was not cytotoxic up to 5000 µg/plate in a range-finding experiment. A slight increase of revertants (in the presence of S9 mix) was observed, but the incidence did not reach a factor of 2 when compared with concurrent controls. In summary, flumioxazin did not display mutagenic potential under the conditions of this test. Satisfactory positive control results were obtained (Kogiso, 1989).

Flumioxazin technical (purity 99.6%) was tested for its mutagenic potential at the *hprt* locus in V79 cells at concentrations up to 450 µg/mL in the presence of S9 mix and up to 225 µg/mL without metabolic activation, in an OECD Test Guideline 476–compliant study. The assay was performed in two independent experiments using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. The maximum concentration of the pre-experiment (3600 µg/mL) was equal to a concentration of approximately 10 mmol/L (the maximum recommended dose, in accordance with the guideline). No cytotoxic effects were observed under the experimental conditions. The concentration ranges of the main experiments were limited by the solubility of the test article in aqueous medium. No substantial and reproducible dose-dependent increase in mutation frequency was observed in either main experiment. The positive controls (ethyl methanesulfonate in the absence of S9 and 7,12-dimethylbenz(*a*)anthracene in the presence of S9) induced marked increases in mutant frequency, thereby demonstrating the sensitivity of the test system. It is concluded that flumioxazin did not induce mutation at the *hprt* locus of V79 Chinese hamster cells when tested under the conditions employed in this study (Wollny, 2011).

Flumioxazin (purity 98.2%) was tested for its clastogenic potential in Chinese hamster ovary K1 cells at concentrations up to 70.9 µg/mL in the presence and absence of S9 mix. The study was conducted according to USEPA FIFRA Guideline 84-2, but was not compliant with GLP (no authorized protocol, study director not identified in report, quality assurance unit not involved in any part of the study). Cells (10^5/mL) were seeded 24 hours before treatment. In the absence of S9, cells were exposed to flumioxazin for 24 hours. The exposure time in the presence of S9 was 6 hours, after which the cells were washed and cultured in fresh media for a further 18 hours. Appropriate positive controls were used. One hundred metaphases from each dose were examined, and the frequencies of cells with structural aberrations (including and excluding gaps) were recorded. The study design was less powerful than required for a study conducted according to the current test guideline (single cultures only, 200 metaphases scored instead of 300, no short-term treatment). Cell survival was calculated by counting the number of cells 48 hours after exposure in the absence of S9 and 42 hours after cultivation in fresh medium in the presence of S9. Some evidence of clastogenic activity was observed in the presence of metabolic activation (Table 6). In the absence of metabolic activation, no evidence of clastogenicity was observed up to a maximum concentration of 70.9 µg/mL (0.2 mmol/L), a concentration that was limited by cytotoxicity (Kogiso, 1988).

(b) In vivo

Flumioxazin (purity 98.4%) was tested for its in vivo clastogenic potential in the mouse micronucleus assay. Groups of four male ICR mice were administered flumioxazin by intraperitoneal injection at a dose of 0, 300, 1000 or 5000 mg/kg bw. The vehicle was corn oil. The total dosing volume was 20 mL/kg bw. The positive control material was cyclophosphamide. The study was conducted according to USEPA FIFRA Guideline 84-2 but was not GLP compliant (study director not identified in report, quality assurance unit not involved in any part of the study). The bone marrow harvest time was 24 hours after intraperitoneal injection. The incidence of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes and the ratio of polychromatic erythrocytes to whole erythrocytes (polychromatic erythrocytes + normochromatic erythrocytes) were

Table 6. Results of a non-GLP mammalian cell in vitro clastogenicity assay performed with flumioxazin

Chemical	Dose (mol/L)	No. of cells examined	Cells with aberrations (%)[a]		Chromosomal aberrations[b]							Survival (%)
			TAG	TA	ctg	ctb	cte	csb	cse	frg	MT	
−S9												
DMSO[c]	–	100	1	1	0	1	0	0	0	0	0	100.0
Flumioxazin	3×10^{-5}	100	1	1	0	0	1	0	0	0	0	101.3
	1×10^{-4}	100	1	0	1	0	0	0	0	0	0	73.2
	2×10^{-4} [d]	100	1	1	0	0	0	1	0	0	0	44.5
	5×10^{-4} [d]	– [e]	–	–	–	–	–	–	–	–	–	22.7
MMC	1.5×10^{-7}	100	40**	37**	6	13	34	1	1	0	0	66.8
+S9												
DMSO[c]	–	100	9	6	3	4	4	0	0	0	0	100.0
Flumioxazin	3×10^{-5}	100	13	9	4	5	3	0	0	0	0	96.5
	1×10^{-4} [d]	100	34**	26**	13	13	23	0	1	0	0	90.8
	2×10^{-4} [d]	100	56**	53**	28	47	41	0	0	1	0	59.4
	5×10^{-4} [d]	– [e]	–	–	–	–	–	–	–	–	–	21.5
BP	2.5×10^{-5}	100	48**	45**	6	12	58	1	1	0	0	94.0

BP: benzo(*a*)pyrene; DMSO: dimethyl sulfoxide; MMC: mitomycin C; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; chi-squared test)

[a] TAG: aberrations including gaps; TA: aberrations excluding gaps.

[b] ctg: chromatid gaps; ctb: chromatid breaks; cte: chromatid exchanges; csb: chromosome breaks; cse: chromosome exchanges (including chromosome rings and dicentric chromosomes); frg: fragmentation; MT: cells with multiple aberrations (more than 10).

[c] Final concentration in the medium was 0.5%.

[d] A small amount of precipitation was observed in the medium.

[e] No metaphase.

determined for each animal. The study design was less powerful than required for a study conducted according to the current test guideline (only 1000 polychromatic erythrocytes evaluated instead of 2000, no second evaluation after 48 hours). In this study, there was no increase in the incidence of micronucleated polychromatic erythrocytes following sampling at 24 hours post-dosing of male mice when tested up to a dose of 5000 mg/kg bw. The ratio of polychromatic erythrocytes to whole erythrocytes was reduced (Hara & Kogiso, 1988).

Flumioxazin technical (purity 94.8%) was tested for its clastogenic potential in the in vivo chromosomal aberration assay in rat bone marrow. Groups of five male and five female Sprague-Dawley rats were administered flumioxazin by oral gavage at a dose of 1250, 2500 or 5000 mg/kg bw. The vehicle was corn oil. The total dosing volume was 20 mL/kg bw. The positive control material was cyclophosphamide. The bone marrow harvest time was 24 hours after oral administration. A time course experiment was also performed in which groups of five males were administered 5000 mg/kg bw and groups of five females received 4400 mg/kg bw (the actual dose for

female rats was due to an error and should have been 5000 mg/kg bw) and sacrificed at 6, 12, 24 or 48 hours after administration. Rats received an intraperitoneal dose of colchicine in saline 2 hours prior to sacrifice. After sacrifice, bone marrow cells were collected from the femur, and slides were prepared. Slides were examined blind to treatment. One thousand erythrocytes were counted to determine the ratio of polychromatic erythrocytes to whole erythrocytes. At least 500 cells per animal were analysed to determine the mitotic index. Fifty metaphases with $2n \pm 1$ chromosomes were analysed from each animal. The study design was less powerful than required for a study conducted according to the current test guideline (100 metaphases scored instead of 200). There were no signs of toxicity on administration of test substance, although body weight gain was suppressed in treated animals at 24 hours after treatment. Suppression of the mitotic index was not seen in males, but a decrease in mitotic index was observed at 6 and 24 hours in females, followed by an increase at 48 hours. A decrease in the ratio of polychromatic erythrocytes to normochromatic erythrocytes was seen at 12, 24 and 48 hours after treatment at 5000 mg/kg bw in males and at 24 and 48 hours after treatment at 4400 mg/kg bw in females, demonstrating exposure of the bone marrow in this assay. Under the conditions of this study, there was no increase in the incidence of chromosomal aberrations in the bone marrow cells following sampling of male and female rats at 6, 12, 24 and 48 hours post-dosing when tested up to a dose of 5000 mg/kg bw (Hara, 1990).

Flumioxazin technical (purity 94.8%) was tested for its DNA damaging potential in an in vivo unscheduled DNA synthesis assay. The study was conducted according to USEPA FIFRA Guideline 84-4. Groups of three male Sprague-Dawley rats were gavaged with 5000 mg/kg bw per day (the vehicle was corn oil, the dosing volume was 20 mL/kg bw), and the occurrence of unscheduled DNA synthesis in hepatocytes was examined 3, 12 and 24 hours after administration (time course study). Groups of three males were also dosed with 1250, 2500 or 5000 mg/kg bw and examined for the occurrence of unscheduled DNA synthesis in hepatocytes 12 hours after administration (dose–response study). At the allotted time, rats were sacrificed, and the hepatocytes were isolated. The hepatocytes were cultured in a medium containing ^{3}H-labelled thymidine for 4 hours. To determine the occurrence of unscheduled DNA synthesis, incorporation of the radiolabelled thymidine was detected by autoradiography and evaluated in 50 cells per animal. The study design was less powerful than required for a study conducted according to the current test guideline, which requires 100 cells per animal from at least two slides. No increase in the incidence of unscheduled DNA synthesis in hepatocytes was observed in this study following sampling of males at 3, 12 and 24 hours post-dosing when tested up to a dose of 5000 mg/kg bw (Kogiso, 1990).

2.5 Reproductive and developmental toxicity

(a) One- and two-generation studies

In a dose range–finding one-generation study for a two-generation reproductive toxicity study, Sprague-Dawley rats were fed diets containing 0, 100, 500, 1000 or 5000 ppm flumioxazin technical (purity 94.8%; during premating period, equal to 0, 5.6, 27.5, 54.9 and 263.9 mg/kg bw per day for males and 0, 6.2, 30.9, 61.4 and 306.0 mg/kg bw per day for females, respectively). The male rats were given diets containing the test material for a total of 63 days, beginning 35 days prior to cohabitation. The female rats were provided the diet containing the test material for 35 days prior to cohabitation, through cohabitation, gestation and delivery, and for 21 days postpartum.

No male rats died; however, three females from the 5000 ppm group died during gestation. Other signs of toxicity in both males and females, including clinical signs, gross lesions (e.g. enlarged spleen) and reduced body weights and/or feed consumption, were observed at doses of 100 ppm and above. Females were most significantly affected during gestation at doses of 500 ppm and above. Concentrations as high as 5000 ppm did not affect mating performance or fertility; however, no live litters were produced in the 500, 1000 or 5000 ppm groups (total resorptions), precluding evaluation of the lactation period for these groups. All pregnant dams in the control and 100 ppm groups delivered litters. The 100 ppm concentration did not affect the averages for the duration of gestation, litter size, pup sex ratio or pup body weight. Similarly, this concentration did not affect the viability or lactation indices or the external or necropsy observations for the pups (Hoberman, 1991a).

Based on the results of this first study, a second pilot study was initiated using 500 ppm as the highest test concentration. In the second study, rats were exposed for a similar period of time to a dose of 0, 100, 200, 300, 400 or 500 ppm flumioxazin (purity 94.8%) in the diet (during premating period, equal to 0, 5.6, 11.3, 17.1, 22.2 and 28.4 mg/kg bw per day for males and 0, 6.9, 13.6, 19.7, 27.1 and 32.3 mg/kg bw per day for females, respectively). The male rats were given diets containing the test material for a total of 72 days, beginning 28 days prior to cohabitation. The female rats were provided with diet containing the test material for 28 days prior to cohabitation, throughout cohabitation, gestation and delivery, and for 21 days postpartum.

Decreased body weight gains and feed consumption values occurred in the 300, 400 and 500 ppm group female rats, and decreases in the gestation indices, litter sizes, average number of pups per litter and pup survival tended to occur in the 300 and 400 ppm groups. No live litters were produced in the 500 ppm group, and the number of implantation sites was reduced in this dose group. Gestation length tended to be increased in the 400 ppm dose group. Based on these data, concentrations as high as 300 ppm were recommended for the multigeneration rat reproductive toxicity study (Hoberman, 1991b).

A two-generation (one litter per generation) reproductive toxicity study was conducted in which flumioxazin technical (purity 94.8%) was administered to Sprague-Dawley rats at dietary levels of 0, 50, 100, 200 and 300 ppm (for test substance intake, see Table 7). F_0 rats (30 of each sex) were exposed continuously for approximately 12 weeks prior to cohabitation and throughout breeding, gestation and lactation, until scheduled sacrifice. The second generation (F_1) (adjusted to 30 rats of each sex) was exposed for a minimum of 86 days before cohabitation and throughout breeding, gestation and lactation, until scheduled sacrifice. Body weights and feed consumption were determined weekly throughout the study until termination for F_0 and F_1 males. For F_0 and F_1 females, body weights and feed consumption were recorded weekly during the premating periods, on days 0, 6, 10, 15, 20 and 25 of gestation, and on days 1, 4, 7, 10, 14, 16, 18 and 21 of lactation. Feed consumption during lactation for the F_0 and F_1 generations was recorded up to postpartum day 14. Estrous cycling of F_0 and F_1 animals was evaluated for 1 week before cohabitation. Rats were cohabited for mating until the presence of sperm or a copulatory plug was observed (designated day 0 of gestation) or until completion of the 21-day cohabitation period. The day of parturition was defined as day 1 of lactation. F_1 and F_2 pups were evaluated daily for external abnormalities, and individual pup body weights were recorded on days 1, 4, 7, 14 and 21 of lactation. Where appropriate, litters were culled (random pup selection) to four male and four female pups after weighing on day 4 of lactation. Culled pups were necropsied. At least one F_1 generation pup of each sex (whenever possible) per litter was selected at weaning for continuation as the F_1 generation (30 pups of each sex). F_1 pups not selected for continued observation were sacrificed and necropsied on postpartum day 21. Organ weights and histopathological evaluation were limited to reproductive organs, mammary gland (females only), pituitary and other organs with gross findings.

In F_0 males, there were no adverse effects on clinical signs, body weight or body weight gain (Table 8), feed intake or findings during necropsy (including histopathological evaluation) in any dose group. No effects on F_0 male mating performance or fertility at concentrations up to and including the highest concentration tested (300 ppm) were reported. The average duration of cohabitation, the number of male rats mating and the number of male rats siring litters were similar among the groups.

In F_1 males, clinical observations, significantly reduced terminal body weight, reduced absolute weights of the left and right epididymides, left and right testes, and brain, reduced weight of the right epididymis relative to brain weight, and a tendency to reduced absolute weight of the prostate were observed at 300 ppm in male rats. There was also one male death at 300 ppm on day 3 post-weaning, which was attributed to treatment, because the rat shared treatment-related signs with two other rats in the same group (appearing pale). The rat that died also appeared dehydrated and had urine-stained abdominal fur and remarkably reduced body weight before death. Necropsy showed black ingesta in the stomach and intestines. Histopathology showed hypoplasia of the coagulating gland, epididymides, prostate, seminal vesicles and testes. The 300 ppm group also tended to have

Table 7. Mean test substance intake in reproductive toxicity study in rats

	Mean test substance intake (mg/kg bw per day)							
	50 ppm		100 ppm		200 ppm		300 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_0 generation								
Premating (days 1–83)	3.2	3.8	6.3	7.6	12.7	15.1	18.9	22.7
Gestation	–	3.3	–	6.7	–	13.0	–	20.8
Lactation	–	5.9	–	12.3	–	24.1	–	29.6
F_1 generation								
Premating (days 1–99)	3.7	4.3	7.5	8.5	15.0	17.2	22.4	25.6
Gestation	–	3.3	–	6.5	–	13.3	–	20.2
Lactation	–	5.7	–	12.0	–	23.0	–	30.5

bw: body weight; ppm: parts per million
Source: Hoberman (1992)

Table 8. Body weight gain in F_0 and F_1 generations during selected study periods

	Body weight gain (g)									
	0 ppm		50 ppm		100 ppm		200 ppm		300 ppm	
	M	F	M	F	M	F	M	F	M	F
F_0 generation										
Days 1–83 premating	393.4	169.3	408.8	175.2	384.9	166.5	385.9	172.1	403.0	169.4
Day 1–termination	489.3	–	517.4	–	487.0	–	486.0	–	498.6	–
Days 0–20 gestation	–	137.6	–	140.6	–	137.2	–	133.0	–	95.5**
Days 1–4 lactation	–	2.5	–	1.7	–	1.4	–	−1.8	–	−10.8
Days 1–21 lactation	–	−3.6	–	−0.8	–	3.7	–	−5.1	–	4.7
F_1 generation										
Days 1–99 premating	544.8	290.9	584.4	300.7	574.1	291.0	553.2	288.7	514.0*	274.7
Day 1–termination	631.3	–	669.4	–	663.9	–	629.6	–	591.4	–
Days 0–20 gestation	–	145.8	–	123.8*	–	139.8	–	125.7*	–	104.8**
Days 1–4 lactation	–	−2.3	–	−1.9	–	4.1	–	−24.5	–	−6.3
Days 1–21 lactation	–	6.3	–	1.7	–	16.0	–	−7.5	–	3.4

F: females; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; Dunnett's test, Dunn's test or Fisher's test)
Source: Hoberman (1992)

reduced numbers of rats that mated and a reduced proportion of pregnant rats in cohabitation. Necropsy of four rats in the 300 ppm group that did not sire a litter revealed changes in one or both testes and epididymides (small, purple or flaccid testes and small, purple or thread-like epididymides; one of these rats also had a small prostate and a small right seminal vesicle).

In F_0 generation female rats, a concentration of 300 ppm produced statistically significant effects on body weight gains and body weights during gestation (Table 8) and on absolute or relative feed consumption values during gestation (increased values) and lactation (decreased values). The averages for the duration of cohabitation, the number of female rats that mated and the number of mated female rats that were pregnant were similar among the groups. At 300 ppm, significantly increased numbers of F_0 generation rats with red vaginal substance present during gestation were observed; this observation was assumed to be related to resorption of litters. Five F_0 generation female rats in the 300 ppm group were pregnant but did not deliver until sacrificed on day 25 of gestation. Organ weights were not affected in F_0 generation female rats.

In the F_1 generation, a statistically significantly increased number of deaths and clinical, necropsy and histopathological observations were seen in adult animals at 300 ppm. During necropsy of deceased females, livers were described as yellow and revealed bile stasis and/or necrosis upon histopathological examination. Dose-related reductions (sometimes statistically significant) in body weight gains (Table 8), body weights, and absolute and relative feed consumption were observed in F_1 females from the 300 ppm group. Lower body weight gain in the 200 ppm dose group was restricted to the late gestational period. The F_1 generation female rats in the 100 and 200 ppm groups tended to have transient reductions in feed consumption values on days 4–7 of lactation; however, these observations did not persist. The averages for the duration of cohabitation, the number of female rats that mated and the number of mated female rats that were pregnant were similar among the groups.

The gestation index in F_0 and F_1 generation female rats was reduced at 300 ppm (Table 9) and was statistically significantly reduced in the F_1 females, which was related to the fetal deaths in utero. This concentration also significantly increased the number of dams in the F_0 generation with all F_1 pups dying on days 1–21 postpartum and the number of dams in the F_0 generation that did not deliver a litter.

The 200 ppm concentration of the test substance significantly reduced F_1 generation pup body weights on day 1 postpartum. The 100 ppm concentration and above tended to increase the number of dying pups per litter on days 1 and 4 (preculling) postpartum and reduced viability index in the F_1 generation. In the 200 ppm dose group, F_1 pup weight per litter was reduced. The 300 ppm concentration of the test substance tended to reduce or significantly reduced the number of pups delivered, number of liveborn pups, viability index, litter size at weighing, average pup weight per litter and surviving pups per litter in both generations; additionally, clinical and necropsy observations related to pup morbidity (reduced live births and viability) were reported in both generations (Table 9). Offspring in the 300 ppm F_1 group appeared weak upon clinical observation.

The NOAEL for parental toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on clinical signs of toxicity and reductions in body weight, body weight gain, feed consumption and organ weights.

The NOAEL for reproductive toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on reduced gestation index in both the F_0 and F_1 generations and an increased number of F_0 dams that did not deliver a litter.

The NOAEL for offspring toxicity was 50 ppm (equal to 3.2 mg/kg bw per day), based on increased postnatal pup mortality in the F_1 generation (Hoberman, 1992).

(b) Developmental toxicity

Rats

A non-GLP pilot dose range–finding study was conducted in pregnant rats to determine appropriate doses for the oral teratology study. Flumioxazin (purity 98.2%) was suspended in a 0.5% aqueous solution of carboxymethyl cellulose and administered by gavage at a dose of 0, 30, 100, 200 or 500 mg/kg bw per day to six pregnant rats per dose on days 6 through 15 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean

Table 9. Mating and gestation parameters in the rat dietary two-generation reproductive toxicity study with flumioxazin

	F_0 generation					F_1 generation				
	0 ppm	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**	**0 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**
Pregnancy rate, *N*/*N* (%) (fertility index)	23/28 (82.1)	22/28 (78.6)	26/28 (92.8)	28/30 (93.3)	21/27 (77.8)	23/28 (82.1)	21/29 (72.4)	19/26 (73.1)	18/26 (69.2)	18/22 (81.8)
Mated within days 1–7, 1st male, *N* (%)	24 (85.7)	22 (81.5)	24 (88.9)	27 (90.0)	23 (92.0)	23 (85.2)	23 (82.1)	22 (88.0)	19 (76.0)	15 (75.0)
Mated days 8–14, 1st male, *N* (%)	2 (7.1)	2 (7.4)	3 (11.1)	0 (0.0)	1 (4.0)	3 (11.1)	0 (0.0)	2 (8.0)	2 (8.0)	1 (5.0)
Mated with 2nd male, days 15–21, *N* (%)	2 (7.1)	3 (11.1)	0 (0.0)	3 (10.0)	1 (4.0)	1 (3.7)	5 (17.8)	1 (4.0)	4 (16.0)	4 (20.0)
Pregnant *N*/*N* cohabited (%)	23/30 (76.7)	22/30 (73.3)	26/30 (86.7)	28/30 (93.3)	21/30 (70.0)	23/30 (76.7)	21/30 (70.0)	19/30 (63.3)	18/30 (60.0)	18/26 (69.2)
Gestation index: Live litter delivered / pregnant rats, *N*/*N* (%)	23/23 (100)	22/22 (100)	25/26 (96.2)[a]	28/28 (100.0)	16/21[##] (76.2)	23/23 (100.0)	20/21 (95.2)	19/19 (100.0)	18/18 (100.0)	16/18 (88.9)
Dams that did not deliver a live litter, *N* (%)	0	0	1 (3.8)[a]	0	5 (23.8)[##]	0	1 (4.8)	0	0	2 (11.1)
Duration of gestation (days)	23.0	22.8	23.1	22.8	23.2	22.9	23.0	22.8	22.9	23.0
Day of delivery[b,c]										
Day 21	3	3	3	4	1	2	1	3	3	0
Day 22	17	18	18	24	10	16	12	10	8	10
Day 23	3	0	3	0	2	4	5	5	6	4
Day 24	0	0	1	0	1	0	1	0	0	0
Implantation sites per delivered litter	16.1	15.9	15.5	16.5	15.0	17.6	15.4	17.3	15.4	16.2
Total implantation sites[d]	16.1	15.9	15.5	16.5	15.4	17.6	15.1	17.3	15.3	16.3
Dams with stillborn pups, *N* (%)	3 (13.0)	3 (13.6)	3 (11.5)	2 (7.1)	1 (4.8)	2 (8.7)	3 (15.0)	2 (10.5)	4 (22.2)	2 (12.5)
Dams with all stillborn pups, *N* (%)	0	0	1 (3.8)	0	0	0	0	0	0	0
Dams delivering one or more liveborn pups, *N* (%)	23 (100.0)	22 (100.0)	25 (96.2)[a]	28 (100.0)	16 (100.0)	23 (100.0)	20 (100.0)	19 (100.0)	18 (100.0)	16 (100.0)

	F_0 generation					F_1 generation				
	0 ppm	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**	**0 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**
Dams with all liveborn pups dying days 1–21 postpartum, *N* (%)	0	0	0	0	2 (12.5)[##]	0	0	0	0	0[e]
Pups delivered *N*	322	324	360	409	115	363	271	298	242	153
Pups delivered/ dam	14.4	15.2	13.8	14.6	7.2[##]	15.8	13.6	15.7	13.4	9.6[##]
Pups dying[j]										
Day 1	2	2	3	5	5[##]	0	0[f]	2	1	5[g##]
Days 2–4	0	3	10[##]	15[##]	9[##]	5	6[f]	6	7	26[g##]
Days 5–7	1	0	0	0	1	2	1	0	0	1
Days 8–14	0	0	0	0	0	2	0	0	0	0
Viability index[h] *N*/*N* (%)	324/326 (99.4)	321/326 (98.5)	341/354[#] (96.3)	387/407[##] (95.1)	98/112[##] (87.5)	353/358 (98.6)	245/251[f] (97.6)	288/296 (97.3)	222/230 (96.5)	118/149[g] (79.2[##])
Lactation index[i] *N*/*N* (%)	176/177 (99.4)	172/172 (100.0)	192/192 (100.0)	215/215[e] (100.0)	80/81 (98.8)	179/183 (97.8)	139/140 (99.3)	152/152 (100.0)	138/138 (100.0)	93/94 (98.9)
Live litter size										
Day 1	14.1	14.7	14.0	14.4	6.7[##]	15.6	13.2	15.5	12.7	9.1[##]
Day 4 precull	14.1	14.6	13.6	13.8	7.0[##]	15.3	12.9	15.2	12.3	7.9[##]
Day 4 postcull	7.7	7.8	7.7	7.7	5.8[##]	8.0	7.4	8.0	7.7	6.3[##]
Day 21	7.6	7.8	7.7	7.7	6.2[#]	7.8	7.3	8.0	7.7	6.2[#]
Pup weight per litter (g)										
Day 1	6.9	6.5	6.7	6.1**	5.7**	6.4	6.7	6.3	6.1	5.6**
Day 4 precull	10.5	9.7	10.1	9.5	8.5**	9.0	9.8	8.9	9.2	8.0*
Day 4 postcull	10.6	9.8	10.2	9.6	8.6**	9.2	9.9	9.1	9.3	8.0*
Day 7	17.9	16.8	17.3	16.3	14.1*	15.7	16.6	15.3	15.2	13.1**
Day 14	35.3	33.2	36.3	33.9	29.5	33.8	34.7	32.8	33.1	30.1**
Day 21	54.0	51.7	56.1	52.7	47.8	52.8	55.3	52.9	53.5	50.0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (significantly different from the vehicle control group; Dunnett's test, Dunn's test or Fisher's test); [#]: $P < 0.05$; [##]: $P < 0.01$ (significantly different from the vehicle control group; Dunn's test)

[a] One dam delivered a litter consisting of only two stillborn pups.

[b] Restricted to rats with confirmed mating dates.

[c] Excludes values for one dam, which delivered a litter on day 18 of gestation (the mating date was incorrectly identified).

[d] Includes pregnant rats that did not deliver a litter and were sacrificed on day 25 of gestation.

[e] Excludes value for one pup missing on day 18 postpartum. It was found on the floor on day 20 postpartum and sacrificed.

[f] Excludes values for dam found dead on day 2 of lactation. There were three pups stillborn, one pup found dead, and 13 pups sacrificed on the same day.

[g] Excluded value for one dam found dead on day 2 of lactation. The one surviving pup was sacrificed on the same day.

[h] No. of live pups on day 4 (preculling) postpartum/no. of liveborn pups on day 1 postpartum.

[i] No. of live pups on day 21 postpartum/no. of live pups on day 4 (postculling) postpartum.

[j] Historical control data: mean mortality postnatal days 1–4: 2.6% (range: 0–8.7%).

Source: Hoberman (1992)

section; hence, the study design may have been less sensitive than required according to current standards.

No animals died during the course of the study, and maternal toxicity was limited to decreased weight gain in all treatment groups. No changes in feed intake were reported. High embryolethality was observed in all dose groups. Litters from dams exposed to 200 and 500 mg/kg bw per day were completely resorbed, and only four live fetuses were produced in the 100 mg/kg bw per day group. Fetuses from 30 mg/kg bw per day dams had significantly reduced weights and were found to have both skeletal and visceral abnormalities, primarily wavy ribs and ventricular septal defects (Table 10). One fetus in the low-dose group was reported to have curvature of scapula. Because of the high degree of embryolethality at doses of 100 mg/kg bw per day and above, the highest dose selected for the definitive study was 30 mg/kg bw per day (Kawamura, 1989).

Flumioxazin technical (purity 94.8%) was administered by gavage to four groups of 22 pregnant rats (Slc:SD) at a dose of 1, 3, 10 or 30 mg/kg bw per day during organogenesis (days 6 through 15) to evaluate the effects on dams and fetuses. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The material was suspended in a 0.5% aqueous methyl cellulose vehicle. A control group received vehicle only. Animals were mated overnight, and the day on which sperm were observed in the vaginal smear was designated day 0 of gestation. All females were observed daily for mortality and clinical signs. Each female was weighed on days 0, 6, 9, 12, 15 and 20 of gestation. Terminal body weight was the weight of the animal at sacrifice without the gravid uterus. Feed consumption was measured for days 6, 9, 12, 15 and 20 of gestation. On the morning of day 20, all dams were sacrificed and examined grossly for abnormalities of the thoracic, abdominal and pelvic viscera. Corpora lutea were counted. The uteri were weighed, including contents, and examined for the number of implantations and the numbers and sites of live fetuses and dead embryos/fetuses. Each live fetus was sexed, weighed and observed externally, including the oral cavity. The fetuses with external abnormalities were fixed in formalin and were not examined for skeletal or visceral abnormalities. Approximately one half of all externally normal fetuses from each litter were randomly selected, fixed and stained with alizarin red S and examined for skeletal abnormalities, variations and ossification. The remaining live fetuses were fixed in Bouin's solution and examined for visceral abnormalities or variations. Statistical analyses were performed using appropriate methods.

No maternal deaths were observed in any dose group, and no treatment-related effects on clinical signs or feed consumption were noted. A decrease in maternal body weight gain was reported for the 30 mg/kg bw per day dose group (only on gestation day [GD] 20); this was related to a decrease in the number of live fetuses and fetal weights. Necropsy findings included dark reddish fluid or material in the uterus of dams receiving 30 mg/kg bw per day, which was also considered to be related to the death of the litter. The numbers of live fetuses and fetal weights were decreased in the 30 mg/kg bw per day group, and the incidence of embryo mortality tended to be higher in this dose group, but was not statistically significant. No effects on the number of implantations, sex ratios or external abnormalities were found. The incidence of fetuses with cardiovascular abnormalities (i.e. ventricular septal defects) was increased in the 30 mg/kg bw per day group (Table 10). The incidence of cardiovascular abnormalities in the 10 mg/kg bw per day group was not significantly different from the incidence in controls, but was above the historical control range. Double aortic branch was observed in three fetuses at 30 mg/kg bw per day. Other developmental effects observed at 30 mg/kg bw per day included an increase in the incidence of wavy ribs and curvature of the scapula and ulna and a decrease in the number of ossified sacrococcygeal vertebral bodies.

The NOAEL for embryo and fetal toxicity was 3 mg/kg bw per day, based on an increased incidence of malformations (cardiac ventricular septal defects) in the rat fetuses. The maternal toxicity NOAEL was 30 mg/kg bw per day, the highest dose tested (Kawamura, 1990a).

Table 10. Incidence of selected fetal findings with laboratory historical control data (affected fetuses/litters[a])

Kawamura (1989): range-finding study	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**200 mg/kg bw per day**	**500 mg/kg bw per day**
Visceral examination					
No. of examined fetuses	38	25	2	0	0
No. of examined litters	6	5	2	0	0
Heart: ventricular septal defects	0/0	11**/5[##]	1/1	–	–
Skeletal examination					
No. of examined fetuses	42	28	2	–	–
No. of examined litters	6	6	1	–	–
Curvature of scapula	0/0	1/1	0/0	–	–
Wavy ribs	0/0	9/3	1/1	–	–
Kawamura (1990a): main study	**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**
Visceral examination					
No. of examined fetuses	147	138	144	144	102
No. of examined litters	22	21	21	22	18
Heart: ventricular septal defects	2/2	1/1	2/2	6/5	26**/12[##]
	HC[b]: 21 studies with no affected fetuses 4 studies with 1 fetus (in 1 litter) affected 1 study with 2 affected fetuses (in 2 litters) Maximum: 2.1% of fetuses / 10% of litters				
Artery: double aortic branch	0/0	0/0	0/0	0/0	3/2
Skeletal examination					
No. of examined fetuses	154	144	152	153	111
No. of examined litters	22	21	21	22	18
Curvature of scapula	0/0	0/0	0/0	0/0	10/4[#]
Curvature of ulna	0/0	0/0	0/0	0/0	3/2
Wavy ribs	1/1	1/1	7/3	0/0	27**/12[##]
Kawamura (1991): dermal study	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	
Visceral examination					
No. of examined fetuses	153	133	144	88	
No. of examined litters	23	22	22	17	
Heart: ventricular septal defects	1/1	1/1	2/2	13**/9[##]	
	HC[b]: see above (Kawamura, 1990a)				
Skeletal examination					
No. of examined fetuses	159	136	149	90	
No. of examined litters	23	22	22	17	
Curvature of scapula	0/0	0/0	0/0	2/1	

Table 10 (continued)

Kawamura (1991): dermal study	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
Wavy ribs	0/0	0/0	2/1	18**/10[##]

bw: body weight; HC: historical control; *: $P < 0.05$; **: $P < 0.01$ (Wilcoxon's rank sum test); [#]: $P < 0.05$; [##]: $P < 0.01$ (Fisher's exact test)

[a] The numbers of affected litters were not included in the study reports but were summarized by the sponsor's representative, Exponent (email dated 24 July 2015).

[b] These 26 studies were conducted from 1982 to 1989; four studies with nine or fewer litters were excluded from the total list of 30 studies submitted; the study reports did not include HC data on further fetal findings.

Apparently, parts of the results of the study by Kawamura (1990a) were also published in the open literature (Kawamura et al., 1995).

The developmental toxicity of flumioxazin in rats was evaluated in a dermal teratology study compliant with USEPA FIFRA Guideline 83-3. On days 6–15 of gestation, pregnant rats (SLc:SD) were exposed dermally to flumioxazin technical (purity 94.8%) in corn oil at a dose level of 30, 100 or 300 mg/kg bw per day, applied to dorsal skin clipped free of hair and secured under a non-occlusive dressing for 6 hours. After 6 hours, the dressing was removed, and the application site was wiped clean of material. A control group received a dermal dose of corn oil alone. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The day of successful mating (sperm in vaginal smear) was designated as day 0 of gestation. All females were observed daily for mortality and clinical signs. Each female was weighed on days 0, 6, 9, 12, 15 and 20 of gestation. Feed consumption of each female was measured over a 1-day period on days 6, 9, 12, 15 and 20 of gestation. On the morning of day 20 of gestation, all dams were sacrificed and examined for gross abnormalities of the thoracic, abdominal and pelvic viscera. Corpora lutea were counted. Uteri were weighed, including contents, and examined for the number of implantations and the number and site of live fetuses and dead embryos/fetuses. Each live fetus was weighed and observed externally, including the oral cavity. The fetuses with external abnormalities were fixed in 10% formalin and not examined for skeletal or visceral abnormalities. Approximately one half of all externally normal fetuses from each litter were randomly selected, fixed in alcohol and stained with alizarin red S. All specimens were examined for skeletal abnormalities or variations and ossification. The remaining live fetuses were fixed in Bouin's solution and examined for visceral abnormalities or variations. Statistical analyses were performed using appropriate methods.

No adverse effects were observed in the dams throughout the study. Uterine weight was reduced in dams of the top-dose group. Increased fetal mortality was accompanied by decreases in the number of live fetuses and fetal weights at 300 mg/kg bw per day. Reddish material was reported in top-dose dams. No external abnormalities were observed at any dose level. An increase in cardiovascular abnormalities (i.e. ventricular septal defect), an increase in wavy ribs and curvature of scapula and a decrease in the number of ossified sacrococcygeal vertebral bodies were observed at 300 mg/kg bw per day (Table 10).

The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, based primarily on increased incidences of cardiac ventricular septal defects, wavy ribs and curvature of scapula and a decrease in the number of ossified sacrococcygeal vertebral bodies in the fetuses of rats. The maternal toxicity NOAEL was 300 mg/kg bw per day, the highest dose tested (Kawamura, 1991).

Apparently, the results of the study by Kawamura (1991) were also published in the open literature (Kawamura et al., 2014).

Rabbits

A pilot dose range–finding study was conducted in pregnant rabbits to determine appropriate doses for the oral teratology study. The study was not GLP compliant, but was quality assured. Flumioxazin technical (purity 94.8%) was administered to New Zealand White rabbits on days 7 through 19 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The test material was suspended in aqueous 0.5% (w/w) methyl cellulose and given via oral intubation at a dose of 0, 300, 500, 1000 or 1500 mg/kg bw per day to five or six rabbits per dose. Clinical observations were recorded at least twice daily during the dosing period and at least once daily during the post-dosing period. Body weights were recorded on day 0 and days 7 through 29 of gestation. Feed consumption was recorded daily. On day 29 of gestation, animals in all dose groups were sacrificed, caesarean sectioned and examined for gross lesions, number of corpora lutea, number and placement of implantation sites, early and late resorptions, and live and dead fetuses.

No deaths, abortions or premature deliveries occurred during this study. Doses of flumioxazin technical as high as 1500 mg/kg bw per day did not result in clinical or necropsy observations, nor did they affect maternal body weight gains or feed consumption values. Similarly, there were no adverse effects of doses of flumioxazin technical up to 1500 mg/kg bw per day on embryo/fetal viability, sex ratios, body weights or external morphology (Hoberman, 1991c).

Based on the results of the pilot study, groups of 20 pregnant New Zealand White rabbits were administered flumioxazin technical (purity 94.8%) at a dose of 0, 300, 1000 or 3000 mg/kg bw per day by oral gavage. The highest dose was well in excess of the 1000 mg/kg bw per day limit dose for developmental toxicity studies. The vehicle used was 0.5% methyl cellulose. The rabbits were artificially inseminated. Rabbits were administered the test material on days 7–19 of gestation. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. The rabbits were observed twice daily during the dosing period and once daily during the post-dosing period for clinical signs of toxicity, abortions, premature deliveries and deaths. Body weights were recorded on the day of insemination and daily during the dosing and post-dosing periods. The rabbits' feed consumption was measured daily, starting with the day of insemination and continuing through the dosing and post-dosing periods. On day 29 of gestation, all rabbits were sacrificed. Caesarean sections were performed, corpora lutea were counted and the uterine contents were examined for the number and placement of implantation sites, live and dead fetuses, early and late resorptions, and any abnormalities. All live fetuses were weighed, sexed and examined for external alterations. Live fetuses were sacrificed and examined for soft tissue alterations and subsequently were eviscerated, stained with alizarin red S and examined for skeletal alterations. Gross lesions in the fetuses and does were preserved in formalin. Adult tissues and carcasses that appeared normal were discarded. Statistical analyses were performed using appropriate methods.

The 3000 mg/kg bw per day dose tended to reduce maternal body weight gains and absolute and relative feed consumption values, which reached statistical significance at certain time ranges. No gross lesions were produced at any dose level. The 3000 mg/kg bw per day dose group litters tended to have reduced fetal weights, but these differences were not statistically significantly different. No fetal external, soft tissue or skeletal findings were attributable to the treatment with the test substance.

Based on the absence of adverse effects, the NOAEL for embryo and fetal toxicity was 3000 mg/kg bw per day, the highest dose tested. The maternal toxicity NOAEL was 1000 mg/kg bw per day, based on reductions in maternal body weight gains and absolute and relative feed consumption values (Hoberman, 1991d).

Apparently, parts of the results of the study by Hoberman (1991d) were also published in the open literature (Kawamura et al., 1995).

2.6 Special studies

(a) Neurotoxicity

Flumioxazin does not belong to a class of chemicals known to be associated with neurotoxicity, and no signs of neurotoxicity were reported in acute or repeated-dose toxicity studies. According to the sponsor, acute and subchronic neurotoxicity studies of flumioxazin were performed in order to comply with the USEPA's data requirements for registration. A delayed neurotoxicity study was not considered necessary, as flumioxazin has no organophosphate or organocarbamate structural groups. It is noted that the purity of the flumioxazin tested was greater than that of the technical material. The respective dose range–finding studies were not submitted by the sponsor, but were cited in the study reports.[1]

In an acute neurotoxicity study, flumioxazin (purity 99.6%) in aqueous 0.5% methyl cellulose was administered once via gavage at a dose of 0, 200, 700 or 2000 mg/kg bw to groups of young male and female adult rats (12 animals of each sex per group); treatment was followed by a 14-day observation period. A neurobehavioural test battery consisting of motor activity and functional observational battery assessments was conducted on all rats prior to test substance administration to obtain baseline measurements and then repeated again on study day 0 (approximately 8 hours post-administration) and on study days 7 and 14. On study day 15, all surviving rats underwent whole-body in situ perfusion under anaesthesia. Brain weights and brain dimensions (excluding olfactory bulbs) were recorded. Central and peripheral nervous system tissues from six rats of each sex in the control and 2000 mg/kg bw groups were processed for histopathological examination.

There were no deaths or clinical signs of toxicity. Mean body weights and body weight gains were unaffected by treatment. No treatment-related effects were observed in functional observational battery measurements or motor activity assessments on the day of dosing or subsequently during the observation period. A few statistically significant differences in brain weights and measurements (Table 11) were considered to be unrelated to dosing with flumioxazin, because they were within or very close to historical control ranges and, with the exception of male brain width measurements, showed no dose–response relationship. Furthermore, there were no associated morphological changes in the brain, and no similar finding was observed in the 90-day repeated-dose neurotoxicity study.

There were no further treatment-related effects on brain parameters or histopathological changes in the nervous system. In this study, the NOAEL for acute neurotoxicity of flumioxazin was 2000 mg/kg bw, the highest dose tested (Herberth, 2011a).

In a subchronic dietary neurotoxicity study, flumioxazin (purity 99.6%) was administered orally via the diet at a dose of 0, 500, 1500 or 4500 ppm over 90 days to groups of young male and female adult rats (12 animals of each sex per group). The mean daily intakes of flumioxazin over study days 0–91 were equal to 0, 37, 110 and 323 mg/kg bw per day for males and 0, 41, 124 and 358 mg/kg bw per day for females, respectively. A neurobehavioural test battery consisting of motor activity and functional observational battery assessments was conducted on all rats prior to test material administration to obtain baseline measurements, and the testing was repeated in weeks 3, 7 and 12 of dietary administration. Ophthalmic examinations were conducted before and near the end of the administration period. After completion of the final neurobehavioural test in week 13, all surviving animals underwent whole-body in situ perfusion under anaesthesia. Brain weights and brain dimensions (excluding olfactory bulbs) were recorded. Central and peripheral nervous system tissues

[1] Herberth MT (2010). An oral (gavage) dose range–finding acute neurotoxicity study of flumioxazin in rats. Unpublished report from WIL Research Laboratories, LLC, Ashland, OH, USA (Report no. WIL-194032).

Crittenden PL (2011). Flumioxazin – A 28-day dietary dose range–finding study in Sprague Dawley rats for immunotoxicity and neurotoxicity studies. Unpublished report from WIL Research Laboratories, LLC, Ashland, OH, USA (Report no. WIL-194034).

Table 11. Summary of brain weights and measurements with laboratory historical control data

	Control	200 mg/kg bw	700 mg/kg bw	2 000 mg/kg bw	HC mean (range)[a]
Males					
Weight (g)	1.97	2.05	2.03	2.01	2.0 (1.80–2.12)
Length (mm)	20.0	19.9	20.6*	20.3	20.9 (19.7–25.2)
Width (mm)	15.1	15.4*	15.5**	15.6**	15.1 (14.7–15.5)
Females					
Weight (g)	1.94	1.88	1.90	1.86*	1.9 (1.67–1.98)
Length (mm)	20.0	19.5	19.8	19.7	20.4 (19.2–24.3)
Width (mm)	15.1	15.0	15.0	14.9	14.7 (14.2–15.2)

bw: body weight; HC: historical control; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
[a] From studies conducted in 2004–2008.
Source: Herberth (2011a)

from six rats of each sex in the control and 4500 ppm groups were processed for histopathological examination.

An isolated mortality in the female high-dose group was considered not to be related to treatment with flumioxazin. Mean body weights and body weight gains were not consistently affected by treatment. Haematological changes characteristic of regenerative microcytic, hypochromic anaemia were observed at 500 ppm (males only), 1500 ppm (both sexes) and 4500 ppm (both sexes). The sponsor considered the haematological changes in males at 500 ppm as not adverse, because they were of low magnitude and were within the historical control range. However, as no details on the historical control data (e.g. underlying studies, dates of studies, individual data, age of animals) were provided and because of the clear dose–response relationship and the similarity to the findings in other studies, these findings in males at 500 ppm are considered to be adverse. No treatment-related effects were reported in ophthalmic, functional observational battery or motor activity assessments during the study. There were no consistent treatment-related effects on brain parameters or histopathological changes in the nervous system. There was no indication of neurotoxicity up to 4500 ppm.

Therefore, under the conditions of the study, the NOAEL for subchronic neurotoxicity was 4500 ppm (equal to 323 mg/kg bw per day for males and 358 mg/kg bw per day for females), the highest dose tested. Haematological changes were reported for all dose groups from 500 ppm (equal to 37 mg/kg bw per day) (Herberth, 2011b).

(b) Immunotoxicity

A study was performed to evaluate the potential immunotoxic effects of flumioxazin when administered orally via the diet to female Sprague-Dawley rats. The assessment of immunotoxicity was based primarily on the results of a splenic antibody-forming cell (AFC) assay to assess the T cell–dependent antibody response (TDAR) to sheep red blood cells (sRBCs). Groups of 15 animals were treated with diet containing flumioxazin (purity 99.6%) at 0, 500, 1500 or 4500 ppm (equal to 0, 42, 126 and 371 mg/kg bw per day, respectively) for 28 consecutive days. In addition, cyclophosphamide, a known immunosuppressant, was administered to a further group for 4 consecutive days (study days 24–27) via intraperitoneal injection at 50 mg/kg bw per day. Ten animals of each group received an intravenous immunization injection of sRBCs on study day 24 (TDAR groups). The remaining five animals from each group were used for haematological evaluation at the scheduled necropsy. On study day 28, all rats were euthanized. During the in-life period, all animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights were recorded twice weekly. Feed consumption and water consumption were recorded weekly. Serum samples for possible

immunoglobulin M (IgM) antibody analysis were collected from all rats in the TDAR groups at the scheduled necropsy. Complete necropsies were conducted on all animals, and the spleen, lymph nodes, bone marrow, adrenal glands, Peyer's patches and thymuses were collected. Spleen and thymus weights were obtained from all rats in the TDAR groups, and spleens were collected for the AFC analysis.

There were no test substance–related effects on survival, clinical observations, body weight, feed consumption, water consumption or macroscopic findings in any of the test substance–treated groups.

In the haematology groups, test substance–related alterations in haematology parameters (white blood cells, haemoglobin, haematocrit, mean cell volume [MCV], mean corpuscular haemoglobin [MCH], mean corpuscular haemoglobin concentration [MCHC], and reticulocyte, neutrophil and lymphocyte counts) were noted in the 1500 and/or 4500 ppm groups. Lower MCV and MCH values were noted in the 1500 and 4500 ppm groups when compared with the vehicle control group. Higher white blood cell count, absolute and per cent reticulocyte counts, and absolute neutrophil and lymphocyte counts and lower haemoglobin, haematocrit and MCHC values were also noted in the 4500 ppm group when compared with the vehicle control group.

In the TDAR groups, there were no test substance–related effects on thymus weights (absolute or relative) or spleen cell numbers in any of the treated groups. Higher absolute and relative spleen weights were noted for the 4500 ppm group.

There were no test substance–related effects on the AFC response, as measured by the AFC IgM specific activity (AFC/10^6 spleen cells) and total spleen activity (AFC/spleen), at any dose level tested. A higher AFC response was noted for the 500 ppm group; however, this result was not considered test substance related based on the presence of one high-responding individual in the 500 ppm group. In addition, statistically significant differences in specific and/or total spleen activity were not noted in the 1500 or 4500 ppm groups when compared with the vehicle control group.

Under the conditions of the study, haematological changes were reported at 1500 and 4500 ppm. No adverse effects on AFC response were reported up to the highest dietary concentration of 4500 ppm (equal to 371 mg/kg bw per day) (Crittenden, 2011).

(c) Mechanistic data regarding developmental toxicity in rats

Species differences were clearly evident in the developmental toxicity studies. Flumioxazin caused embryolethality, teratogenicity (e.g. ventricular septal defects, curvature of the scapula and ulna and wavy ribs) and growth retardation in rats at 30 mg/kg bw per day in the absence of maternal toxicity, but not in rabbits at the 100-fold higher and maternally toxic dose of 3000 mg/kg bw per day. A research programme was initiated by the sponsor to elucidate the reasons for the species differences and to support the assessment of the human relevance of these findings. The results of the submitted studies are presented below.

Critical period of embryonic sensitivity

To determine the critical period of embryonic sensitivity, pregnant rats were administered a single dose of flumioxazin (purity 94.8%) in 0.5% methyl cellulose by gavage at 400 mg/kg bw on 1 day of gestation beginning on day 11 through day 15. Groups of 4–5 animals were treated per gestation day. This 400 mg/kg bw dose was selected as one that would produce fetuses with ventricular septal defects following a single dose, without producing excessive fetal deaths. Rats were sacrificed on day 20, and uterine and fetal examinations were performed. Live fetuses were sexed, weighed, examined externally and fixed in Bouin's solution for examination for ventricular septal defects. The study was not GLP compliant (no authorized protocol, quality assurance unit not involved in any part of the study). GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, fetal weight reduction and incidence of ventricular septal defects (Kawamura, 1993).

Apparently, some of the results of the study by Kawamura (1993) were also published in the open literature (Kawamura et al., 1995).

Pathogenesis of developmental toxicity in rats

Flumioxazin (purity 94.8%) was given to groups of six female rats at a dose of 0, 3000 or 10 000 ppm in the diet for a maximum of 5 weeks. The effects of flumioxazin on the haematological system (haematological end-points, blood chemistry, urinary coproporphyrin, free erythrocyte protoporphyrin) were examined periodically to elucidate the mechanism by which it induces anaemia. The study was not conducted according to GLP principles and was not quality assured.

Throughout the treatment period, no deaths occurred, nor were there any treatment-related changes in body weight or gross pathology. Feed consumption decreased minimally at 3000 ppm and in the higher-dose groups, but this change was transient. The haematological changes at both dose levels included decreases in erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC and bone marrow myeloid/erythroid ratio and increases of siderocytes, erythroblasts and platelets. The neutrophils and reticulocytes decreased during the early treatment period, but increased thereafter. The urinary coproporphyrin and erythrocyte protoporphyrin levels were increased in the 3000 ppm group (but were not measured for the 10 000 ppm group). In the blood biochemistry tests, the abnormal findings in treated groups included increases in serum iron, total cholesterol, blood urea nitrogen, sodium and potassium levels, as well as decreases in aspartate aminotransferase activity and uric acid, calcium and triglyceride levels. Increased liver and spleen weights were observed in treated groups.

These findings suggest that flumioxazin-associated anaemia in rats can be classified as sideroblastic anaemia, resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis. Increased blood porphyrin suggested that flumioxazin induces porphyria in rats (Yoshida, 1996).

A study was designed to investigate the ontogeny and pathogenesis of a variety of developmental end-points in rat embryos and fetuses. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Flumioxazin (purity 94.8%) was administered to pregnant rats by gavage on day 12 of gestation at 400 mg/kg bw, and embryos/fetuses were removed from the uterus on days 13 through 20 of gestation (number of treated animals was eight on GD 13, 16 on GDs 14, 15, 17 and 20, and 18 on GD 16). A control group received vehicle only (number of treated animals was seven on GD 13 and eight on GDs 14, 15, 16, 17 and 20). Embryos/fetuses were examined externally for enlargement of heart and oedema of the whole body. One half of each litter was examined internally for closure of interventricular foramen of the heart. The other half was used to measure the number of red blood cells, haemoglobin and serum protein and was then examined for wavy ribs.

The indicators of developmental toxicity were noted remarkably on day 14, when treated embryos were observed to have enlarged heart, oedema and anaemia (decreased red blood cell count and haemoglobin). These effects were also observed on days 15 and 16 of gestation, after which the values for treated litters were similar to control values. Beginning on day 15 and continuing to day 20, mortality in the treated litters was increased. The mortality rate was relatively constant on GDs 17 and 20, indicating that all deaths occurred during the earlier period. This is consistent with the early resorptions observed in other developmental toxicity studies with flumioxazin. Closure of interventricular foramen began on day 16 of gestation in control fetuses (72.7% closed). Closure of interventricular foramen did not occur in any treated fetuses until day 16, and even then, the percentage with closure on day 17 was well below control values (89.7% control versus 23.9% treated). On day 20, the foramen of 95.2% of the control fetuses had closed, with only 57.7% of the treated fetuses reaching this milestone. Serum protein concentration was reduced on days 15 and 16 of gestation in treated animals and recovered by day 17 to values similar to control values. In treated litters, evidence of incomplete chondrification of the ribs was observed on day 16, delayed

ossification was observed on day 17, and wavy ribs and other skeletal findings (bent scapula, radius and ulna) were observed on day 20.

These data suggest that the enlarged heart, oedema and anaemia preceding the occurrence of the fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding (Kawamura, 1997a).

Comparative histopathological changes in rat and rabbit embryos

An examination of the histopathological changes in rat and rabbit embryos was undertaken for fetuses exposed to flumioxazin at the most sensitive point of fetal development. Pregnant rats and rabbits were administered flumioxazin (purity 94.8%) in 0.5% methyl cellulose at 1000 mg/kg bw on day 12 of gestation. Control animals received 0.5% methyl cellulose on day 12 of gestation. One to four animals were used per treatment regimen. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Rats were sacrificed at 6, 12, 24, 36 or 48 hours after treatment, and rabbits at 6, 24 or 48 hours. Embryos were examined externally and then subjected to examinations of umbilical blood smears and light or electron microscopic examination of tissues. Umbilical blood smears were stained with Berlin blue for detection of cellular iron. Sagittal or transverse sections of the thoraco-abdominal region of the fetuses were prepared and stained with haematoxylin and eosin for light microscopic examinations. Some sections with heart and liver were stained with Berlin blue and examined. The hearts and livers of some fetuses were prepared for examination by electron microscopy.

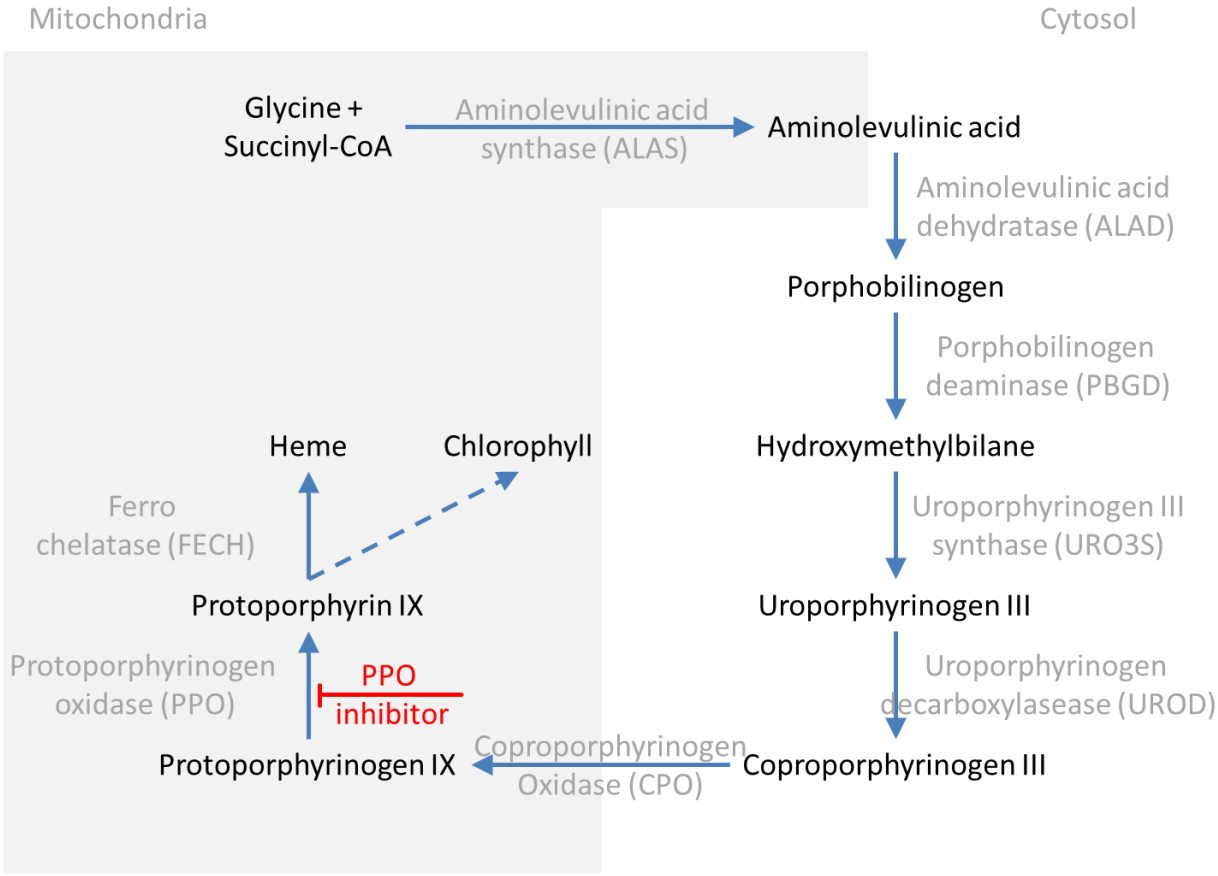
No embryonic deaths were observed in rats at 24 hours after treatment. The first intrauterine deaths were observed at 36 hours, and embryonic mortality increased to 93.2% at 48 hours after treatment. A primary effect was observed in circulating erythroblasts. Mitochondrial iron deposits in polychromatophilic erythroblasts, dilatation of the mitochondrial matrix in polychromatophilic erythroblasts at 6 hours post-dosing, and erythroblastic cell death evident after the appearance of the mitochondrial lesions were observed. These findings were suggested to reflect the observed anaemia in rat embryos. No histopathological signs in the embryonic rat heart were observed up to 24 hours after treatment, and no cell death in the embryonic rat heart was observed up to 48 hours after treatment, suggesting that no primary injury to the embryonic heart was produced by flumioxazin. Histological changes in the rat embryonic hearts at 36 or 48 hours after treatment included thin ventricular wall, poorly developed ventricular trabeculae, and hypoplasia of the muscular septum and endocardial cushions of the atrioventricular canal. In rat fetuses, dilatation of hepatic sinusoidal vessels was observed at 24 and 36 hours; at 48 hours, hepatic necrosis was reported. No treatment-related changes in the external appearance of embryos or intrauterine deaths were reported in rabbits. Likewise, no iron deposits in erythroblasts of rabbit embryos or histopathological changes similar to those produced in rat embryos were seen in the rabbit embryo (Kawamura & Yoshioka, 1997).

Apparently, parts of the results of the study by Kawamura & Yoshioka (1997) were also published in the open literature (Kawamura et al., 1996a).

Protoporphyrin IX accumulation

According to the sponsor, flumioxazin is a photo-bleaching agent whose herbicidal activity is considered to derive from inhibition of porphyrin biosynthesis, a key step in chlorophyll production in plants (Fig. 3).

Fig. 3. Haem and chlorophyll biosynthesis pathway



Source: Adapted from Ajioka, Phillips & Kushner (2006) and Nordmann & Puy (2002)

The sponsor stated that treatment of plants with flumioxazin resulted in the accumulation of protoporphyrin IX (PPIX) in plant cells, probably due to inhibition of PPO and auto-oxidation of protoporphyrinogen IX to PPIX. Porphyrin biosynthesis is common to plants and animals as part of chlorophyll and haem synthesis, respectively. Therefore, considering the biological activity of flumioxazin in plants and indications of haematotoxicity in rats in subchronic and chronic toxicity studies, mechanistic studies were conducted examining the accumulation of PPIX in animal embryos exposed to flumioxazin.

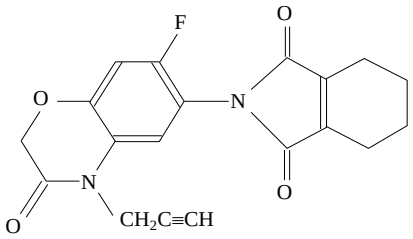
To determine whether a species difference exists in accumulation of PPIX in rat and rabbit embryos, pregnant rats and rabbits were administered a single oral dose of 1000 mg/kg bw flumioxazin in 0.5% methyl cellulose, and the concentrations of PPIX in embryos and maternal livers were measured with a high-performance liquid chromatographic (HPLC) method at 2, 6, 12, 18 and 24 hours after administration for rats and at 2, 6, 12, 24 and 48 hours after administration for rabbits. Two to four rats and 2–3 rabbits were used per treatment regimen. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). The concentrations of PPIX in rat embryos and maternal livers were much higher than those in the corresponding tissues of the rabbit, with peak concentrations observed at 12 hours for embryos (concentration change from pretreatment to maximum concentration; rats: 0.15 versus 19 µg/g tissue; rabbits: not detected versus 0.1 µg/g tissue) and 6–12 hours for maternal livers (rats: 0.1 versus approximately 1.3 µg/g tissue; rabbits: 0.05 versus 0.22 µg/g tissue) (Kawamura, 1996a).

Apparently, parts of the results of the study by Kawamura (1996a) were also published in the open literature (Kawamura et al., 1996b).

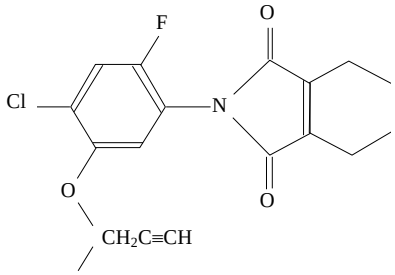
To allow further comparisons, a selection of structurally related chemicals was included in the investigations: S-23121 and S-23031 (chemical structures are depicted in Fig. 4).

Fig. 4. Chemical structures of the three related chemicals (flumioxazin, S-23121 and S-23031) of the N-phenylimides family

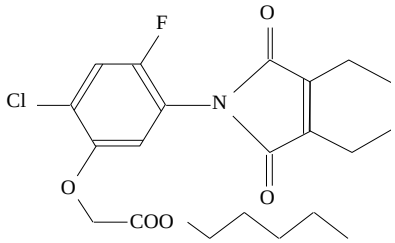
Flumioxazin



S-23121



S-23031



Source: Holmes (2014)

Both flumioxazin (Kawamura, 1990a) and S-23121 (Kawamura, 1990b) were found to produce the same pattern of developmental toxicity in rats at a dose level of 10 or 20 mg/kg bw per day, respectively, with no evidence of developmental effects when tested in rabbits (S-23121: highest dose tested was 15 mg/kg bw per day, Hoberman, 1990; flumioxazin: Hoberman, 1991d). The maternal NOAEL in rabbits treated with S-23121 was 2 mg/kg bw per day, based on abortions and decreased body weight, body weight gain and feed intake at 4 mg/kg bw per day and above. S-23031, however, did not produce maternal or developmental toxicity, even when tested up to 1500 mg/kg bw per day in rats (Lemen, 1991a), nor did it produce developmental toxicity when tested up to 800 mg/kg bw per day in rabbits, a dose that produced maternal toxicity (mortality) with a NOAEL of 400 mg/kg bw per day (Lemen, 1991b).

Apparently, parts of the results of the studies by Kawamura (1990b), Hoberman (1990) and Lemen (1991a,b) were also published in the open literature (Kawamura et al., 2013).

To investigate whether PPIX accumulation in rat embryos was different for the three similar compounds, pregnant rats were administered flumioxazin, S-23121 or S-23031 at 1000 mg/kg bw on day 12 of gestation, and PPIX accumulation was measured 14 hours later in maternal livers and in embryos with an HPLC method. The 14-hour time period approximates the peak PPIX accumulation time point reported previously. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). Both flumioxazin

and S-23121 induced PPIX accumulation to a similar extent in rat embryos, about 250 times that of control fetuses (approximately 25 µg/g versus 0.095 µg/g tissue). The PPIX concentration in maternal livers of animals treated with flumioxazin or S-23121 was approximately 3 times that observed in livers of control animals (0.7–0.8 µg/g versus 0.236 µg/g). The PPIX concentration in embryos of S-23031-treated rats was similar to the value for control embryos, whereas the concentration in maternal livers was slightly higher than the control value (0.379 µg/g versus 0.236 µg/g) (Kawamura, 1996b).

To investigate the possible link between developmental toxicity and PPO inhibition, the critical period for PPIX accumulation, presumably resulting from PPO inhibition, in rat and rabbit embryos and maternal livers was investigated. Pregnant rats and rabbits (3–5 animals per group) were administered a single oral gavage dose of flumioxazin (purity 94.8%) at 400 mg/kg bw (rats) or 1000 mg/kg bw (rabbits) at 19:30 on 1 day of gestation from day 10 through 15. A control group of pregnant rats received 0.5% methyl cellulose in the same dosing scheme. The study lacked control animals for rabbits. The study was not conducted according to GLP principles (no authorized protocol, quality assurance unit not involved in any part of the study). PPIX was extracted from embryos and maternal livers 14 hours after treatment and quantified with an HPLC method. The most sensitive period for inducing the highest accumulation of PPIX in rat embryos was treatment with flumioxazin at 19:30 on GD 11 to 19:30 on GD 12. PPIX accumulation was not observed at any developmental stage in the rabbit. Accumulation of PPIX was not observed in maternal rat or rabbit liver at the doses used in this study. However, reported results for rabbits need to be evaluated with caution, taking into account the missing control animals (Kawamura, 1997b).

Apparently, parts of the results of the study by Kawamura (1997b) were also published in the open literature (Kawamura, Kato & Fantel, 2014).

Species differences in the accumulation of PPIX due to the inhibition of PPO by flumioxazin were investigated in vitro using cryopreserved primary hepatocytes from female rats, rabbits, monkeys and humans. The study was not conducted according to GLP principles (the report contained neither GLP nor quality assurance statements). Cells were thawed and seeded at 0.5×10^6 cells/mL in a 24-well plate for approximately 24 hours. Flumioxazin was dissolved in dimethyl sulfoxide (DMSO) and added at final concentrations of 0, 0.01, 0.03, 0.1 and 0.3 µg/mL. No fewer than six replicates of each concentration were prepared, with each experiment conducted in triplicate for each lot of hepatocytes. Following exposure to flumioxazin for 24 hours, the medium was removed, and cells were harvested and processed for potential PPIX accumulation using analysis by liquid chromatography/mass spectrometry (LC/MS). The concentration of PPIX was expressed relative to protein amount. The basal PPIX concentration in rat hepatocytes was 293 pg/mg protein and was dose-dependently increased by the addition of flumioxazin, with approximately a 10-fold increase above the basal level when cells were exposed to 0.3 µg/mL of flumioxazin. The basal PPIX concentration in rabbit hepatocytes was similar to that in the rat; however, the PPIX concentration was not increased in rabbit hepatocytes when exposed to flumioxazin. A similar effect was seen in monkey hepatocytes: whereas the basal PPIX concentration was much lower (42 pg/mg protein), no accumulation of PPIX was observed when monkey hepatocytes were exposed to flumioxazin. In all three batches of human hepatocytes, basal PPIX levels were lower than those of the rat and rabbit, and the mean PPIX concentration of the three lots in the control samples was 180 pg/mg protein. An approximate 4.4-fold increase in mean PPIX concentration was observed following exposure to flumioxazin up to 0.3 µg/mL. In two of the three human hepatocyte samples, the increase was approximately 6-fold (with individual experiments reaching 10-fold increases).

In conclusion, the induction ratios of PPIX following treatment with flumioxazin at 0.3 µg/mL were 10.3-, 1.1-, 1.4- and 4.4- to 6-fold in primary hepatocytes of rat, rabbit, monkey and human, respectively. When judging the validity of the experiments, it should be taken into account that in the report, no data on the viability of the hepatocytes were given, and the interindividual variability was not evaluated for rat, rabbit or monkey samples. Human hepatocyte samples were taken from Caucasian women 64, 38 and 68 years of age (Abe, 2011a).

Table 12. Mean IC_{50} values for test chemicals in PPO assays

	Mean (range) IC_{50} values (µmol/L)						
	Rat liver			Rabbit liver			Human liver
Chemical	**Study 1 Green & Dabbs (1993)**	**Study 2 Green & Dabbs (1996)**	**Study 3 Noda (1995)**	**Study 1 Green & Dabbs (1993)**	**Study 2 Green & Dabbs (1996)**	**Study 3 Noda (1995)**	**Study 2 Green & Dabbs (1996)**
Flumioxazin	0.008 08 (0.005 62–0.010 9)	0.007 15 (0.005 69–0.009 50)	0.023	0.051 9 (0.019 8–0.069 9)	0.138 (0.076 4–0.220)	0.30	0.017 3 (0.012 2–0.020 5)
S-23121	0.010 8 (0.009 75–0.011 8)	–	0.036	1.56 (0.540–2.57)	–	0.690	–
S-23031	0.793 (0.212–1.27)	–	2.23	4.75 (0.413–9.06	–	12.50	–

IC_{50}: median inhibitory concentration; PPO: protoporphyrinogen oxidase

Inhibition of protoporphyrinogen oxidase

Inhibition of PPO activity by flumioxazin and two chemically related compounds in rat, rabbit and human liver mitochondria. As discussed previously, PPO is a key enzyme in haem synthesis, catalysing the transformation of protoporphyrinogen IX to PPIX, which proceeds to formation of protohaem (see Fig. 3 above). Three in vitro studies were initiated to determine whether flumioxazin would inhibit PPO activity in adult liver and/or whole embryos. They were not conducted according to GLP principles or quality assured. In the first study, flumioxazin and two chemically related compounds (S-23121 and S-23031; see Fig. 4 for structures) were tested for their effects on PPO activity in adult liver, day 12 embryos and day 15 embryos of rats and rabbits. Viable and metabolically active mitochondria were prepared from each of these tissues and incubated with substrate, protoporphyrinogen and selected concentrations of each test chemical (flumioxazin: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L; S-23121: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L; S-23031: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/L).

The mean relative potency for inhibition of PPO from mitochondria of all tissues was flumioxazin > S-23121 >> S-23031 (Table 12). A difference in sensitivity between the two species was observed, with rat tissue exhibiting a greater sensitivity (100-fold) to PPO inhibition by the test chemicals compared with rabbit tissues. Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test chemicals (Green & Dabbs, 1993).

Because rat mitochondria from adult liver showed properties similar to those of embryonic tissue in the prior study, only adult liver samples were used in the two following studies. The effect of flumioxazin on PPO activity in adult female human liver samples, in addition to specimens from rats and rabbits, was investigated. Mitochondria were prepared from the livers of adult female rats, rabbits and humans (six Caucasian donors, aged 15–71 years). The inhibition of PPO activity was studied in freshly prepared mitochondria. The final test concentrations covered a broad range when tested in specimens from humans (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/L) or from rats and rabbits (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L).

The calculated median inhibitory concentration (IC_{50}) values are shown in Table 12. The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit. The mean IC_{50} for human liver PPO was 0.0173 ± 0.0044 µmol/L. The IC_{50} determined in the current study for rat liver mitochondria agreed very closely with that determined in the previous study

(0.007 15 ± 0.0021 µmol/L compared with 0.008 08 ± 0.0027 µmol/L). The IC_{50} for flumioxazin to rabbit liver PPO determined in the present study was slightly higher than the values obtained previously (0.138 ± 0.0739 µmol/L compared with 0.0519 ± 0.028 µmol/L). IC_{50} values in human samples were 2.4-fold higher than those determined in rat samples. Interindividual differences in the enzyme kinetic parameters K_m (Michaelis-Menten constant) and V_{max} (maximum velocity) were in the range of 4- to 6-fold, respectively, in liver samples from humans (Green & Dabbs, 1996).

The third study (Noda, 1995) examined the effects of flumioxazin and the other two structurally related chemicals on PPO activity in adult female rat and rabbit liver. IC_{50} values are summarized in Table 12. Whereas the individual experiments by Noda (1995) were done in triplicate, they were not reproduced in a second experiment. The experiments by Green & Dabbs (1993, 1996) were repeated, to gain a total of three experiments each with triplicate determinations.

Although there were some differences in absolute values between studies, the relative potency for inhibition of PPO from mitochondria of all tissues was flumioxazin > S-23121 > S-23031 (Green & Dabbs, 1993). The relative sensitivity of the species to PPO inhibition by flumioxazin was rat (0.007 15 µmol/L) > human (0.0173 µmol/L) > rabbit (0.138 µmol/L) or rat (1) > human (2.4) > rabbit (19.3) (Green & Dabbs, 1996). The relative relationship of PPO inhibition between the three species tested is rat > human > rabbit, with the two agents (flumioxazin and S-23121) that produced developmental effects in rats both producing the greatest inhibition of PPO (Noda, 1995).

Inhibition of PPO activity by flumioxazin and its major metabolites in rat liver mitochondria. The PPO IC_{50} was determined for flumioxazin and its major metabolites – 3-OH-flumioxazin, 4-OH-flumioxazin and APF – in rat liver mitochondrial fraction in a non-GLP study. The assay was conducted in a 96-well format. Serial dilutions of the test substances were made in DMSO (or DMSO alone as a vehicle control) and were aliquoted in duplicate along with diluted mitochondrial solution into wells. To correct for the auto-oxidation rate of PPIX, heat-inactivated mitochondria (denatured at 80 °C for 15 minutes) were also assayed in parallel with the samples. Following preincubation and the addition of protoporphyrinogen IX, the 96-well plate was loaded into a plate reader, and changes in fluorescence (excitation wavelength, 410 nm; emission wavelength, 630 nm) derived from the produced PPIX were measured automatically for 60 minutes at 1-minute intervals. The results suggest that flumioxazin had the highest PPO inhibitory activity among the four substances tested, followed by 3-OH-flumioxazin and 4-OH-flumioxazin (Table 13). APF had only slight inhibitory activity against PPO up to 100 µmol/L (below 20%) in this study; therefore, no IC_{50} value could be determined (Abe, 2011b).

Table 13. Results for PPO inhibition by flumioxazin and its major metabolites

	Flumioxazin	**3-OH-flumioxazin**	**4-OH-flumioxazin**	**APF**
IC_{50} (µmol/L)	0.004 55	0.062 5	0.667	ND
Fold change relative to flumioxazin	1	13.7	147	ND

ND: not determined
Source: Abe (2011b)

In vitro studies for effects of flumioxazin on haem synthetic pathway and cell proliferation in human and rat cells

Effects of flumioxazin on haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effect of flumioxazin on the haem synthetic pathway in the human K562 cell line (derived from a patient with chronic myeloid leukaemia in the acute phase); this cell line can differentiate into erythroid cells by treatment with sodium butyrate. The study was not conducted according to GLP principles. K562 cells were plated at 1×10^5 cells/mL per well

(24-well plate) in RPMI medium containing sodium butyrate and flumioxazin (dissolved in DMSO). Flumioxazin was examined in the presence of sodium butyrate at concentrations of 0, 0.01, 0.1, 1 and 5 µmol/L. Cell densities were determined on days 2, 4, 6 and 8 (following subculturing at day 4); PPIX and haem content were determined at the same intervals using LC/MS or liquid chromatography with ultraviolet (UV) detection, respectively. The effect of flumioxazin treatment on K562 cells in the absence of sodium butyrate was not reported. No positive control substances were included in the investigations.

In conclusion, PPIX accumulation in K562 cells at 1 µmol/L and above was observed in a dose-dependent manner, with no effect on cell proliferation or haem synthesis up to the highest dose of 5 µmol/L (Kawamura, 2012a).

Effects of metabolites of flumioxazin on haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effects of selected metabolites of flumioxazin (3-OH-flumioxazin, 4-OH-flumioxazin and APF) on the haem synthetic pathway in K562 cells. The study was not conducted according to GLP principles. K562 cells were plated onto 6 cm dishes at 5×10^5 cells/5 mL per dish in RPMI medium containing flumioxazin or its metabolites at a concentration of 5 µmol/L and sodium butyrate. Test substances and sodium butyrate were dissolved in DMSO. Cell densities were determined on days 2, 4, 6 and 8 (following subculturing at day 4), with PPIX and haem content determined at the same intervals using LC/MS or LC/UV detection, respectively. The effects of the treatment with flumioxazin or metabolites on K562 cells in the absence of sodium butyrate were not reported. No positive control substances were included in the investigations. A possible dose–response relationship was not determined.

In conclusion, flumioxazin and the tested metabolites had no effect on haem synthesis or proliferation of K562 cells. PPIX was increased in K562 cells treated with flumioxazin, but this was not observed with any of the tested metabolites (Kawamura, 2012b).

Effects of flumioxazin on haem synthetic pathway and cell proliferation in rat erythroleukaemia cells. The inhibition of haem synthesis by flumioxazin and accumulation of PPIX were investigated in a study with a rat erythroleukaemia (REL) cell line (derived from transplantable tumours from 7,12-dimethylbenz(*a*)anthracene-induced erythroleukaemia in the Long-Evans rat) that had been induced to differentiate into an erythroid form by treatment with hexamethylenebisacetamide (HMBA). The study was not conducted according to GLP principles. REL cells were suspended in RPMI medium containing flumioxazin (at 5, 1, 0.3, 0.1, 0.01 or 0 µmol/L) and HMBA (both were dissolved in DMSO), plated onto 60 mm dishes at 5×10^5 cells/5 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until determination of haem and PPIX content. On days 2, 4 and 6, the cells were also subcultured at a concentration of 1×10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/UV and LC/MS, respectively. The effect of flumioxazin treatment on REL cells in the absence of HMBA was not reported. No positive control substances were included in the investigations.

There was no effect on cell proliferation up to the highest dose of 5 µmol/L. PPIX accumulated in cells at 0.1 µmol/L and above in a dose-dependent manner from day 2. The accumulation of PPIX reached a maximum at day 4. Haem synthesis was inhibited in REL cells at 0.01 µmol/L and above in a dose-dependent manner from day 4. The inhibition of haem synthesis reached a maximum at day 6 (Kawamura, 2013a).

Effects of flumioxazin on haem synthetic pathway and cell proliferation in human CD36+ cells. The inhibition of haem synthesis by flumioxazin and accumulation of PPIX were investigated in a study with human CD36+ cells, which were derived from human cord CD34+ cells in culture. The study was not conducted according to GLP principles. Human CD36+ cells were cultured in haematopoietic progenitor growth medium supplemented with stem cell factor, erythropoietin,

interleukin-3 (IL-3) and IL-6 to induce differentiation into erythroid cells, which were then treated with flumioxazin (at 5, 1, 0.1, 0.01 or 0 µmol/L, dissolved in DMSO), plated onto 30 mm dishes at 6 × 10^5 cells/3 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until measurement. On days 2, 4 and 6, the cells were also subcultured at a concentration of 2 × 10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/UV and LC/MS, respectively. The effect of flumioxazin treatment on cells in the absence of the differentiation-inducing agents was not reported. No positive control substances were included in the investigations.

PPIX was accumulated slightly in human erythroidal CD36+ cells at 0.1 µmol/L and clearly at 1 µmol/L and above in a dose- and time-dependent manner. There was no effect on cell proliferation and haem synthesis up to the highest dose of 5 µmol/L (Kawamura, 2013b).

Inhibition of PPO activity by flumioxazin and its major metabolites in human liver mitochondria. The PPO IC_{50} was determined for flumioxazin and selected metabolites 3-OH-flumioxazin, 4-OH-flumioxazin and APF in human liver mitochondria in a non-GLP study. Human microsomes were a mix derived from five donors, but no further details (e.g. age, sex or genetic background/race) were included in the report. The assay was conducted in a 96-well format. Serial dilutions of the test substances were made in DMSO (or DMSO alone as a vehicle control, 10-fold spacing) and were aliquoted in duplicate along with diluted mitochondrial solution into wells. To correct for the auto-oxidation rate of PPIX, heat-inactivated mitochondria (denatured at 80 °C for 15 minutes) were also assayed in parallel with the samples. Following preincubation and the addition of protoporphyrinogen IX, the 96-well plate was loaded into a plate reader, and changes in fluorescence (excitation wavelength, 410 nm; emission wavelength, 630 nm) derived from the produced PPIX were measured automatically for 60 minutes at 1-minute intervals.

The results suggest that the PPO inhibitory activity of the two metabolites (3-OH-flumioxazin and 4-OH-flumioxazin) was weaker than that of the parent compound, flumioxazin (Table 14). An IC_{50} value for APF was not obtained in this study. APF inhibited PPO activity by approximately 40% up to 100 µmol/L. Only transformed values were given in the report (Abe, 2014).

Table 14. IC_{50} values for the inhibition of human liver mitochondrial PPO by flumioxazin and selected metabolites

	Flumioxazin	3-OH-flumioxazin	4-OH-flumioxazin	APF
IC_{50} (µmol/L)	0.021 ± 0.003 7	0.104 ± 0.019 4	0.893 ± 0.402 4	> 100

Source: Abe (2014)

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in K562 cells. The objective of this study was to investigate the effects of flumioxazin and dihydroartemisinin (DHA), which causes fetal anaemia, leading to a similar pattern of developmental toxicity in rats, on the haem synthetic pathway in K562 cells. The study was not conducted according to GLP principles. K562 cells were plated onto 6 cm dishes at 5 × 10^5 cells/5 mL per dish in RPMI medium containing flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L) and sodium butyrate. Test substances were dissolved in DMSO. Cell densities were determined on days 2, 4, 6 and 8, with PPIX and haem content determined at the same intervals using LC/MS or LC/UV detection, respectively.

PPIX accumulation in K562 cells was observed with flumioxazin; however, there was no effect on cell proliferation or haem content per cell. DHA caused no accumulation of PPIX in K562 cells, but caused a reduction of haem content and a severe inhibition of cell proliferation at the highest concentration of 2 µmol/L, but not at lower concentrations. The impact of inhibition of cell proliferation on the results is unclear (Kawamura, 2015b).

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in rat erythroleukaemia cells. The objective of this non-GLP study was to investigate the effects of flumioxazin and DHA on the haem synthetic pathway in REL cells. REL cells were induced to differentiate into erythroid cells using HMBA. REL cells were plated onto 6 cm dishes at 5×10^5 cells/5 mL per dish in RPMI medium containing flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L) and HMBA. Cell densities were determined on days 2, 4, 6 and 8. On days 2, 4 and 6, the cells were subsequently subcultured at a concentration of 1×10^5 cells/mL in new dishes at 5 mL/dish. At 2 µmol/L DHA, the number of cells was too low to subculture at a concentration of 1×10^5 cells/mL on days 4 and 6, so 1 mL of test medium was added after 1 mL of sampling. PPIX and haem content were determined using LC/MS or LC/UV detection, respectively.

PPIX accumulation and reduction of haem content per cell were observed with flumioxazin; however, there was no effect on cell proliferation. DHA caused no accumulation of PPIX in REL cells, but caused a reduction of haem content and inhibition of cell proliferation at concentrations of 0.5 and 2 µmol/L. At the concentration of 2 µmol/L, cell proliferation was practically completely inhibited. The impact of this high level of inhibition of cell proliferation on the results is unclear (Kawamura, 2015a).

Comparative effects of flumioxazin and dihydroartemisinin on the haem synthetic pathway and cell proliferation in human CD36+ cells. The inhibition of haem synthesis by flumioxazin and DHA and accumulation of PPIX were investigated in a study with human CD36+ cells. The study was not conducted according to GLP principles. Human CD36+ cells were cultured in haematopoietic progenitor growth medium supplemented with stem cell factor, erythropoietin, IL-3 and IL-6 to induce differentiation into erythroid cells, which were then treated with flumioxazin (5 µmol/L) or DHA (2, 0.5, 0.125 or 0 µmol/L, both dissolved in DMSO), plated into 30 mm dishes at 6×10^5 cells/3 mL per dish. The seeding day was defined as day 0. Control cell suspension at day 0 was analysed for haem and PPIX content. The cells were counted on days 2, 4, 6 and 8, after which cells were stored at −80 °C until measurement. On days 2, 4 and 6, the cells were also subcultured at a concentration of 2×10^5 cells/mL. Concentrations of haem and PPIX were analysed using LC/MS and LC/UV, respectively.

PPIX accumulation in human CD36+ cells was observed with flumioxazin; however, there was no effect on cell proliferation or haem content per cell. DHA caused no accumulation of PPIX in human CD36+ cells, but caused a reduction of haem content and inhibition of cell proliferation at ≥ 0.125 µmol/L and ≥ 0.5 µmol/L, respectively. At the concentrations of 0.5 and 2 µmol/L, cell proliferation was practically completely inhibited. The impact of such high levels of inhibition of cell proliferation on the results is unclear (Kawamura, 2015c).

Haematological investigations in developing rats

Additional study to evaluate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. A study was designed to investigate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. A previous study demonstrated that flumioxazin induced embryo/fetal lethality and ventricular septal defects when administered at 30 mg/kg bw per day on GDs 6–15. This study was conducted to assess whether anaemia also occurs in the embryo at this dose. The study was not conducted according to GLP principles. Mated female Sprague-Dawley rats (20 per dose) were gavaged with flumioxazin at 0 (vehicle, 0.5% aqueous methyl cellulose), 15, 30 or 60 mg/kg bw per day on days 6–15 of gestation. Embryos from 10 dams per group were examined on day 14 of gestation to identify embryonic parameters related to anaemia; fetuses from another 10 dams per group were examined on day 20 of gestation to confirm fetal lethality and ventricular septal defects. Dams showed lower body weight and body weight gain at 60 mg/kg bw per day on GD 20, but not at earlier time points. Dose-dependent anaemia was confirmed in GD 14 embryos from dams dosed at 15, 30 and 60 mg/kg bw per day. Anaemia was characterized by pale yolk sacs and pale embryos, histopathologically decreased erythroblast content in the heart, iron deposits in erythroblasts and degenerative erythroblasts. Most of these findings showed a dose–

response increase in incidence and severity. Embryo/fetal lethality (and fewer live fetuses) was observed at 60 mg/kg bw per day (increased rate of late postimplantation loss at GD 14 and of early postimplantation loss at GD 20). Slightly increased incidences of ventricular septal defects were reported at 15 mg/kg bw per day and clearly at 30 and 60 mg/kg bw per day. Increased incidence and severity of thinning of ventricular walls and of atrium dilatation were reported in fetuses at 30 and 60 mg/kg bw per day. Fetal livers showed dilatation of sinusoidal vessels and hepatocytic necrosis in the peripheral region of moderate severity at 60 mg/kg bw per day. Lower fetal body weights were reported at 60 mg/kg bw per day and to a slight extent also at 30 mg/kg bw per day.

In summary, adverse findings were reported in fetuses in all dose groups starting at the lowest dose of 15 mg/kg bw per day (Hosokawa, 2015).

Chronological changes of morphology and population of circulating erythroblasts in rat embryos during yolk sac haematopoiesis. The differentiation of circulating erythroblasts in rat embryos (Crl:CD(SD)) was investigated at daily intervals from embryonic development day 11 through day 14 in a non-GLP study. Timed pregnant females (three per time point) were anaesthetized in the morning of the scheduled necropsy, and uteri and embryos were removed. Embryos were exsanguinated from severed umbilical cord, and embryonic blood cells from the same litter were pooled. On day 11 of embryonic development, more than 95% of circulating erythroblasts were basophilic erythroblasts. From day 12 to 13 of embryonic development, the predominant cell population was the polychromatophilic erythroblast (approximately 95%). At day 14, the population of polychromatophilic erythroblasts was markedly reduced (approximately 8%), with the orthochromatophilic erythroblasts as the predominant cell type (approximately 90%). Additionally, enucleated reticulocyte populations were detected.

The results of this study show that differentiation of circulating erythroblasts in rat embryos from embryonic days 11 to 14 was synchronized (Ihara, 2011).

Placental transfer studies

The placental transfer of flumioxazin was investigated in rats and mice, and in rats and rabbits, in two GLP-compliant studies. Data indicate that flumioxazin does cross the placenta and that both parent and various metabolites are present in measurable quantities in the fetus (Isobe, 1992, 1993). At the same dose level, the concentrations of ^{14}C and of flumioxazin are higher in rat fetuses than in rabbit fetuses, but lower than in the mouse (Isobe, 1993). No clear pattern of absorption, distribution, metabolism or excretion was evident that could account for the species differences in developmental toxicity observed in rats and rabbits.

Rats at day 12 of gestation and mice at day 10 of gestation were given a single oral dose of [phenyl-^{14}C]flumioxazin (for chemical structure, see Fig. 1) at 30 mg/kg bw (dissolved in corn oil). Concentrations of ^{14}C in maternal tissues and the fetus were examined at 1, 2, 4, 8, 24 and 72 hours after administration, and the excretion of ^{14}C into faeces and urine during 24 hours (mice) or 72 hours (rats) after administration was examined. Groups of four animals per time point were treated. Metabolites in excreta, blood cells, plasma, liver and fetus (mice only) were analysed. Metabolites were separated by thin-layer chromatography using several different mobile phases, the retention times were compared with synthetic reference standards and spots were detected using autoradiography. The metabolites were quantified by liquid scintillation counting.

Concentrations of ^{14}C in maternal tissues reached maxima more rapidly and decreased more rapidly in mice than in rats. Concentrations of ^{14}C in the fetus reached maxima 1 hour after administration for both species and decreased rapidly thereafter, with half-lives of 14 and 5 hours for rats and mice, respectively. Maximum ^{14}C concentrations were 1.05 and 1.72 ppm for rats and mice, respectively. ^{14}C was almost completely excreted within 3 and 1 days for rats and mice, respectively. The total ^{14}C excretion was 95.7% (faeces, 74.7%; urine, 21.0%) for rats and 95.8% (faeces, 72.9%; urine, 22.9%) for mice. The metabolism of flumioxazin seemed to be qualitatively the same in pregnant rats and mice. However, it was suggested that 3-hydroxylation activity might be higher in

pregnant rats than in pregnant mice. The major metabolite in the fetus of mice at 1 hour after administration was 4-OH-flumioxazin (Isobe, 1992).

A single oral dose of [phenyl-^{14}C]flumioxazin at 30 mg/kg bw was administered to pregnant rats (four animals per time point) and rabbits (two animals per time point) on day 12 of gestation. Maternal tissues and fetuses were obtained at 1, 2, 4 and 24 hours after dosing for analysis of ^{14}C concentrations and identification of metabolites in bone marrow, fat, fetus, kidney, liver, ovary, placenta, spleen, uterus and blood. After 24 hours, the ^{14}C excretion was found to be much slower in rabbits (30.2%: urine, 12%; faeces, 18.3%) than in rats (76.6%: urine, 21.7%; faeces, 54.9%). Concentrations of ^{14}C in the fetus, amniotic fluid and maternal tissues were higher in the rat than in the rabbit, with liver and kidney accounting for the highest concentrations in each species. Maximum ^{14}C concentrations in maternal tissues were higher than those observed in the fetus or amniotic fluid in both species. Maximum ^{14}C concentrations in the fetus were 0.782 and 0.2 ppm for rat and rabbit, respectively, observed at 4 hours after dosing for both species. For those time points, where sufficient fetal tissue was available for metabolite identification (1 and 24 hours for rats and 2 and 24 hours for rabbits), the highest concentration of parent flumioxazin was 0.06 ppm (at 1 hour after dosing) and 0.02 ppm (at 2 hours after dosing) in fetal tissue for rats and rabbits, respectively. Ratios between the ^{14}C concentrations in fetuses and those in maternal plasma were 0.21–0.26 in rats and 0.09–0.14 in rabbits (Isobe, 1993).

Pharmacokinetics and metabolism in rats and rabbits, and human PBPK model development

The pharmacokinetics of ^{14}C-labelled flumioxazin was investigated in groups of three pregnant rats and pregnant rabbits after repeated oral administration. Non-fasted pregnant rats and pregnant rabbits were administered ^{14}C-labelled flumioxazin at 30 mg/kg bw per day for 7 days by oral gavage from GDs 6 to 12. The study was not conducted according to GLP and was not quality assured. Concentrations of radioactivity in blood and plasma, excretion in urine and faeces, tissue concentrations, tissue distribution and transfer to female reproductive tissues were investigated. Blood, plasma and excreta were obtained daily 2 and 24 hours post-dosing. To complete a time course, blood and plasma were also collected 2, 4, 6, 8 and 24 hours after the final administration. Tissues were also collected at 7 and 24 hours (rats) or 3 and 24 hours (rabbits) after the final dose. Tissues collected included kidney, liver, spleen, fat, ovaries, placenta, uterus and fetus, along with amniotic fluid, to determine the concentration of radioactivity. Concentrations of flumioxazin and its metabolites were determined in pooled samples of urine, faeces, plasma, blood cells, liver, fetus and amniotic fluid.

Flumioxazin was excreted rapidly in pregnant rabbits (urine and faeces) and pregnant rats (faeces).

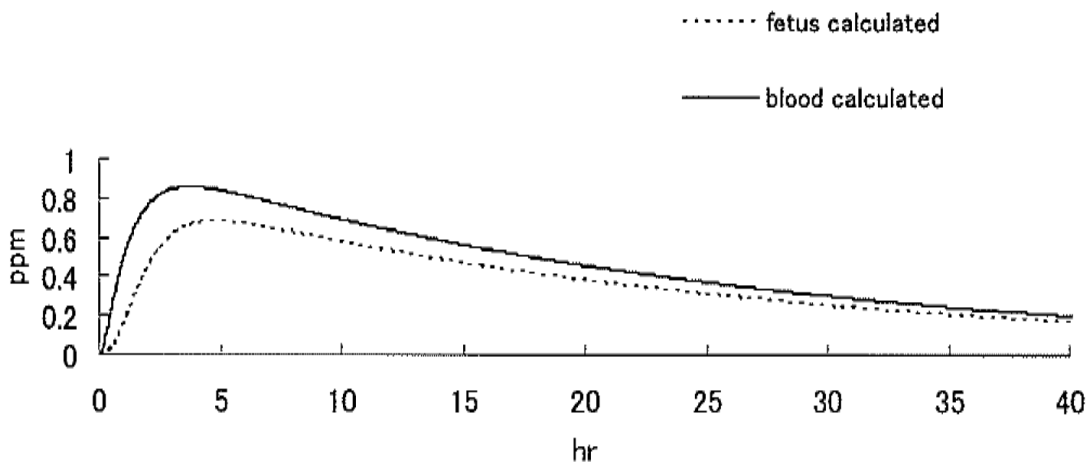
The concentration of radioactivity in blood and plasma almost reached steady state on day 4 and day 2, respectively, for rats. After the final administration to rats (day 7), the concentration of radioactivity in plasma increased gradually to reach 4.49 µg eq/mL at 8 hours after administration and then decreased to 1.71 µg eq/mL at 24 hours after administration, whereas the concentrations in both blood and plasma of rabbits did not reach steady state within the 7-day dosing period. After the final administration to rabbits on day 7, the concentration of radioactivity in plasma increased gradually to reach 4.19 µg eq/mL at 4 hours after administration, and then decreased gradually to 2.24 µg eq/mL 24 hours after administration. In rats, flumioxazin was distributed at higher concentrations in all tissues, except for the uterus, amniotic fluid and fetus, compared with plasma. In rabbits, flumioxazin was distributed at lower concentrations in all tissues, except for liver and kidney, than in plasma when compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma. Flumioxazin, APF, 3-OH-flumioxazin, 4-OH-flumioxazin, 3-OH-flumioxazin-SA, 4-OH-flumioxazin-SA and Ac-APFA were detected in excreta and tissues of pregnant animals and in fetuses. (An exception to this was for 3-OH-flumioxazin-SA and 4-OH-flumioxazin-SA in the fetuses of pregnant rabbits.) Concentrations of flumioxazin in the plasma and

blood cells of pregnant rats were generally similar to the concentrations in fetuses and amniotic fluid. In rat fetuses, 1.14 and 0.73 µg/g of radioactivity were detected after 7 and 24 hours, respectively. The concentration of flumioxazin in fetuses of rabbits was half that in rat fetuses: 0.32 and 0.20 µg/g of radioactivity were detected after 3 and 24 hours, respectively. When amniotic fluid of rabbits was analysed, no flumioxazin was detected. Tissue concentrations of flumioxazin and most metabolites were highest in the liver of pregnant rats compared with other tissues in the rat and compared with tissue concentrations in the rabbit. Whereas APF was identified at higher concentrations than other metabolites in most tissues of rats and in rabbit liver, it should be noted that most of the radioactivity in the evaluated rat and rabbit samples could not be identified (Shirai, 2009).

The biliary excretion of [phenyl-^{14}C]flumioxazin was investigated in female rats to determine oral absorption rate. Five bile duct–cannulated female rats received a single oral dose of [phenyl-^{14}C]flumioxazin at 1000 mg/kg bw. Recovery of ^{14}C in the bile, urine and faeces was measured during 0–72 hours. The study was not conducted according to GLP and was not quality assured. At termination, ^{14}C remaining in the gastrointestinal tract and the carcass was measured. Data from the three rats surviving to study termination were analysed (two rats died before the scheduled kill). The results demonstrated that most of the dosed radiocarbon was eliminated in the faeces and accounted for 84.7% of the dose by 72 hours post-dosing. The ^{14}C excretion in urine and bile accounted for 6.8 ± 1.9% and 5.2 ± 4.1%, respectively. In addition, residues remaining in the contents of the gastrointestinal tract and the residual carcass accounted for 0.6% and 0.3%, respectively. Total ^{14}C recoveries accounted for 97.6% of the dose. The majority of the administered compound was excreted within 24–48 hours. The absorption of [phenyl-^{14}C]flumioxazin following a single oral dose of 1000 mg/kg bw was calculated to be 12.3 ± 2.6% (amount in urine, bile and carcass; Takaku, 2012a).

A physiologically based pharmacokinetic (PBPK) model was developed to predict parent flumioxazin concentrations in the blood and fetus of pregnant humans based on data obtained in the rat. This work was not conducted according to GLP and was not quality assured. An in vitro metabolism study using rat and human liver microsomes was carried out to determine any species differences in the metabolism of flumioxazin between rat and human. Human microsomes were pooled from eight males and seven females, but no further details were included in the report (e.g. age, sex or genetic background/race). Physiological data for humans were cited from the literature, and the human model was developed to predict the pharmacokinetics in humans in several tissues. Although it is impossible, experimentally, to measure human fetal concentrations of flumioxazin, development of the PBPK model in the pregnant rat was scaled to humans to provide an estimate of the disposition of flumioxazin in pregnant humans. The metabolites produced by human microsomes were nearly identical to those produced by female rat liver microsomes; it was therefore concluded that there was no species difference in the metabolism of [phenyl-^{14}C]flumioxazin. The parameters for the rat PBPK model were manually optimized to reproduce the blood (predicted maximum concentration: approximately 0.08 ppm), liver (predicted maximum concentration: approximately 2.5 ppm) and fetus (predicted maximum concentration: approximately 0.06 ppm) concentrations of flumioxazin after a single oral dose of 30 mg/kg bw reported by Isobe (1992) and using 50% fraction absorbed (the values needed to be estimated from the figures in the study report, because the respective data were not reported). As no other experimental data were available for flumioxazin, the model could not be tested. The rat model was extrapolated to the pregnant human based on human physiological data and the in vitro metabolism data. The fraction of dose absorbed at 1000 mg/kg bw in rats was 12%. These values were then combined to develop the PBPK model at a dose of 1000 mg/kg bw. Simulated blood and fetal concentrations of flumioxazin at an oral dose of 1000 mg/kg bw in pregnant women were estimated to be 0.86 ppm and 0.68 ppm (maximum concentrations), respectively (Fig. 5). The predicted concentration in human liver was not reported. The part describing the in vitro metabolism results in the study report is too short to allow a proper evaluation of the results. AUC as a further toxicokinetic parameter was not reported (Takaku, 2012b).

Fig. 5. Predicted blood and fetal concentrations of flumioxazin at the dose of 1000 mg/kg bw in pregnant human



Source: Takaku (2012b)

Apparently, parts of the results of the study by Takaku (2012b) were also published in the open literature (Takaku, Nagahori & Sogame, 2014).

Discussion on human relevance of the developmental effects induced by flumioxazin in rats

Flumioxazin caused embryolethality, teratogenicity (especially ventricular septal defects) and growth retardation in rats at 30 mg/kg bw per day without maternal toxicity, but not in rabbits at the maternally toxic dose of 3000 mg/kg bw per day. The sponsor carried out a programme of research with flumioxazin to elucidate the mechanism of the developmental toxicity observed in rats. The assessment of the proposed mode of action for the induction of ventricular septal defects and of its relevance for humans is presented in Appendix 1.

(d) Literature review

A literature review was commissioned by the sponsor to assess published information on flumioxazin to support the European Union Plant Protection Product Regulation renewal procedure. The review focused on literature published between 1999 (the date of first inclusion into Annex I of the previous Plant Protection Product Directive) and the end of 2011, when the review was conducted. Information was sought that was relevant to the OECD data requirements for toxicological and toxicokinetic studies, residues in or on treated products (food and feed), fate and behaviour in the environment, and ecotoxicological studies. Where information with possible pertinence to other data requirements within the dossier was brought up as part of this search, this too was reviewed. A number of search terms were used separately or in conjunction across a range of databases sufficient to assess the entirety of literature available. Following a rapid assessment of the results that were obtained, 15 papers were identified as requiring detailed assessment (two for toxicology, four for environmental fate and nine for ecotoxicology). Following a detailed assessment, it was concluded that none of the papers had an impact on the human health risk assessment for flumioxazin (Hall, 2012).

A further literature search was commissioned by the sponsor to identify information published after 1 January 2005 on 1) substances inducing ventricular septal defects and 2) reports on the relationship between PPO (or porphyrinogen or porphyrin) and ventricular septal defects (Pharis, 2015). This was accomplished by four different search statements, which were used to query several relevant databases (e.g. Medline, CAplus and Biosis previews). In total, two articles were identified. One was not relevant for the intended question (a case report about a calf with ventricular septal

defect). The other article (Aasa et al., 2014) was included in Appendix 1 in the discussion of the human relevance of ventricular septal defects.

(e) Metabolites reported in residues

Some metabolites were reported to occur in residues in plant or animal metabolism studies. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported to be major residues in goat and poultry matrices when animals were evaluated with a relatively short post-administration period.

Besides limited mechanistic in vitro data, no toxicological data on these metabolites were submitted. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were observed in rats in amounts above 10%, when further downstream metabolites observed in toxicokinetic studies are included.

Flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported as Cramer class III (modules: Cramer rules; Cramer rules, with extensions), when evaluated with Toxtree (v. 2.6.13; Patlewicz et al., 2008).

When evaluated with the module "in vitro mutagenicity (Ames test) alerts by ISS" (Benigni & Bossa, 2011; Benigni, Bossa & Tcheremenskaia, 2013), flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin raised alerts for *Salmonella typhimurium* mutagenicity. It is noted that the same groups led to the alerts in all three chemicals; in the available studies, however, flumioxazin was unlikely to be genotoxic in vivo.

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

Manufacturing records of flumioxazin technical material and the health status of workers engaged in the manufacturing operations were reviewed. Flumioxazin technical material was manufactured at factories in Japan during 2000–2010. During this period of operation, production volume rose from 50 to approximately 700 tonnes per year. During this time, 14–15 workers were engaged in the manufacture of flumioxazin. Under Japanese law, every company employee is required to undergo annual medical examinations. These examinations include investigation of medical, work, smoking and medication histories; presence/absence of subjective and/or objective symptoms; body measurements (height, weight, abdominal circumference, body mass index); eyesight and audibility; chest X-ray examination; blood pressure measurement; haematology (red and white blood cell counts, haematocrit, haemoglobin content, platelet count); liver function test (aspartate transaminase, alanine transaminase, gamma-glutamyltranspeptidase); blood lipid examination (total cholesterol, low-density/high-density lipoprotein cholesterol, triglyceride); blood sugar and haemoglobin A1c; electrocardiogram; urine analysis (sugar, protein, occult blood, urobilinogen); and renal function test (urea nitrogen, creatinine, uric acid). At the manufacturing site of flumioxazin technical material, no adverse health effects have been reported from the results of the annual medical examinations (Nishioka, 2011).

3.2 Variegate porphyria

The disease variegate porphyria is a disorder of hepatic haem biosynthesis, which is seen in humans carrying autosomal dominant mutations in the gene for PPO (Gross, Hoffmann & Doss, 2000; Nordmann & Puy, 2002; Ajioka, Phillips & Kushner, 2006; Phillips & Anderson, 2010). At least 100 mutations have been identified. These mutations can inhibit the function of the enzyme, which catalyses one of the last steps in the haem biosynthesis pathway of protoporphyrinogen IX to PPIX (see Fig. 3 above). Most haem synthesized in liver is required for cytochrome P450 synthesis. Patients with this disease present with neurovisceral symptoms and/or blistering skin lesions induced by photosensitivity. In faeces, coproporphyrin III and protoporphyrin levels are increased. A characteristic marker for variegate porphyria would be fluorescence maximum in plasma at

approximately 626 nm. The risks for chronic hypertension, renal disease and hepatocellular carcinoma are increased. Unrelated individuals with the same mutation may present with different clinical phenotypes.

According to published literature, there are several cases with heterozygous variegate porphyria, but only a few cases with the homozygous type. The latter cases are often of higher severity.

A 6-year-old boy and his 8-month-old sister were diagnosed with homozygous variegate porphyria based on analysis of plasma, urine and faeces for porphyrins and precursors and measurement of PPO activity. They had serious photodermatosis and ocular nystagmus. Both children were mentally retarded (Korda et al., 1984).

According to a case report, homozygous variegate porphyria might be associated with retarded growth and/or dwarfism. One adult patient had marked retarded bone age in X-rays of the hands (Murphy et al., 1986).

According to another case report, a boy diagnosed with homozygous variegate porphyria showed, in addition to severe skin lesions, unusually short fingers and toes (Mustajoki et al., 1987):

> Motor development was slower than usual: the patient learnt to sit at 10 months and to walk at 18 months, and was clumsy in his movements. His EEG [electroencephalogram] at the age of three years was slightly abnormal, with occasional slow waves and irregularity but without focal findings. In psychological tests mental development and speech were found to be normal. His rate of growth has followed a curve 1 SD [standard deviation] below the mean values for weight and height.

A 14-year-old girl was described as mentally subnormal and attending a special school. Her "skin of face, backs of hands and knees was dry with crusts, erosions, milia and multiple superficial scars.…Perioral radial scarring was present, as was clinodactyly and brachydactyly of the fifth fingers and flexion deformities of other fingers." She was diagnosed with homozygous variegate porphyria (Norris, Elder & Hawk, 1990).

In a further report (Hift et al., 1993), the case of a 6-year-old South African girl diagnosed with homozygous variegate porphyria was described:

> born to non-consanguineous parents, developed severe blistering of the face and hands within days of birth.…She has never developed symptoms of the acute porphyric attack. She has markedly foreshortened, stubby fingers and toes, resulting in severe functional impairment such that she cannot grasp a pencil or use a pair of scissors. Radiographs demonstrate brachydactyly with broad, short metacarpals and phalanges, and a delayed bone age. She has severe skin disease, manifested by blistering, erosions, crusting, pigmentation and milia. The lesions have a wider distribution than those of heterozygous VP [variegate porphyria]. In addition to typical blistering and scarring of sun-exposed areas, the skin in non-exposed areas of the arms, legs and trunk is thickened and infiltrated, and clearly abnormal.…She has severe myopia and a pendular nystagmus. Neurological development is delayed. Her perceptual and motor development are about one year behind the expected stage of development. Verbal skills are appropriate for age and she appears to be of normal intelligence. Since the age of 3 she has suffered infrequent complex partial seizures; an electroencephalogram was, however, normal. Over the [previous] 2 years she has developed a gross sensory neuropathy of the hands and feet, manifesting with painless trauma to the hands. Electrophysiological studies of nerve conduction have demonstrated a reduced amplitude of conduction in the right and left median sensory nerves in keeping with a sensory neuropathy. No motor abnormalities were demonstrable. Sural nerve conduction studies were normal, yet abnormalities were shown on histological examination of a sural nerve biopsy specimen.

Regarding another case, it was stated (Hift et al., 1993) that a

> male infant, born to consanguineous parents, developed epilepsy at 5 months and showed developmental delay, nystagmus and clinodactyly. Skin manifestations were noted from the age of 6 months. He presented to a dermatology clinic at the age of 6 with blistering and fragility of the skin of his hands, face and ears. Examination revealed hypertrichosis, hyperpigmentation, scarring and milia of these areas.

A 7-year-old Chilean boy had severe photosensitivity at the age of 6 months and also blisters and erosions developing recurrently. At the age of 7 years, pronounced scarring in sun-exposed body areas was additionally described, as well as a short stature (2 standard deviations below reference height) and shortening of fingers. He attended school regularly and did not reveal mental retardation or any neurological disturbances. Via polymerase chain reaction analysis, he was diagnosed with homozygous variegate porphyria (Poblete-Gutiérrez et al., 2006).

When the genes for PPO in five patients with homozygous variegate porphyria were sequenced, two had homoallelic mutations (from consanguineous parents), and three had heteroallelic mutations (from unrelated parents) (Roberts et al., 1998).

Porteous (1963) described a women with idiopathic porphyria delivering a live girl but developing a psychotic state with transfer to a mental hospital afterwards. She showed signs of severe peripheral neuritis and renal failure. Six years later, she was pregnant a second time, delivering in week 36 a live boy who died after 1 day due to atelectasis. The third pregnancy resulted in early delivery of a healthy girl in week 37. Following each pregnancy/delivery, the mother's condition was aggravated, but could be treated.

Porteous (1963) summarized the knowledge on pregnancy in patients with porphyria:

> Neilson and Neilson (1958) analyzed 40 cases of porphyria complicated by pregnancy which were reported in the world literature. Ninety-five per cent of these deteriorated during pregnancy and the overall maternal mortality was 42.5 per cent. Only 60.5 per cent of the pregnancies proceeded to foetal viability and in those which did there was a foetal mortality of 63 per cent. One of the 40 patients had the erythropoietic or congenital form of porphyria where the defect is in the bone marrow. The other 39 patients suffered from the hepatic form of porphyria. Most of the latter were affected by the acute intermittent type of hepatic porphyria but one patient had the chronic cutaneous type of hepatic porphyria, and four patients suffered from the mixed type of hepatic porphyria.

Similarly, Dean (1953) described "during pregnancy the patient is usually much worse, perhaps partly because she is then given sedatives. There is often a history that previous pregnancies were terminated because of pains, vomiting, and hysteria." However, it is not totally clear to which types of porphyria the findings relate, but a relevance for variegate porphyria cannot be excluded. Other authors concluded that pregnancy does not influence the course of variegate porphyria, nor would variegate porphyria influence pregnancy (Bloch, 1965).

Hift et al. (1993) summarized the symptoms in the known cases of patients with homozygous variegate porphyria:

> It is an interesting observation that all the parents of the cases described thus far appear to have been latent carriers as none are reported to have expressed VP clinically. A review of the eight reported cases shows them, with one exception, to be remarkably similar. Both sexes are affected and the condition presents with photosensitivity within days to months of birth. Many have been mentally retarded. Delayed development is common and most will suffer seizures. Nystagmus may be noted. Structural abnormalities of the hands are frequently reported, ranging from clinodactyly to severe deformity. Growth retardation and a retarded bone age are frequent and perhaps invariable.

The inhibition of PPO would be similar to the herbicidal mechanism of action proposed by the sponsor.

Comments

Biochemical aspects

Experiments in bile duct–cannulated rats given 1 mg/kg bw orally showed that absorption of flumioxazin was at least 80%, with similar amounts in urine and bile (Gibson et al., 1997). Radioactivity in the urine of rats given [^{14}C]flumioxazin was 31–43% at the low dose (1 mg/kg bw) and 13–23% at the high dose (100 mg/kg bw). Flumioxazin was widely distributed. Radioactivity was detected mainly in excretory organs and was eliminated rapidly, showing that flumioxazin and its

metabolites do not accumulate in tissues. Radioactivity in faeces of rats given [^{14}C]flumioxazin was 56–72% at the low dose and 78–88% at the high dose. Excretion was rapid, with 69–87% of the dose being eliminated in urine and faeces within 24 hours of dosing. Urinary excretion of radioactivity was higher in females than in males in all groups, and pretreatment with unlabelled flumioxazin had little effect on the route or rate of excretion (Matsunaga, 1993; Shiba, 1994).

Flumioxazin was metabolized extensively. Seven of the 35 metabolites detected and quantified when dosing with [phenyl-^{14}C]flumioxazin were identified, and 10 of the 29 metabolites detected and quantified when dosing with [phthalimide-^{14}C]flumioxazin were identified. The main metabolic reactions were hydroxylation of the cyclohexene ring of the tetrahydrophthalimide moiety, cleavage of the imide linkage, cleavage of the amide linkage in the benzoxazine ring, reduction of the double bond in the tetrahydrophthalimide ring, acetylation of the amino group of the aniline derivative and incorporation of a sulfonic acid group in the tetrahydrophthalimide ring (Matsunaga, 1993; Shiba 1994).

Toxicological data

Flumioxazin was not acutely toxic to rats, with no mortality at limit doses after oral (LD_{50} > 5000 mg/kg bw; Hiromori, 1990a) or dermal (LD_{50} > 2000 mg/kg bw; Hiromori, 1990b) exposure. In rats exposed to flumioxazin via inhalation, an LC_{50} greater than 3.93 mg/L was reported (Kawaguchi, 1990). Flumioxazin was not irritating to the skin (Nakanishi, 1989) and caused only minimal eye irritation in rabbits (Nakanishi, 1989). It was not a skin sensitizer in a maximization test (Nakanishi, 1990).

Short-term toxicity studies were conducted in the mouse, rat and dog. In all three species, liver was a target organ. Anaemia was also observed in the rat.

In a 13-week study, mice received flumioxazin in the diet at a concentration of 0, 100, 1000, 3000 or 10 000 ppm (equal to 0, 16.3, 164, 459 and 1062 mg/kg bw per day for males and 0, 18.6, 202, 595 and 2163 mg/kg bw per day for females, respectively). The NOAEL was 3000 ppm (equal to 459 mg/kg bw per day), based on increased liver weight and decreased ovary weight at 10 000 ppm (equal to 1062 mg/kg bw per day) (Hagiwara, 1990).

In a 90-day study in rats, flumioxazin was offered to groups of rats at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 2.28, 20.7, 69.7 and 244 mg/kg bw per day for males and 0, 2.21, 21.7, 71.5 and 230 mg/kg bw per day for females, respectively). The NOAEL was 300 ppm (equal to 20.7 mg/kg bw per day), based on changes in haematological and clinical chemistry parameters at 1000 ppm (equal to 69.7 mg/kg bw per day) (Hagiwara, 1989).

In a second 90-day study in rats, flumioxazin was offered to animals at a dietary concentration of 0, 30, 300, 1000 or 3000 ppm (equal to 0, 1.9, 19.3, 65.0 and 197 mg/kg bw per day for males and 0, 2.2, 22.4, 72.9 and 218 mg/kg bw per day for females, respectively). The NOAEL was 30 ppm (equal to 2.2 mg/kg bw per day), based on haematological changes (including anaemia and extramedullary haematopoiesis) in females at 300 ppm (equal to 22.4 mg/kg bw per day), although the effects were marginal. For males, the NOAEL was 300 ppm (equal to 19.3 mg/kg bw per day), based on increases in liver, heart, kidney and thyroid weights at 1000 ppm (equal to 65.0 mg/kg bw per day) (Adachi, 1991).

In dog studies, flumioxazin was administered in gelatine capsules to groups of dogs of both sexes at a dose of 0, 10, 100 or 1000 mg/kg bw per day for 90 days (Nakano, 1993) or for 1 year (Nakano, 1992). The NOAELs for both the 90-day and 12-month studies were 10 mg/kg bw per day, based on increases in total cholesterol and phospholipid levels and elevated alkaline phosphatase activity at 100 mg/kg bw per day in the 90-day study and similar changes (elevated alkaline phosphatase activity and increased liver weights) at 100 mg/kg bw per day in the 12-month study.

In a 78-week study in mice, flumioxazin was administered at a dietary concentration of 0, 300, 3000 or 7000 ppm (equal to 0, 31, 315 and 754 mg/kg bw per day for males and 0, 37, 346 and

859 mg/kg bw per day for females, respectively). The incidence of centrilobular hepatocyte hypertrophy was increased in males receiving 3000 and 7000 ppm; at these doses, there was an increase in diffuse hypertrophy and single-cell necrosis of hepatocytes in females. The NOAEL was 300 ppm (equal to 37 mg/kg bw per day), based on non-neoplastic changes in the liver in females. In this study, no increases in tumour incidence were reported (Seki, 1993a).

In a 24-month rat study, flumioxazin was administered at a dietary concentration of 0, 50, 500 or 1000 ppm (equal to 0, 1.8, 18 and 36.5 mg/kg bw per day for males and 0, 2.2, 21.8 and 43.6 mg/kg bw per day for females, respectively). At the highest dose tested (1000 ppm), anaemia was the most significant toxicological finding; it was also apparent in rats receiving 500 ppm flumioxazin. The NOAEL was 50 ppm (equal to 1.8 mg/kg bw per day), based on an increase in anaemia. Under the conditions of the study, tumour incidence was unaffected by treatment (Seki, 1993b).

The Meeting concluded that flumioxazin is not carcinogenic in mice or rats.

Flumioxazin was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. Flumioxazin was negative in in vitro studies (Kogiso, 1989; Wollny, 2011), but resulted in an increase in chromosomal aberrations (Kogiso, 1988). However, there was no genotoxic activity in follow-up in vivo studies (Hara & Kogiso, 1988; Hara, 1990; Kogiso, 1990).

The Meeting concluded that flumioxazin is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and the absence of carcinogenicity in mice and rats, the Meeting concluded that flumioxazin is unlikely to pose a carcinogenic risk to humans from the diet.

A two-generation reproductive toxicity study in rats was conducted with flumioxazin using dietary concentrations of 0, 50, 100, 200 and 300 ppm (during the pre-mating period, equal to 0, 3.2, 6.3, 12.7 and 18.9 mg/kg bw per day for males and 0, 3.8, 7.6, 15.1 and 22.7 mg/kg bw per day for females, respectively). The NOAEL for parental toxicity was 200 ppm (equal to 12.7 mg/kg bw per day), based on clinical signs of toxicity and reductions in body weight, body weight gain, feed consumption and organ weights. The reproductive NOAEL was also 200 ppm (equal to 12.7 mg/kg bw per day), based on reduced gestation index in both the F_0 and F_1 generations and on an increase in the number of F_0 dams that did not deliver a litter. The NOAEL for offspring toxicity was 50 ppm (equal to 3.2 mg/kg bw per day), based on increased postnatal pup mortality in the F_1 generation (Hoberman, 1992).

Flumioxazin was administered by gavage to groups of pregnant rats at a dose of 0, 1, 3, 10 or 30 mg/kg bw per day during organogenesis (days 6 through 15). The NOAEL for embryo and fetal toxicity in rats was 3 mg/kg bw per day, based on an increased incidence of malformations (cardiac ventricular septal defects) in the fetuses at 10 mg/kg bw per day. The NOAEL for maternal toxicity was 30 mg/kg bw per day, the highest dose tested (Kawamura, 1990a).

Studies have been conducted to elucidate the mechanism of formation of ventricular septal defects reported in rats as a possible consequence of PPO inhibition. GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, reductions in fetal weight and the incidence of ventricular septal defects (Kawamura, 1993). Effects were seen in offspring within hours after administration (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997; Hosokawa, 2015) and progressed through the development of the pups/fetuses (Kawamura, 1997a). In studies comparing effects seen in rats and in rabbits, fewer or no effects were seen in rabbits (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997).

Flumioxazin inhibits a key enzyme, PPO, in the rat, thereby interfering with normal haem synthesis (Green & Dabbs, 1993, 1996; Noda, 1995; Abe, 2011a, 2014). Available in vitro investigations in specimens and cell lines from different species indicated (slightly) higher sensitivity of rats compared with rabbits or humans (Green & Dabbs, 1993, 1996; Noda, 1995; Abe, 2011a; Kawamura, 2012a, 2013a,b, 2015a,b,c).

The disease variegate porphyria is a disorder of hepatic haem biosynthesis, which is seen in humans carrying autosomal dominant mutations in the gene for PPO (Gross, Hoffmann & Doss, 2000; Nordmann & Puy, 2002; Ajioka, Phillips & Kushner, 2006; Phillips & Anderson, 2010). Children with homozygous variegate porphyria were reported to exhibit various findings, such as photosensitivity, retarded mental development, delayed development, seizures, nystagmus or structural abnormalities of the hands (Hift et al., 1993).

The sponsor proposed a case that the induction of ventricular septal defects in rats is not relevant for humans. However, the Meeting considered that the case is not robust enough to support the mode of action in rats and to demonstrate the non-relevance for humans.

Pregnant rabbits were administered flumioxazin by oral gavage at 0, 300, 1000 or 3000 mg/kg bw per day on days 7−19 of gestation. The NOAEL for embryo and fetal toxicity was 3000 mg/kg bw per day, the highest dose tested. The NOAEL for maternal toxicity was 1000 mg/kg bw per day, based on reductions in maternal body weight gains and feed consumption (Hoberman, 1991d).

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

In an acute neurotoxicity study, flumioxazin was administered once via gavage at a dose of 0, 200, 700 or 2000 mg/kg bw to groups of rats of both sexes. The NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested, based on the absence of specific neurotoxic effects (Herberth, 2011a).

In a subchronic neurotoxicity study, flumioxazin was administered to groups of rats via the diet at a dose of 0, 500, 1500 or 4500 ppm (equal to 0, 37, 110 and 323 mg/kg bw per day for males and 0, 41, 124 and 358 mg/kg bw per day for females, respectively) for 90 days. The NOAEL for subchronic neurotoxicity was 4500 ppm (equal to 323 mg/kg bw per day), the highest dose tested. Haematological changes were reported for all dose groups from 500 ppm (equal to 37 mg/kg bw per day) (Herberth, 2011b).

The Meeting concluded that flumioxazin is not neurotoxic.

In a 28-day immunotoxicity study in female rats, groups of animals were treated with diet containing 0, 500, 1500 or 4500 ppm flumioxazin (equal to 0, 42, 126 and 371 mg/kg bw per day, respectively). No adverse effects on antibody-forming cell response were reported up to 4500 ppm (equal to 371 mg/kg bw per day), the highest dose tested. Haematological changes were reported from 1500 ppm (equal to 126 mg/kg bw per day) (Crittenden, 2011).

The Meeting concluded that flumioxazin is not immunotoxic.

Toxicological data on metabolites and/or degradates

Some metabolites were reported to occur as residues in plant metabolism studies. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported to be major residues in goat and poultry matrices when animals were evaluated after a relatively short post-administration period.

Besides mechanistic in vitro data on PPO inhibition, no toxicological data were submitted on some of these metabolites. The metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were observed in rats in amounts above 10%, when further downstream metabolites observed in toxicokinetic studies are included. Flumioxazin and the metabolites 4-OH-flumioxazin and 3-OH-flumioxazin were reported as Cramer class III (modules: Cramer rules; Cramer rules, with extensions), when evaluated with Toxtree (v. 2.6.13; Patlewicz et al., 2008).

Acute and chronic exposures to 3-OH-flumioxazin and 4-OH-flumioxazin (up to 9 µg/day) are below the threshold of toxicological concern (TTC) for Cramer class III compounds (90 µg/day). Hence, no safety concern is anticipated.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted (Nishioka, 2011). No information on accidental or intentional poisoning in humans is available.

The Meeting concluded that the existing database on flumioxazin 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 0–0.02 mg/kg bw on the basis of a NOAEL of 1.8 mg/kg bw per day for anaemia in a long-term study in rats, with application of a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.03 mg/kg bw on the basis of a NOAEL of 3 mg/kg bw per day for malformations in a developmental toxicity study in rats, with application of a safety factor of 100. This ARfD applies to women of childbearing age only.

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

Levels relevant to risk assessment of flumioxazin

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	300 ppm, equal to 37 mg/kg bw per day	3 000 ppm, equal to 346 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 754 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 1.8 mg/kg bw per day	500 ppm, equal to 18 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 36.5 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	200 ppm, equal to 12.7 mg/kg bw per day	300 ppm, equal to 18.9 mg/kg bw per day
		Parental toxicity	200 ppm, equal to 12.7 mg/kg bw per day	300 ppm, equal to 18.9 mg/kg bw per day
		Offspring toxicity	50 ppm, equal to 3.2 mg/kg bw per day	100 ppm, equal to 6.3 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	30 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	3 mg/kg bw per day	10 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day	3 000 mg/kg bw per day
		Embryo and fetal toxicity	3 000 mg/kg bw per day[b]	–
Dog	Ninety-day and 1-year studies of toxicity[d,e]	Toxicity	10 mg/kg bw per day	100 mg/kg bw per day

[a] Dietary administration.

[b] Highest dose tested.
[c] Gavage administration.
[d] Gelatine capsule administration.
[e] Two or more studies combined.

Estimate of acceptable daily intake (ADI)

0–0.02 mg/kg bw

Estimate of acute reference dose (ARfD)

0.03 mg/kg bw (applies to women of childbearing age)

Unnecessary (for the general population)

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

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

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

Absorption, distribution, excretion, and metabolism in mammals	
Rate and extent of oral absorption	> 80% in the rat
Dermal absorption	Not given
Distribution	Widely distributed
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Rapidly excreted, 30−40% via urine and 60% via faeces within 7 days
Metabolism in animals	Extensively metabolized; hydroxylation of cyclohexene ring and cleavage of the imide linkage
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.93 mg/L air (maximal attainable concentration)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Minimally irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman test)
Short-term studies of toxicity	
Target/critical effect	Haematotoxicity (rat), liver (mouse, rat)
Lowest relevant oral NOAEL	2.2 mg/kg bw per day (90 d rat)
Lowest relevant dermal NOAEL	300 mg/kg bw (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematotoxicity (rat), liver (mouse)

Lowest relevant oral NOAEL	1.8 mg/kg bw per day (2-year rat)
Carcinogenicity	Not carcinogenic in mice or rats[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	Impairment of reproductive capacity at dose levels associated with systemic toxicity; increased postnatal pup mortality in the absence of parental toxicity
Lowest relevant parental NOAEL	12.7 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	3.2 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	12.7 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Malformations (ventricular septum defects)
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	3 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	323 mg/kg bw per day (highest dose tested; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Mechanism studies	Mechanistic studies related to developmental toxicity in rats
Studies performed on metabolites or impurities	In vitro studies with metabolites were performed within the mechanistic studies for the proposed mode of action for developmental toxicity in rats
Immunotoxicity NOAEL	371 mg/kg bw per day (highest dose tested; rat)
Medical data	
	No evidence of adverse health effects attributed to flumioxazin during manufacturing operations reported

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

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year toxicity study (rat)	100
ARfD[a]	0.03 mg/kg bw	Developmental toxicity study (rat)	100

[a] Applies to women of childbearing age only; no ARfD necessary for the general population.

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Appendix 1. Mode of action analysis using the WHO/IPCS framework

When the sponsor was asked to provide an assessment of the human relevance of the malformations observed in rats based on the World Health Organization (WHO)/International Programme on Chemical Safety (IPCS) human relevance framework (Meek et al., 2014), a statement was provided, which is taken into account and analysed in the following.

Problem formulation

In rat developmental toxicity studies with flumioxazin, increases of ventricular septal defects were reported in offspring. The mode of action and the possible relevance of this effect for humans are analysed below.

Hypothesized mode of action statement

The sponsor (C. Harris, personal communication, 2015) proposed the following mode of action:

> Mechanistic research has established that toxic effects observed in the developmental studies and to a lesser extent the repeat dose studies (haematotoxicity) result from inhibition of the enzyme protoporphyrinogen oxidase (PPO) (PPO is responsible for the 7th step in haem production, by removing hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX). The effects reported in the rat developmental study were observed in the absence of maternal toxicity. In the rabbit developmental study, whilst the administered dose was 100-fold greater than in the rat study and maternal toxicity was observed, no embryolethal or teratogenic effects were observed. There is convincing evidence for a single mode of action causing the developmental toxicity in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal haem synthesis, which causes loss of blood cells leading to embryo-fetal anaemia, embryolethality and the development of malformations. Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in erythroblasts. This leads to anaemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure.

Summary of data for use in mode of action analysis

In Table A1, the data used in the mode of action analysis are summarized.

Table A1. Summary of data for use in mode of action analysis

Species	Route	Route/dose (mg/kg bw per day)	Incidence of VSD (affected fetuses/ litters)	Comments	Reference
Rat	Oral	0	2/2	–	Kawamura (1990)
		1	1/1		
		2	2/2		
		10	6/5		
		30	26**/12##		
Rat	Oral	0	0/0	Range-finding study	Kawamura (1989)
		30	11**/5##		
		100, 200, 500	Not analysable	Dose groups 100, 200 and 500 mg/kg bw per day had too few offspring to analyse	
Rat	Dermal	0	1/1	–	Kawamura (1991)
		30	1/1		
		100	2/2		
		300	13*/9##		
Rat	Oral	400	GD 11: 5/2	One treatment on different GDs only, 5 dams per group	Kawamura (1993)
			GD 12: 6/3		
			GD 13: 3/2		
			GD 14: 3/2		
			GD 15: 1/1		

bw: body weight; GD: gestation day; VSD: ventricular septal defects; *: $P < 0.05$; **: $P < 0.01$ (Wilcoxon's rank sum test); ##: $P < 0.01$ (Fisher's exact test)

Listing of key events identified for a specific mode of action

According to the WHO/IPCS mode of action framework, key events are measurable events that are critical to the induction of the (adverse) effect. The key events are listed in order from earliest to latest key event; the last key event should be the (adverse) effect.

The identified key events for flumioxazin's mode of action are listed in Table A2.

Table A2. Listing of key events identified for a specific mode of action

Key event	Description
1	Inhibition of PPO
2	Anaemia

PPO: protoporphyrinogen oxidase

Bradford Hill considerations for weight of evidence analysis of available data/information for mode of action analysis in experimental species

Dose–response relationships and temporal association

In this section, concordance of dose–response relationships together with temporal association are presented.

Dose–response relationship

Only doses inducing malformations were tested for accumulation of PPIX, but not doses below this dose level. Additionally, mainly single dose levels were used in the in vivo mechanistic studies (Kawamura, 1996a,b, 1997a).

A study was designed to investigate the potential of flumioxazin to cause fetal anaemia at developmentally toxic doses in rats. Mated female Sprague-Dawley rats were gavaged with flumioxazin at 0 (vehicle, 0.5% aqueous methyl cellulose), 15, 30 or 60 mg/kg bw per day on days 6–15 of gestation. Dams showed lower body weight and body weight gain at 60 mg/kg bw per day on GD 20, but not at earlier time points. Dose-dependent anaemia was confirmed in GD 14 embryos from dams dosed at 15, 30 or 60 mg/kg bw per day. Anaemia was characterized by pale yolk sacs and pale embryos, histopathologically decreased erythroblast content in the heart, iron deposits in erythroblasts and degenerative erythroblasts. Embryo/fetal lethality (and fewer live fetuses) was observed at 60 mg/kg bw per day (increased rate of late postimplantation loss at GD 14 and of early postimplantation loss at GD 20). Slightly increased incidences of ventricular septal defects were reported at 15 mg/kg bw per day and clearly at 30 and 60 mg/kg bw per day. Increased incidence and severity of thinning of ventricular walls and of atrium dilatation were reported in fetuses at 30 and 60 mg/kg bw per day. Fetal livers showed dilatation of sinusoidal vessels and hepatocytic necrosis in the peripheral region of moderate severity at 60 mg/kg bw per day. Lower fetal weights were reported at 60 mg/kg bw per day and, to a slight extent, also at 30 mg/kg bw per day. Adverse findings were reported in fetuses in all dose groups starting at the lowest dose of 15 mg/kg bw per day (Hosokawa, 2015).

Hence, a dose–response relationship for an increase in PPIX concentration cannot be established, whereas it can be seen for the induction of anaemia.

Temporal association

To examine the accumulation of PPIX in rat and rabbit embryos, pregnant rats and rabbits were administered a single oral dose of flumioxazin in 0.5% methyl cellulose at 1000 mg/kg bw, and

the concentrations of PPIX in embryos and maternal livers were measured at 2, 6, 12, 18 and 24 hours after administration for rats and at 2, 6, 12, 24 and 48 hours after administration for rabbits. The concentrations of PPIX in rat embryos and maternal livers were much higher than in the corresponding tissues of the rabbit, with peak concentrations observed at 12 hours for embryos and 6–12 hours for maternal livers (Kawamura, 1996a).

In a follow-up study, the development of fetuses was examined 6, 12, 24, 36 or 48 hours after treatment with flumioxazin at 1000 mg/kg bw per day in rat dams and after 6, 24 or 48 hours in rabbit does. No embryonic deaths were observed in rats at 24 hours after treatment. The first intrauterine deaths were observed at 36 hours, and embryonic mortality increased to 93.2% at 48 hours after treatment. A primary effect was observed in circulating erythroblasts. Observations included mitochondrial iron deposits in polychromatophilic erythroblasts, dilatation of the mitochondrial matrix in polychromatophilic erythroblasts at 6 hours post-dosing and erythroblastic cell death evident after the appearance of the mitochondrial lesions. These findings were suggested to reflect the observed anaemia in rat embryos. No histopathological signs in the embryonic rat heart were observed up to 24 hours after treatment, and no cell death in the embryonic rat heart was observed up to 48 hours after treatment, suggesting that no primary injury to the embryonic heart was produced by flumioxazin. Histological changes in the rat embryonic hearts at 36 or 48 hours after treatment included thin ventricular wall, poorly developed ventricular trabeculae and hypoplasia of the muscular septum and endocardial cushions of the atrioventricular canal. In rat fetuses, dilatation of hepatic sinusoidal vessels was observed at 24 and 36 hours; at 48 hours, hepatic necrosis was reported. No treatment-related changes in the external appearance of embryos or intrauterine deaths were reported in rabbits. Likewise, neither iron deposits in erythroblasts of rabbit embryos nor histopathological changes similar to those produced in rat embryos were seen in the rabbit embryo (Kawamura & Yoshioka, 1997).

Flumioxazin was administered to pregnant rats on day 12 of gestation at 400 mg/kg bw by gavage, and embryos/fetuses were removed from the uterus on days 13 through 20 of gestation and examined. Developmental toxicity was noted remarkably on day 14, when treated embryos were observed to have enlarged heart, oedema and anaemia (decreased red blood cell count and haemoglobin). These effects were also observed on days 15 and 16 of gestation, after which the values for treated litters were similar to control values. Beginning on day 15 and continuing to day 20, mortality in the treated litters was increased. The mortality rate was relatively constant on GD 17 and GD 20, indicating that all deaths occurred during the earlier period. This is consistent with the early resorptions observed in other developmental toxicity studies with flumioxazin. Closure of interventricular foramen began on day 16 of gestation in control fetuses (72.7% closed). Closure of interventricular foramen did not occur in any treated fetuses until day 16, and even then, the percentage with closure on day 17 was well below control values (89.7% control versus 23.9% treated). On day 20, the foramen of 95.2% of the control fetuses had closed, with only 57.7% of the treated fetuses reaching this milestone (Kawamura, 1997a).

A similar observation was made for malformations induced in rats by trypan blue. During early pregnancy (GD 11.5), fetuses with malformations were seen, whose rate decreased as pregnancy progressed (up to GD 20.5); however, the rate of resorptions increased concomitantly (Beck & Lloyd, 1963).

Further information on the temporal association can be gained from the study by Hosokawa (2015), which is summarized above.

Consistency and specificity and biological plausibility

Consistency and specificity

The main developmental landmarks in cardiac morphogenesis occur within a relatively short period in rats, between GD 12 and GD 16 (Sissman, 1970). Malformations are considered to be adverse findings that can be induced by a single treatment. This was also demonstrated for

flumioxazin. GD 12 was determined to be the most sensitive day, based on the incidence of embryonic deaths, fetal weight reduction and incidence of ventricular septal defects when dams were evaluated on GD 20 (Kawamura, 1993). Treatment of rats on other days led to a lower effect size.

Fetal findings were not reported in rabbit developmental toxicity studies. Similarly, the mechanistic studies in rabbits did not indicate an effect on the evaluated parameters in fetuses (Kawamura, 1996a, 1997b; Kawamura & Yoshioka, 1997).

Biological plausibility

To support the analysis of biological plausibility, other PPO inhibitors were checked for the induction of similar developmental effects.

Other compounds inhibiting PPO in isolated corn etioplasts or potato mitochondria, yeast mitochondria or mouse liver mitochondria are acifluorfen-methyl (Matringe et al., 1989a) and oxadiazon (Matringe et al., 1989b).

Although some effects of sodium acifluorfen on blood (anaemia) were reported, no induction of ventricular septal defects in rats or rabbits was indicated in a USEPA (2002) report. However, in the two-generation study, increased pup mortality was seen (USEPA, 2002).

Similarly, although some effects of oxadiazon on blood (anaemia) were reported, no induction of malformations or postimplantation losses in rats or rabbits was indicated in the European Union monograph (Ministry of Health, Italy, 2006).

Information on nitrofen, which is also a PPO inhibitor, is summarized in a review. Nitrofen induced a high incidence of diaphragm hernia and Harderian gland alterations in mice and hydronephrosis and respiratory difficulties in rats. According to the review, the effects were mediated by alterations in maternal and fetal thyroid hormone status (Gupta, 2011).

A further review indicated that the PPO-inhibiting herbicides azafenidin, butafenacil, carfentrazone, fluazolate, flufenpyr-ethyl, fluoroglycofen, fomesafen and lactofen would exhibit no teratogenic potential, whereas flumiclorac, flumioxazin, oxyfluorfen and sulfentrazone would have teratogenic potential (Dayan & Duke, 2010).

It might be expected, if PPO is inhibited, that the metabolic substrate protoporphyrinogen IX would increase in concentration. However, in the available studies, the metabolic product PPIX increased in concentration. The sponsor speculated that "accumulating protoporphyrinogen eventually leaves the mitochondria, enters the plasma, and is oxidized nonenzymatically there to PPIX. Because of abnormal subcellular location, the resulting PPIX is beyond reach of ferrochelatase and cannot be transformed to heme" (Kawamura, 2012). However, this hypothesis is not supported by the data.

Apparently, no data on the phenotype of PPO knockout rats (or mice) are available. Such data might support the hypothesis that PPO inhibition might lead to ventricular septal defects in developing fetuses.

In summary, there is no indication that inhibition of PPO is a key event leading to ventricular septal defects in developing rat fetuses.

Qualitative and quantitative human concordance

Human concordance cannot be assessed in a situation where the mode of action in animals has not been established.

Other potential modes of action

Other compounds reported to induce ventricular septal defects in rats are the cyclooxygenase inhibitors diflunisal, ibuprofen and ketorolac. In the same study, diclofenac and diflunisal induced postimplantation losses (Cappon, Cook & Hurtt, 2003). In rabbits, ventricular septal defects and

postimplantation losses were induced by diflunisal, but to a lesser extent than reported for rats (Cappon, Cook & Hurtt, 2003).

Hypoxia (by reduced air pressure in a chamber on 1 day during period of GD 9 to GD 13) can induce ventricular septal defects in rats (Clemmer & Telford, 1966). Similarly, treatment of rats with an atmosphere enriched with carbon dioxide and with reduced oxygen also induced ventricular septal defects; the highest incidence was reported when the dams were treated on the tenth day of pregnancy (Haring, 1965).

The antimalaria drug artesunate induced embryolethality and teratogenicity, including cardiovascular and skeletal defects (White et al., 2006; Clark et al., 2008). White et al. (2006) reported that

> Paling of visceral yolk sacs was observed within 3–6 hr after treatment. Within 24 hr, marked paling and embryonic erythroblast depletion were observed macroscopically, which preceded malformations and embryo death, and persisted through Day 14 pc [post-coitus]. Histologically, embryonic erythroblasts were reduced and cells showed signs of necrosis within 24 hr, were maximally depleted by 48 hr, and had partially rebounded within 3–4 days after treatment (Days 13 and 14 pc). Iron accumulation was evident in treated erythroblasts as early as 6 hr after treatment, suggesting impairment of heme synthesis. Heart abnormalities (swollen or collapsed chambers) were observed within 24 hr in approximately 25–60% of embryos and within 48 hr in 100% of embryos, correlating with histologic signs of cardiac myopathy (thinned and underdeveloped heart walls and enlarged chambers). Delays in limb and tail development occurred by Day 13 pc. Embryos were viable through Day 13 pc, but approximately 77% of embryos had died by Day 14 pc, presumably due to hypoxia and/or cardiac abnormalities.

These observations are remarkably similar to the findings in the studies with flumioxazin.

Clark et al. (2008) compared the developmental toxicity of artesunate with that of the structurally related artemisinins dihydroartemisinin (DHA), artemether and arteether. They summarized:

> Several structurally related artemisinins cause similar developmental toxicity, suggesting an artemisinin class effect. Equally embryotoxic oral and IV [intravenous] treatments of one artemisinin compound (artesunate) produced similar systemic exposure to the artesunate metabolite, DHA, suggesting that DHA may be the proximate developmental toxicant. Embryolethal doses of artesunate only caused minor changes in maternal reticulocyte counts indicating that adult hematology parameters are not as sensitive as embryonic erythroblasts.

A further compound inducing ventricular septal defects is dimethadione (DMO), a metabolite of the anticonvulsant drug trimethadione (Aasa et al., 2014). Starting on GD 8 (19:00) and then every 12 hours thereafter, a total of six doses of 300 mg/kg bw each were administered to rat dams. Aasa et al. (2014) summarized their findings:

> Adult rats exposed to DMO in utero had an increased incidence of arrhythmia, elevated blood pressure and CO [cardiac output], greater left ventricular volume and elevated locomotor activity versus CTL [control]. The mean arterial pressure of DMO-exposed rats was more sensitive to changes in dietary salt load compared with CTL. Importantly, most treated rats had functional deficits in the absence of a persistent structural defect. It was concluded that in utero DMO exposure causes cardiovascular deficits that persist into postnatal life in the rat, despite absence of visible structural anomalies.

For DMO-induced heart defects, there are published hypotheses that DMO acts via radical oxygen species or via drug-induced arrhythmia (summarized in Azarbayjani, 2001).

Such alternative modes of action were not scrutinized sufficiently, especially as reduced oxygen availability might be a common mechanism for hypoxia and anaemia as induced by flumioxazin.

Uncertainties/inconsistencies and identification of data gaps

There is no sufficient indication that inhibition of PPO is a key event leading to ventricular septal defects in developing rat fetuses. Some of the conclusions are based on in vitro or in silico investigations.

It might be expected, if PPO is inhibited, that the metabolic substrate protoporphyrinogen IX would increase in concentration. However, in the available studies, the metabolic product PPIX increased in concentration. This discrepancy was not explained by the data.

Conclusions in relation to problem formulation

The sponsor proposed a new mode of action to explain the induction of ventricular septal defects in rat fetuses. Likewise, it was the aim to demonstrate the non-relevance of this mode of action for humans.

In summary, the Meeting concluded that the case is not robust enough to demonstrate that the proposed mode of action is plausible in rats. Hence, the relevance of this mode of action for humans cannot be assessed.

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FLUPYRADIFURONE

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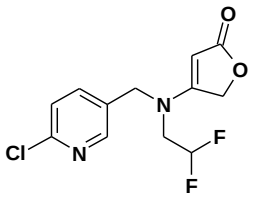
Explanation

Flupyradifurone is the International Organization for Standardization (ISO)–approved common name for 4-[(6-chloro-3-pyridylmethyl)(2,2-difluoroethyl)amino]furan-2(5*H*)-one (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 951659-40-8. Flupyradifurone is a butenolide insecticide that works by binding to insect nicotinic acetylcholine receptors. The chemical structure of flupyradifurone is given in Fig. 1.

Fig. 1. Chemical structure of flupyradifurone (BYI 02960)



Flupyradifurone 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.

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

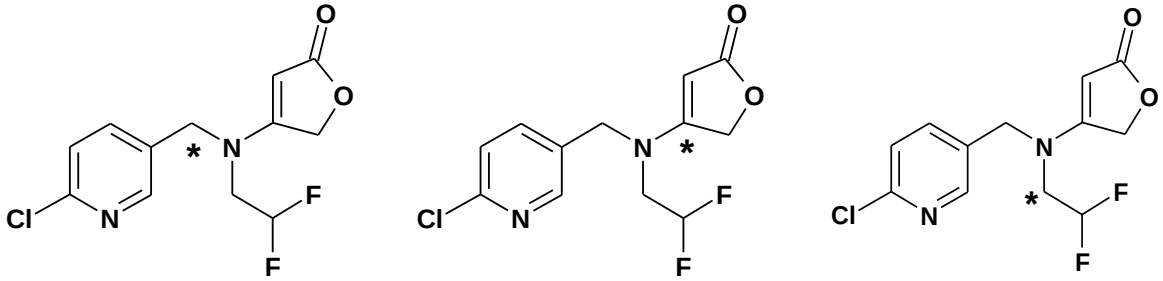
European Union or United States Environmental Protection Agency. Minor deviations from these protocols were not considered to affect the reliability of the studies.

Evaluation for acceptable intake

1. Biochemical aspects

Absorption, distribution, metabolism and excretion (ADME) studies were conducted in rats using flupyradifurone labelled with ^{14}C in the pyridinylmethylene bridge, in the 4-position of the furanone ring or in the 1-position of the ethyl side-chain (Fig. 2).

Fig. 2. Position of ^{14}C label in flupyradifurone used in rat ADME studies



Pyridinylmethyl-^{14}C

* Denotes the position of the radiolabel

Furanone-4-^{14}C

* Denotes the position of the radiolabel

Ethyl-1-^{14}C

* Denotes the position of the radiolabel

1.1 Absorption, distribution and excretion

In a study by Klempner (2012), [pyridinylmethyl-^{14}C]flupyradifurone (99.4% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension was administered to groups of four Wistar Hsd/Cpb:WU rats of each sex at a single gavage dose of 2 mg/kg body weight (bw) (low dose) or 200 mg/kg bw (high dose). An additional group of four male rats was administered a single intravenous dose of [pyridinylmethyl-^{14}C]flupyradifurone in water at 2 mg/kg bw. Urine was collected at 4, 8, 12, 24, 48 and 72 hours after dosing. Faeces were collected at 24, 48 and 72 hours after dosing. Blood was collected at 10, 20 and 40 minutes after dosing and then at 1, 1.5, 2, 3, 4, 6, 8, 24, 28, 32, 48, 52, 56 and 72 hours. Rats were killed after 72 hours, and tissues were sampled. Radioactivity was quantified in plasma, excreta and tissues by liquid scintillation counting (LSC).

The mass balance of radioactivity is summarized in Table 1. Recovery of radioactivity was greater than 96% following oral dosing and 91% following intravenous dosing, with the majority detected in urine. Gastrointestinal absorption was almost complete, as evidenced by the high bioavailability factor (F = 0.93) calculated from a comparison of the dose-normalized area under the plasma concentration–time curve (AUC) for low-dose males after oral and intravenous administration and by the proportion of radioactivity detected in the urine and carcass. The majority (up to approximately 90%) of radioactivity was excreted in urine within 24 hours of dosing.

Pharmacokinetic parameters are summarized in Table 2. Maximum plasma concentrations of radioactivity (C_{max}) were reached 1 hour (T_{max}) after the low oral dose and 2 hours (males) or 4 hours (females) after the high oral dose. At the high oral dose, the dose-normalized C_{max} was approximately half that of the low dose. The AUC did not increase proportionally with dose, indicating non-linear kinetics.

At termination, tissue radioactivity was low, with only trace amounts (< 0.1–0.3% of the administered dose) detected in the body and in the gastrointestinal tract. Radioactivity was detected in most tissues, with higher tissue concentrations determined at the high oral dose (ranging from 0.0859

to 2.345 mg/kg) than at the low oral dose (ranging from 0.0007 to 0.0175 mg/kg), despite the lower plasma concentrations. In both sexes, blood contained the highest concentration of radioactivity, followed by the gastrointestinal tract and eyes (females) (Table 3).

Table 1. Cumulative mass balance of radioactivity in rats following dosing with [pyridinylmethyl-^{14}C]flupyradifurone

	Mean % of the administered radioactive dose				
	2 mg/kg bw, oral		200 mg/kg bw, oral		2 mg/kg bw, intravenous
Sample	**Males**	**Females**	**Males**	**Females**	**Males**
Urine					
4 h	17.25	5.34	14.89	9.50	26.12
8 h	40.99	33.80	31.33	28.56	44.02
12 h	51.67	–	–	–	–
24 h	72.61	86.95	68.26	72.15	73.80
48 h	75.15	89.77	75.56	84.15	75.75
72 h	75.45	90.07	76.26	85.95	76.24
Faeces					
24 h	20.55	6.86	20.22	4.90	13.31
48 h	22.82	7.38	25.84	9.64	14.41
72 h	23.09	7.49	26.14	10.32	14.64
Carcass	0.188	0.074	0.214	0.306	0.227
Balance	**98.73**	**97.63**	**102.60**	**96.57**	**91.11**

Source: Klempner (2012)

Table 2. Mean plasma pharmacokinetic parameters in rats following dosing with [pyridinylmethyl-^{14}C]flupyradifurone

	2 mg/kg bw, oral		200 mg/kg bw, oral		2 mg/kg bw, intravenous
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**
T_{max} (h)	1.0	1.0	2.0	4.0	0.67
C_{max} (µg eq/g)[a]	0.878	0.937	0.497	0.578	1.04
$t_{½}$ (h)	3.9	3.0	3.6	8.1	3.8
$AUC_{(0-\infty)}$ (mg/L·h)	6.10	7.96	6.16	9.73	6.55
MRT_{tot} (h)	6.07	6.69	8.70	13.10	5.71

$AUC_{(0-\infty)}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; eq: equivalents; MRT_{tot}: mean total residence time; $t_{½}$: half-life; T_{max}: time to reach the maximum concentration

[a] Dose normalized.

Source: Klempner (2012)

Table 3. Mean tissue concentrations of radioactivity 72 hours after dosing with [pyridinylmethyl-^{14}C]flupyradifurone

	Mean tissue concentrations of radioactivity (mg eq/kg)				
	2 mg/kg bw, oral		200 mg/kg bw, oral		2 mg/kg bw, intravenous
Tissue	**Males**	**Females**	**Males**	**Females**	**Males**
Blood cells	0.017 5	0.006 7	2.345 0	1.577 0	0.015 8
Plasma	0.002 0	0.001 3	0.298 9	0.296 3	0.002 5
Carcass	0.002 1	0.001 1	0.179 4	0.237 7	0.002 0
Heart	0.002 4	NC	0.264 3	0.332 8	0.002 3
Brain	0.000 8	NC	0.085 9	0.092 2	0.000 8
Kidneys	0.006 4	0.003 3	0.797 5	0.669 1	0.006 7
Liver	0.006 8	0.003 4	0.874 1	0.772 0	0.006 3
Gastrointestinal tract	0.014 1	0.001 9	1.729 0	1.145 0	0.016 7
Testes	0.000 8	–	0.102 0	–	0.001 1
Ovaries	–	NC	–	0.288 0	–
Uterus	–	0.001 6	–	0.459 9	–
Adrenal gland	0.004 8	0.003 2	0.443 6	0.560 6	0.004 5
Harderian gland	0.005 0	0.002 2	0.410 1	0.721 8	0.003 4
Thyroid	NC	NC	NC	NC	NC
Spleen	0.003 0	0.001 7	0.358 6	0.339 8	0.003 2
Lung	0.006 0	0.003 5	0.664 8	0.566 3	0.005 4
Eyes	0.006 4	0.013 3	0.599 6	1.343 0	0.006 6
Skin	0.001 8	0.001 4	0.235 4	0.971 0	0.002 4
Bone (femur)	NC	0.002 3	0.177 1	0.279 4	0.002 4
Fat (perirenal)	0.001 8	NC	NC	0.124 5	0.003 0
Muscle (leg)	0.000 8	0.000 7	0.120 6	0.209 8	0.001 2

bw: body weight; eq: equivalents; NC: not calculated
Source: Klempner (2012)

Koester & Weber (2011) investigated the distribution of flupyradifurone and its metabolites in rats by quantitative whole-body autoradiography. Eight male and eight female Wistar Hsd/Cpb:WU rats were administered a single gavage dose of [pyridinylmethyl-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension at 5 mg/kg bw. A separate control male and female rat each received unlabelled flupyradifurone at 5 mg/kg bw. Urine was collected at 1, 4, 8 and 24 hours and every 24 hours thereafter until 168 hours. Faeces were collected at 24-hourly intervals until 168 hours after dosing. Expired air was collected from four rats of each sex at 0–24 and 24–48 hours. Radioactivity was quantified in excreta by LSC. One treated male and one treated female rat were killed at 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing for whole-body autoradiography.

In the majority of tissues, maximum concentrations of radioactivity were reached at 1 hour after dosing and thereafter rapidly declined to less than 5% of the maximum concentration 1 day after dosing and less than 1% of the maximum 2 days after dosing. The tissue/blood–concentration ratios

were comparable between males and females and were highest for the liver (1.78), adrenal gland (1.76) and kidney (1.68). Ratios for myocardium, thyroid, Harderian gland, salivary gland and pancreas were approximately 1.2–1.4. The lowest values were for the spinal cord (0.37), brain (0.35) and perirenal fat (0.15). There was no evidence of any accumulation or retention of radioactivity in any tissue. The majority of radioactivity was excreted in urine (up to 85% in males and 93% in females), with the remainder eliminated in faeces. Less than 0.1% of the administered radioactivity was expired. The majority (> 90%) of radioactivity was excreted within 24 hours of dosing, with 95–100% of radioactivity excreted by 48 hours.

In a study by Weber (2011a), [furanone-4-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension was administered by gavage to groups of four Wistar Hsd/Cpb:WU rats of each sex at a single dose of 2 mg/kg bw. Urine was collected at various intervals from 4 to 168 hours after dosing. Faeces were collected at 24-hourly intervals to 168 hours after dosing. Blood was collected at multiple time points from 10 minutes to 168 hours after dosing. Rats were killed after 168 hours, and tissues were sampled. Radioactivity was quantified in plasma, excreta and tissues by LSC.

Recovery of radioactivity was approximately 100%, with the majority detected in urine (Table 4). Based on the concentration of radioactivity in urine and the carcass, gastrointestinal absorption was at least 79% in males and 91% in females. Maximum plasma concentrations of radioactivity were reached at 1.5 hours after dosing, with elimination following a biphasic decline; a short first half-life of approximately 3 hours was mainly attributable to the elimination of the parent compound, and a significantly longer second half-life of 53 hours was probably related to the incorporation of radioactivity into biomolecules (Table 5). The major route of excretion was renal, with the majority (up to approximately 87%) of radioactivity excreted in urine within 24 hours of dosing.

At termination, 0.5% of radioactivity was detected in the carcass of male rats, and approximately 0.2% was detected in female rats. Tissue concentrations of radioactivity ranged from 0.0025 to 0.0336 mg equivalents (eq)/kg in males and from 0.0012 to 0.0131 mg eq/kg in females (Table 6), with the lowest concentrations detected in plasma and the highest detected in the thyroid. In most tissues, radioactivity was approximately 2–3 times higher in males than in females. This difference may be attributable to the higher rate of incorporation of radioactivity into biomolecules in males.

Koester (2011a) investigated the distribution of flupyradifurone and its metabolites in rats by quantitative whole-body autoradiography. Eight male and eight female Wistar Hsd/Cpb:WU rats were administered a single gavage dose of [furanone-4-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension at 5 mg/kg bw. A separate control male and female rat each received unlabelled flupyradifurone at 5 mg/kg bw. Urine was collected at 1, 4, 8 and 24 hours and every 24 hours thereafter until 168 hours. Faeces were collected at 24-hourly intervals until 168 hours after dosing. Expired air was collected from four rats of each sex at 0–24 and 24–48 hours. Radioactivity was quantified in excreta by LSC. One treated male and one treated female rat were killed at 1, 4, 8, 24, 48, 72, 120 and 168 hours after dosing for whole-body autoradiography.

Radioactivity was rapidly absorbed and distributed to the entire body. In the majority of organs and tissues, the T_{max} was 1 hour after dosing, whereas in female rats, the olfactory bulb reached its maximum concentration only after 8 hours. The mean tissue/blood–concentration ratios at T_{max} were highest for the adrenal gland (1.90), liver (1.80), kidney (1.67), olfactory bulb (1.64), thyroid and Harderian gland (1.41), myocardium (1.33), salivary gland (1.35) and pancreas (1.30). The lowest ratios were for the spinal cord (0.38), brain (0.34) and perirenal fat (0.15). A similar pattern of distribution of radioactivity was evident at 168 hours after dosing.

Table 4. Cumulative mass balance of radioactivity in rats following a single oral [furanone-4-^{14}C]flupyradifurone dose of 2 mg/kg bw

	Mean % of administered radioactivity	
Sample	**Males**	**Females**
Urine		
4 h	8.74	6.52
8 h	33.86	18.32
12 h	39.55	–
24 h	75.04	87.31
48 h	78.40	90.44
72 h	78.75	90.89
96 h	78.86	91.14
120 h	78.91	91.24
144 h	78.94	91.33
168 h	78.96	91.37
Faeces		
24 h	14.76	9.45
48 h	16.40	10.29
72 h	16.50	10.33
96 h	16.54	10.35
120 h	16.56	10.36
144 h	16.57	10.37
168 h	16.59	10.38
Carcass	0.48	0.17
Gastrointestinal tract	0.02	0.01
Total body	0.49	0.18
Balance	**96.05**	**101.90**

Source: Weber (2011a)

Table 5. Mean plasma pharmacokinetic parameters in rats following a single oral [furanone-4-^{14}C]flupyradifurone dose of 2 mg/kg bw

Parameter	**Males**	**Females**
T_{max} (h)	1.5	1.5
C_{max} (µg eq/g)	1.457	1.912
$t_{½}$ elimination 1 (h)	3.07	2.88
$t_{½}$ elimination 2 (h)	53.1	53.6
$AUC_{(0-\infty)}$ (mg/kg·h)	16.0	18.2

$AUC_{(0-\infty)}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; eq: equivalents; $t_{½}$: half-life; T_{max}: time to reach the maximum concentration
Source: Weber (2011a)

Table 6. Mean tissue concentrations of radioactivity 162 hours after a single oral [furanone-4-^{14}C]flupyradifurone dose of 2 mg/kg bw

	Mean tissue concentrations of radioactivity (mg eq/kg)	
Tissue	**Males**	**Females**
Blood cells	0.008 3	0.003 8
Plasma	0.002 5	0.001 2
Carcass	0.007 9	0.003 1
Heart	0.006 5	0.002 9
Brain	0.007 2	0.003 3
Kidneys	0.010 4	0.004 5
Liver	0.012 8	0.008 1
Testes	0.005 9	–
Ovaries	–	0.003 9
Uterus	–	0.003 5
Adrenal gland	0.020 0	0.011 4
Harderian gland	0.024 1	0.009 1
Thyroid gland	0.033 6	0.013 1
Spleen	0.008 1	0.003 2
Lung	0.007 5	0.004 8
Eye	0.005 3	0.004 8
Skin	0.011 1	0.004 7
Bone femur	0.008 5	0.004 9
Fat (perirenal)	0.011 8	0.005 8
Muscle (leg)	0.006 9	0.002 3

bw: body weight; eq: equivalents
Source: Weber (2011a)

Tissue concentrations of radioactivity declined following biphasic kinetics, with the second and slower decline phase starting after 24 hours in males and after 48 hours in females. Excretion of radioactivity via urine and faeces was almost complete after 2 days, with renal excretion the predominant route (> 80% of the dose was detected in urine). Between 0.96% (females) and 3.25% (males) of the dose was exhaled as $^{14}CO_2$ during a sampling period of 48 hours, indicating that for a small portion of the dose, the furanone ring of the molecule underwent extensive biotransformation to C1- and C2-fragments. At the end of the study, tissue radioactivity was 1.4–4.7 times higher in males than in females, presumably due to quantitative differences in metabolism leading to more C1- and C2-fragments and also higher incorporation of these components into the endogenous carbon pool in male rats. There was no evidence of any accumulation or retention of radioactivity in any tissue.

In a study by Koester (2011b) designed to examine the absorption, distribution and excretion of flupyradifurone over 6 hours, [furanone-4-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension was administered by gavage to groups of four Wistar Hsd/Cpb:WU rats of each sex at a single dose of 3 mg/kg bw. Urine and faeces were collected at 0–6 hours. Blood and samples of liver, kidney, muscle and fat were collected at termination (6 hours). Radioactivity was quantified in pooled plasma, excreta and tissues by LSC. Metabolites were

analysed in excreta and tissues by high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy.

Table 7 summarizes the mass balance of radioactivity. Recovery of radioactivity was approximately 100%, with the majority detected in the urine and carcass. Of the analysed tissues, the skin contained the highest proportion of radioactivity, followed by the liver, muscle, kidney and fat.

Table 7. Mass balance of radioactivity in rats 6 hours after a single oral [furanone-4-^{14}C]flupyradifurone dose of 3 mg/kg bw

	% of administered dose	
Sample	**Males**	**Females**
Urine	36.64	42.82
Plasma	0.70	0.55
Carcass	24.00	26.73
Kidneys	0.73	1.04
Liver	3.56	3.61
Gastrointestinal tract + faeces	23.63	12.57
Skin	9.18	9.42
Fat (perirenal)	0.06	0.09
Muscle (leg)	2.09	1.26
Total	**100.60**	**98.09**

bw: body weight
Source: Koester (2011b)

In a study by Weber (2011b), [ethyl-1-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension was administered by gavage to four male Wistar Hsd/Cpb:WU rats at a single dose of 2 mg/kg bw. Urine was collected at 4, 8, 12, 24, 48 and 72 hours after dosing. Faeces were collected at 24-hourly intervals to 72 hours after dosing. Expired air was collected at intervals of 0–24, 24–48 and 48–72 hours. Blood was collected at multiple time points from 10 minutes to 72 hours after dosing. Rats were killed after 72 hours, and tissues were sampled. Radioactivity was quantified in plasma, excreta and tissues by LSC.

Recovery of radioactivity was almost 100%, with the majority detected in urine (82.24% of the administered dose), followed by the faeces (13.51%), carcass excluding the gastrointestinal tract (3.19%), gastrointestinal tract (0.73%) and exhaled air (0.20%). Based on the proportion of radioactivity detected in urine and the carcass excluding the gastrointestinal tract, absorption was at least 85%. Radioactivity was detected in plasma from 10 minutes after dosing, with the maximum concentration reached 1 hour after dosing. The concentration of radioactivity in plasma decreased to 50% of the C_{max} by 8 hours and 8% of the C_{max} at termination. The $AUC_{(0-\infty)}$ was 45.4 mg/L·h, and the half-life was 49.9 hours. The majority of radioactivity (> 87%) was excreted within 24 hours of dosing. Radioactivity was distributed to all tissues, with concentrations similar across the majority of tissues (0.05–0.1 mg eq/kg); the highest proportion of radioactivity was detected in the skin (0.91%), gastrointestinal tract (0.73%), liver (0.24%) and plasma (0.10%).

In a study by Koester (2011c) designed to examine the absorption, distribution and excretion of flupyradifurone over 24 hours, [ethyl-1-^{14}C]flupyradifurone (> 99% radiochemical purity) in a 0.5% aqueous Tragacanth® suspension was administered by gavage to three groups of four Wistar

Hsd/Cpb:WU rats of each sex at a single dose of 3 mg/kg bw. Urine and faeces were collected at 0–1, 0–6 and 0–24 hours. Four rats of each sex were killed at 1, 6 and 24 hours after dosing, blood was collected, and liver, kidneys and muscle were sampled. Radioactivity was quantified in pooled plasma, excreta and tissues by LSC. Metabolites were analysed in excreta and tissues by HPLC, TLC, MS and NMR spectroscopy.

Table 8 summarizes the mass balance of radioactivity. Recovery of radioactivity was greater than 95% in males and 100% in females. Absorption was rapid, with radioactivity detected in urine and tissues from 1 hour after dosing. Based on the concentration of radioactivity in urine, tissues and carcass, gastrointestinal absorption is estimated to be at least 83% in males and 91% in females. Renal excretion of radioactivity commenced from 1 hour after dosing, with 71.80% and 85.88% excreted within 24 hours of dosing. As a proportion of the dose, the highest levels of radioactivity were detected in the skin and carcass; however, the highest concentrations were detected in the blood, liver and kidneys. Radioactivity was rapidly eliminated from tissues, and there was no indication of any retention of radioactivity in any of the analysed tissues.

To determine the appropriate C_{max} and T_{max} for the collection of blood in repeated-dose toxicity studies, Odin-Feurtet (2010) analysed plasma concentrations of flupyradifurone in Wistar rats (five of each sex) that had been exposed continuously to diets containing 400 parts per million (ppm) flupyradifurone (96.2% purity) (equal to 22.6 mg/kg bw per day for males and 32.4 mg/kg bw per day for females) for 7 days. There were no deaths and no treatment-related clinical signs. In male rats, a C_{max} of 8.3 mg/L was measured at the time of collection (08:00). In female rats, a C_{max} of 9.4 mg/L was measured at the time of collection (14:00). However, in view of the inter-rat variability, the concentrations of flupyradifurone in plasma were considered similar between the three times of blood collection for both sexes (between 7.8 and 8.3 mg/L for males and between 8.8 and 9.4 mg/L for females). On this basis, blood samples collected between 08:00 and 17:00 were considered adequate for measuring flupyradifurone concentrations in plasma around the C_{max} for both sexes.

1.2 Biotransformation

In the study by Klempner (2012) described in section 1.1 above, metabolites were analysed in excreta by HPLC, TLC, MS and NMR spectroscopy; glucuronide conjugates were identified following enzyme treatment with glucuronidase or arylsulfatase. Table 9 summarizes the results of the metabolite analysis of excreta. Parent compound, three major metabolites and five minor metabolites were identified in all samples, with a further 19 unknown metabolites characterized, ranging from less than 0.1% to 0.9% of the administered dose. Flupyradifurone was the main compound in the urine of both sexes. In male faeces, flupyradifurone-OH[1] was more prominent than the parent compound. Two metabolites, 6-chloronicotinic acid and hippuric acid, were also prominent in male rats, but not in females. All other identified and characterized metabolites represented a minor proportion of the dose. The metabolic profiles in urine and faeces were comparable between males and females, with male rats metabolizing flupyradifurone more extensively than females.

In the study by Koester & Weber (2011) described in section 1.1 above, metabolites were analysed in excreta by HPLC, TLC and MS. Table 10 summarizes the results of the metabolite analysis of excreta. Although the metabolite profiles were similar between male and female rats, less parent compound was detected in male excreta than in female excreta (55% versus 76%, respectively). Male rats also showed a higher proportion of the polar metabolite fraction and higher residues in the carcass at the time of termination, suggesting that the furanone-4-^{14}C radiolabel is not completely stable and that a small proportion of the dose underwent biotransformation to C1- and C2-fragments, resulting in the incorporation of radioactivity into biomolecules.

[1] 4-{[(6-Chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-hydroxyfuran-2(5*H*)-one.

Table 8. Cumulative mass balance of radioactivity in rats after a single oral [ethyl-1-^{14}C]flupyradifurone dose of 3 mg/kg bw

	Mean % of administered radioactive dose	
Sample	**Males**	**Females**
Urine		
1 h	6.22	8.76
6 h	36.53	39.70
24 h	71.80	85.88
Plasma		
1 h	1.09	1.27
6 h	0.62	0.61
24 h	0.21	0.13
Carcass		
1 h	38.32	51.19
6 h	24.47	29.57
24 h	4.25	3.08
Gastrointestinal tract + faeces		
1 h	27.24	13.94
6 h	21.18	14.26
24 h	16.61	9.44
Skin		
1 h	14.64	17.54
6 h	9.19	10.48
24 h	2.13	1.45
Liver		
1 h	6.80	7.30
6 h	3.44	3.62
24 h	0.60	0.39
Kidney		
1 h	1.30	1.17
6 h	0.73	0.63
24 h	0.08	0.06
Muscle		
1 h	2.31	2.34
6 h	1.14	1.35
24 h	0.17	0.13
Fat		
1 h	0.10	0.15
6 h	0.04	0.09

Sample	Mean % of administered radioactive dose	
	Males	Females
24 h	0.01	0.01
Balance		
1 h	98.03	103.70
6 h	97.33	100.30
24 h	95.85	100.60

bw: body weight
Source: Koester (2011c)

Table 9. Metabolites identified in urine and faeces following administration of [pyridinylmethyl-^{14}C]flupyradifurone to rats

	% of the administered radioactive dose									
	2 mg/kg bw, oral				200 mg/kg bw, oral				2 mg/kg bw, intravenous	
	Urine		Faeces		Urine		Faeces		Urine	Faeces
Compound	M	F	M	F	M	F	M	F	M	M
Flupyradifurone	37.6	73.8	3.3	6.9	36.1	61.1	3.5	4.4	43.9	3.4
6-Chloronicotinic acid	2.3	0.4	0.1	–	6.0	1.3	0.2	–	2.7	0.1
Hippuric acid	7.4	1.1	0.2	–	10.4	2.2	0.2	0.1	5.1	< 0.1
Flupyradifurone-OH-gluA (isomer 1)	1.8	0.4	–	–	1.6	0.7	–	–	1.6	–
Flupyradifurone-OH-gluA (isomer 3)[2]	2.3	0.4	0.1	–	2.3	1.0	0.1	< 0.1	1.0	–
Flupyradifurone-OH-desfluoroethyl	2.0	2.3	0.2	0.1	1.4	2.5	0.4	0.2	1.5	0.2
Flupyradifurone-OH-SA[3]	0.1	0.2	0.1	0.1	0.2	0.4	0.2	0.1	0.1	0.1
Flupyradifurone-OH	17.8	9.0	11.2	1.8	12.7	11.8	11.3	3.3	15.7	6.6
Flupyradifurone-iso-SA	< 0.1	–	0.4	< 0.1	< 0.1	–	0.4	0.1	0.1	0.4
Total identified	71.4	87.7	15.5	6.0	70.7	81	16.2	8.2	71.7	10.8
Total characterized	0.7	2.0	1.1	0.1	4.8	3.2	1.9	0.3	4.1	0.7

bw: body weight; F: females; M: males
Source: Klempner (2012)

[2] 3-{[(6-Chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl β-D-glucopyranosiduronic acid.
[3] 3-{[(6-Chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-5-oxo-2,5-dihydrofuran-2-yl hydrogen sulfate.

Table 10. Metabolites identified in urine and faeces following a single oral [furanone-4-^{14}C]flupyradifurone dose of 2 mg/kg bw

	% of the administered radioactive dose			
	Males		Females	
Compound	**Urine**	**Faeces**	**Urine**	**Faeces**
Flupyradifurone	48.34	6.34	70.02	5.94
Difluoroethyl-amino-furanone	3.42	0.07	0.96	–
Flupyradifurone-OH-gluA (isomer 1)	1.13	–	0.49	–
Flupyradifurone-OH-gluA (isomer 3)	2.17	–	1.01	–
Flupyradifurone-des-difluoroethyl	1.87	0.24	3.09	0.26
Flupyradifurone-OH-SA	0.16	0.09	0.25	0.09
Flupyradifurone-OH	13.75	6.86	10.65	2.60
Flupyradifurone-iso-OH	–	0.26	–	0.07
Total identified	70.83	13.86	86.46	8.96
Total characterized	7.57	0.43	3.98	0.11

bw: body weight
Source: Koester & Weber (2011)

Flupyradifurone, one major metabolite and six minor metabolites were identified in urine and faeces, with up to a further 17 unknown metabolites characterized, ranging from less than 0.05% to 5.30% of the administered dose. Flupyradifurone was the main compound in urine (approximately 50% of the administered dose in males and 70% in females), with only one major metabolite, flupyradifurone-OH, detected (approximately 14% of the administered dose in males and 11% in females). Other identified urinary metabolites comprised less than 4% of the administered dose and included difluoroethyl-amino-furanone, flupyradifurone-gluA (isomers 1 and 3), flupyradifurone-des-difluoroethyl and flupyradifurone-OH-SA. A very polar metabolite fraction representing 5.22% of the dose in the urine of males and 2.42% in females was also detected. In faeces, flupyradifurone and flupyradifurone-OH were the main compounds identified (6% and 7% of the administered dose, respectively, in males and 6% and 3% of the administered dose, respectively, in females). Other identified faecal metabolites included difluoroethyl-amino-furanone, flupyradifurone-des-difluoroethyl, flupyradifurone-OH-SA and flupyradifurone-iso-OH, which each accounted for less than 0.3% of the administered dose.

In a study by Koester (2011b) described in section 1.1 above, metabolites were analysed in excreta and tissues by HPLC, TLC, MS and NMR spectroscopy. Six hours after administration, [furanone-4-^{14}C]flupyradifurone was incompletely metabolized, with the parent compound comprising the largest proportion of radioactivity measured in urine (60% in males, 88% in females), plasma (83% in males, 96% in females), liver (72% in males, 95% in females), kidney (72% in males, 93% in females), muscle (84% in males, 96% in females) and fat (85% in males, 100% in females). Low concentrations of between two and five identified metabolites and one and eight unknown metabolites were determined, with flupyradifurone-OH and difluoroethyl-amino-furanone the main compounds detected in each sample (Table 11). Metabolism was qualitatively similar in males and females, but was more extensive in males.

Table 11. Metabolite profile in rats 6 hours after a single oral [furanone-4-^{14}C]flupyradifurone dose of 3 mg/kg bw

	% of the total radioactivity in each sample (male/female)					
Metabolite	**Urine**	**Plasma**	**Liver**	**Kidney**	**Muscle**	**Fat**
Flupyradifurone	60/80	83/96	72/95	72/93	84/96	85/100
Difluoroethyl-amino-furanone	4.0/0.5	7.7/0.9	3.7/0.5	4.6/–	6.5/0.6	4.2/–
Flupyradifurone-OH-gluA (isomer 1)	1.0/0	–	0.9/–	0.4/–	–	–
Flupyradifurone-OH-gluA (isomer 3)	2.5/–	–	1.9/0.4	0.7/–	–	–
Flupyradifurone-des-difluoroethyl	2.2/1.9	–	1.2/0.8	1.3/1.1	1.0/0.8	–
Flupyradifurone-OH	19/8	5.2/1.9	8.3/2.6	11.7/4.5	6.0/2.1	7.1/–
Total identified	89/98	96/99	88/99	91/98	97/100	96/100
Total characterized	11/2	4.3/1.2	6.8/0.9	7/1.4	1.9/–	–

bw: body weight
Source: Koester (2011b)

In a study by Weber (2011b) described in section 1.1 above, metabolites were analysed in excreta by HPLC, TLC and MS. The main compound identified in urine was flupyradifurone (52% of the dose). Four metabolites were identified in urine, including flupyradifurone-OH (16.13% of the dose), difluoroacetic acid (5.28% of the dose), difluoroethyl-amino-furanone (3.63% of the dose) and flupyradifurone-OH-gluA (isomers 1 and 3) (1.40% and 1.79% of the dose, respectively). Four unknown metabolites totalling 2% of the administered dose were also characterized in urine. In faeces, the main compounds identified were flupyradifurone-OH (7.60% of the dose) and flupyradifurone (3.79% of the dose), with difluoroacetic acid and flupyradifurone-iso-OH identified at levels less than 0.5% of the dose. Two unknown metabolites different from those detected in urine were characterized at levels of less than 0.2% of the dose.

In a study by Koester (2011c) described in section 1.1 above, metabolites were analysed in excreta and tissues by HPLC, TLC, MS and NMR spectroscopy.

The main component in urine was flupyradifurone (47.7% of the dose in males and 76.5% of the dose in females at 24 hours after dosing). In both sexes, the other main urinary metabolite was flupyradifurone-OH (12.33% of the dose in males and 6.42% of the dose in females at 24 hours after dosing), whereas flupyradifurone-OH-gluA (isomers 1 and 3), difluoroacetic acid and difluoroethyl-amino-furanone were present in the urine of males at between 1% and 5% of the administered dose at 24 hours after dosing. In the urine of females, the only other metabolite identified was difluoroacetic acid (1.70% of the dose at 24 hours after dosing). There were up to 10 unknown metabolites characterized in urine, accounting for 3.52% of the dose in males and 0.42% of the dose in females.

At 1 hour after dosing, flupyradifurone was the main component present in plasma (92.2% and 97.9% of total plasma radioactivity in males and females, respectively), declining to 24 hours after dosing (6.4% and 18.2% of total plasma radioactivity in males and females, respectively). There was a concomitant increase in difluoroacetic acid from 1 to 24 hours after dosing (from 2.7% to 91.2% of total plasma radioactivity in males and from 2.1% to 81.8% of total plasma radioactivity in females). No other plasma metabolites were detected in females, whereas difluoroethyl-amino-furanone and flupyradifurone-OH were identified in male plasma (up to 5.6% and 3.0% of total plasma radioactivity, respectively). Two unknown metabolites were detected only in male plasma at 6 hours after dosing (2.8% of total plasma radioactivity).

In the liver, flupyradifurone accounted for 91.1% and 95.6% of total radioactivity detected at 1 hour after dosing in males and females, respectively, declining to 22.8% and 34.6%, respectively, by

24 hours. Similar to plasma, there was a concomitant increase in difluoroacetic acid from 1 to 24 hours after dosing (from 1.1% to 59.4% of total liver radioactivity in males and from 0.6% to 55.9% of total liver radioactivity in females). Difluoroethyl-amino-furanone and flupyradifurone-OH were detected in the liver of both sexes (up to 3.4% and 6.0% of total liver radioactivity, respectively, in males and up to 0.6% and 2.1% of total liver radioactivity, respectively, in females). Flupyradifurone-OH-gluA (isomer 1) was detected only in males (0.8% of total liver radioactivity at 6 hours after dosing). Five unknown metabolites were characterized in males (totalling 3.2% of total liver radioactivity), and six were characterized in females (totalling 1.7% of total liver radioactivity).

In kidney, flupyradifurone accounted for 89.7% and 95.8% of total radioactivity detected at 1 hour after dosing in males and females, respectively, declining to 25.9% and 38.2%, respectively, by 24 hours. Similar to plasma and liver, there was a concomitant increase in difluoroacetic acid from 1 to 24 hours after dosing (from 0.9% to 63.2% of total kidney radioactivity in males and from 0.8% to 54.1% of total kidney radioactivity in females). Flupyradifurone-OH was identified in both sexes (up to 11.5% of total kidney radioactivity in males and up to 4.1% of total kidney radioactivity in females). Difluoroacetic acid was identified only in males at 1 and 6 hours after dosing (1.8% and 5.3% of total kidney radioactivity, respectively). Three unknown metabolites comprising 3% of total kidney radioactivity were characterized only in males.

Compounds detected in muscle included flupyradifurone (94.2–35.9% of total radioactivity in males from 1 to 24 hours after dosing; 97.4–32.9% of total radioactivity in females from 1 to 24 hours after dosing), difluoroacetic acid (0.9–59.5% of total radioactivity in males from 1 to 24 hours after dosing; 0.8–64.8% of total radioactivity in females from 1 to 24 hours after dosing), difluoroethyl-amino-furanone (up to 5.2% of total radioactivity in males and 1.2% in females) and flupyradifurone-OH (up to 3.6% of total radioactivity in males and 1.6% in females). No unknown metabolites were characterized.

In fat, flupyradifurone accounted for 89.7% and 100% of total radioactivity detected at 1 hour after dosing in males and females, respectively, declining to 28% and 27.1%, respectively, by 24 hours. Difluoroacetic acid was identified from 6 to 24 hours after dosing (from 10.3% to 68.9% of total radioactivity in males and from 4.7% to 68.4% of total radioactivity in females). The only other metabolites detected were in males at 6 hours after dosing and included flupyradifurone-OH (3.7% of total radioactivity) and one unknown metabolite (7.1% of total radioactivity).

The proposed metabolic pathway of flupyradifurone in rats is given in Fig. 3. The principal metabolic reactions of flupyradifurone in rats were:

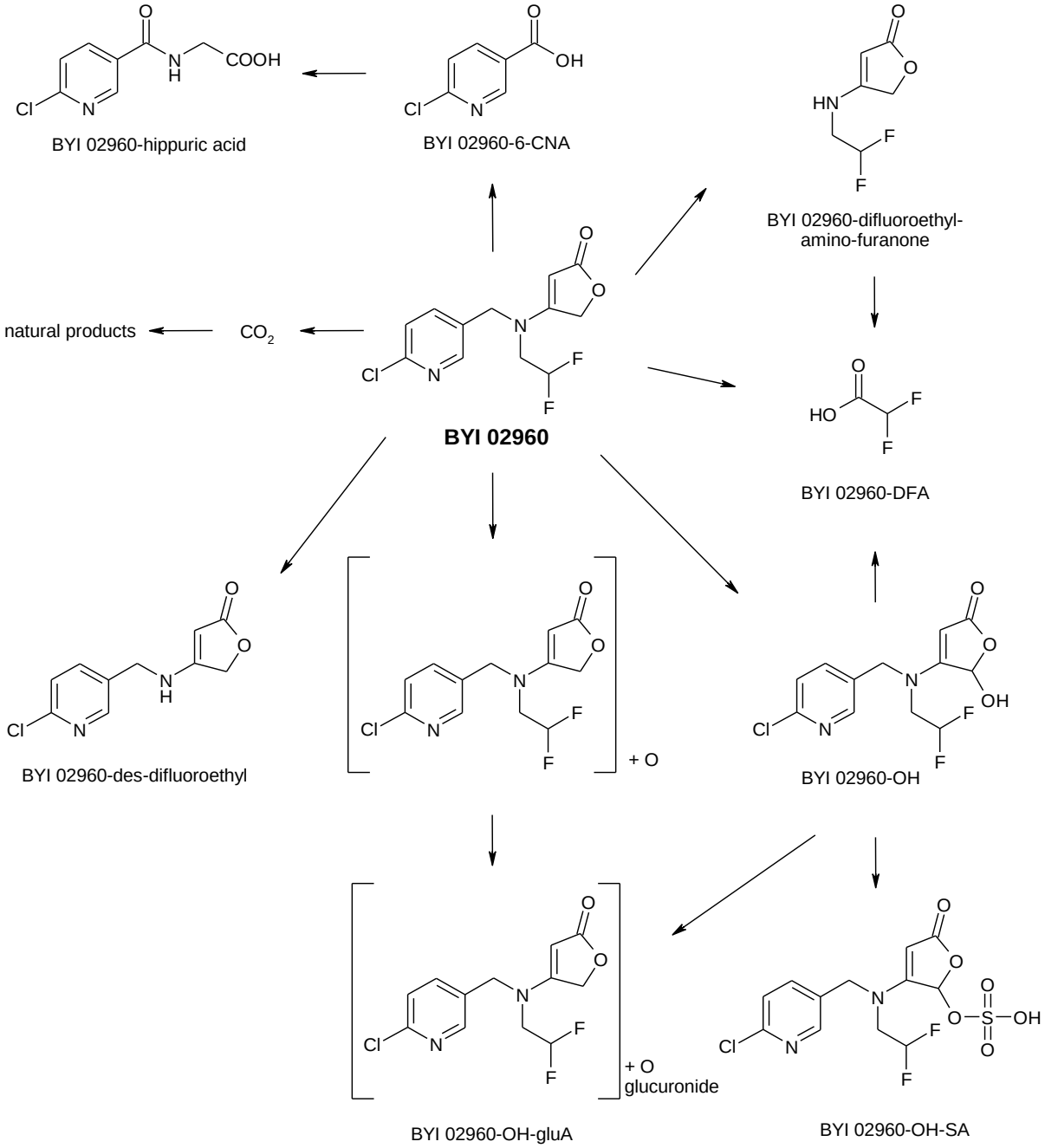
- hydroxylation followed by conjugation with glucuronic acid or sulfate;
- cleavage of the difluoroethyl group, forming flupyradifurone-des-difluoroethyl and difluoroacetic acid; and
- cleavage of the molecule at the pyridinylmethylene bridge, forming 6-chloronicotinic acid, which is further conjugated with glycine to hippuric acid and difluoroethyl-amino-furanone.

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity tests on flupyradifurone, including skin and eye irritation and skin sensitization studies, are summarized in Table 12. In the acute oral study by Gillissen (2009a), clinical signs were observed at 300 mg/kg bw (breathing sounds; all rats from 50 minutes to 7 hours after dosing) and 2000 mg/kg bw (decreased mobility, tremor, piloerection, laboured breathing and clonic cramps from 20 minutes to 5 days after dosing). In the acute inhalational study by Folkerts (2010), transient clinical signs (to 3 days after exposure) were observed at 5 mg/L, which included increased breathing rate, laboured breathing or irregular breathing patterns, piloerection, reduced mobility, tremor, limp, high-legged gait, exophthalmia, red encrustations on the nose, stridor and abdominal position with uncoordinated movements and hypothermia.

Fig. 3. Proposed metabolic pathway of flupyradifurone in rats



2.2 Short-term studies of toxicity

Mice

In a preliminary toxicity study by Blanck (2007), flupyradifurone (99.7% purity) was admixed in the diet at a concentration of 0, 300, 500 or 1200 ppm and fed ad libitum to groups of C57BL/6J mice (five of each sex per group) for 30 days. There was some degradation of the test compound in the 500 ppm diet (18% over 34 days frozen and 8 days at room temperature). The estimated achieved doses following correction for 80% recovery were, respectively, 0, 40, 78 and 207 mg/kg bw per day for males and 0, 47, 98 and 192 mg/kg bw per day for females.

Table 12. Results of studies of the acute toxicity of flupyradifurone

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	HsdCpd:Wu	Female	Oral	96.2	2% Cremophor EL	LD_{50}: > 300–2 000	Gillissen (2009a)
Rat	HsdCpd:Wu	Male and female	Dermal	96.2	Moistened with water	LD_{50}: > 2 000	Gillissen (2009b)
Rat	HsdCpd:Wu	Male and female	Inhalation	96.2	50% (w/w) in PEG 400	LC_{50}: > 4.67	Folkerts (2010)
Rabbit	Crl:KBL(NZW)BR	Female	Skin irritation	96.2	Moistened with water	Not irritating	Gmelin (2009a)
Rabbit	Crl:KBL(NZW)BR	Female	Eye irritation	96.2	Nil (pulverized solid)	Not irritating	Gmelin (2009b)
Mouse	Hsd Win:NMRI	Female	LLNA	96.2	DMF	Not sensitizing up to the maximum tested concentration of 50%	Vohr (2009)

bw: body weight; DMF: *N*,*N*-dimethylformamide; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; LLNA: local lymph node assay; PEG: polyethylene glycol; w/w: weight per weight

There were no deaths or treatment-related clinical signs. At 1200 ppm in males, mean body weight gain over the first week of exposure was significantly lower ($P < 0.05$) than the control value (−0.03 g versus 0.14 g, respectively), with mean body weight slightly lower than the control value on day 8 (6%, not statistically significant) and cumulative body weight gain (to day 29) 15% lower than the control value (not statistically significant). No treatment-related effects on body weight or body weight gain occurred in females or at lower doses. There was no effect on feed consumption. There was no treatment-related effect on clinical chemistry parameters; significant increases in alanine aminotransferase (ALAT) (+43%, $P < 0.05$) and alkaline phosphatase (ALP) (+21%, $P < 0.05$) activities in females at 1200 ppm were attributable to individual animal variation. There were no effects on organ weights, gross findings or histopathological findings.

Flupyradifurone (99.5% purity) was admixed in the diet at a concentration of 0, 100, 500 or 2500 ppm and fed ad libitum to C57BL/6J mice (10 of each sex per group) for at least 90 days. The achieved doses were 0, 15.6, 80.6 and 407 mg/kg bw per day, respectively, for males and 0, 18.8, 98.1 and 473 mg/kg bw per day, respectively, for females. Observations for deaths and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Blood was sampled on the day of termination (day 93, 94 or 95) for the analysis of clinical chemistry parameters. There was no analysis of haematology parameters, and there was no urine analysis. Blood was sampled during week 12 for the analysis of flupyradifurone. Following termination, mice were necropsied, organs weighed and tissues examined histopathologically.

The concentrations of flupyradifurone in plasma collected during week 12 were 1.94, 10.8 and 42.8 mg/L at 100, 500 and 2500 ppm, respectively, in males and 1.23, 8.72 and 34.0 mg/L at 100, 500 and 2500 ppm, respectively, in females.

There were no treatment-related deaths or clinical signs. Significantly lower ($P < 0.01$) body weight (both sexes) and body weight gain (males only) occurred consistently at 2500 ppm (Table 13), with no adverse effect on body weight parameters occurring at lower doses. Over the first 3 weeks of the study, feed consumption was 10–11% lower than the control value only in females at 2500 ppm. At 2500 ppm, there were a number of adverse effects on clinical chemistry parameters, including reduced cholesterol, total protein and albumin levels, increased ALP, ALAT and aspartate aminotransferase (ASAT) activities and increased urea levels (Table 13). At necropsy, the liver of six high-dose females was pale, and this was coincident with significantly elevated ($P < 0.01$) absolute liver weight, relative (to body weight) liver weight and relative (to brain weight) liver weight (Table 13). In high-dose males, relative liver weight was significantly higher ($P < 0.01$) than the control value. Histopathological examination revealed a slight increase in the severity of diffuse hepatocellular vacuolation at 2500 ppm in both sexes (Table 13).

Table 13. Effects in mice exposed to flupyradifurone for 90 days

	Males				**Females**			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**
Body weight (% of control)								
Week 1	0	98	98	93**	0	98	97	93**
Week 4	0	100	98	89**	0	98	97	93**
Week 8	0	100	98	90**	0	100	97	94**
Body weight gain (% of control)								
Weeks 1–4	0	97	79	18**	0	97	98	74
Weeks 1–8	0	96	86	49**	0	104	85	85
Days 1–92	0	96	90	57**	0	104	89	93
Clinical chemistry parameters[a]								
Total cholesterol (mmol/L)	1.91	1.87 (−2%)	1.77 (−7%)	1.34** (−30%)	1.52	1.50 (−1%)	1.42 (−7%)	1.16** (−24%)
ALP (IU/L)	76	74 (−3%)	74 (−3%)	105** (+38%)	131	129 (−2%)	134 (+2%)	144 (+10%)
ALAT (IU/L)	27	32 (+19%)	32 (+19%)	35 (+30%)	36	38 (+6%)	38 (+6%)	74* (+106%)
ASAT (IU/L)	88	91 (+3%)	95 (+8%)	105 (+19%)	130	129 (−1%)	132 (+2%)	177 (+36%)
Urea (mmol/L)	11.14	14.04 (26%)	13.98 (+25%)	16.78** (+51%)	12.76	13.31 (+4%)	14.79 (+16%)	15.23* (+19%)
Total protein (g/L)	59	57 (−3%)	59	56.4* (−4%)	58	58	57 (−2%)	55* (−5%)
Albumin (g/L)	34	34	38 (+12%)	33 (−3%)	36	35 (−3%)	35 (−3%)	33** (−8%)

Table 13 (continued)

Parameter	Males				Females			
	0 ppm	100 ppm	500 ppm	2 500 ppm	0 ppm	100 ppm	500 ppm	2 500 ppm
Organ weights[a]								
Absolute liver weight (g)	0.96	1.02 (+6%)	0.95 (−1%)	1.02 (+6%)	0.82	0.82	0.84 (+2%)	0.92** (+12%)
Relative liver weight (%)	4.17	4.42 (+6%)	4.21 (+1%)	4.99** (+20%)	4.47	4.50 (+1%)	4.61 (+3%)	5.26** (+18%)
Liver to brain weight ratio (%)	214.94	223.16 (+4%)	215.17	229.92 (+7%)	181.52	179.34 (−1%)	191.00 (+5%)	205.395** (+13%)
Diffuse hepatocellular vacuolation (n = 10)								
Minimal	6	3	8	0	7	7	6	2
Slight	4	7	2	6	3	3	3	3
Moderate	0	0	0	4	0	0	0	5
Total	10	10	10	10	10	10	9	10

ALAT: alanine aminotransferase; ALP: alkaline phosphatase; ASAT: aspartate aminotransferase; IU: International Units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Odin-Feurtet (2009a)

The no-observed-adverse-effect level (NOAEL) was 500 ppm (equal to 80.6 mg/kg bw per day for males and 98.1 mg/kg bw per day for females), for reduced body weight and body weight gain, perturbations in clinical chemistry parameters, increased liver weight parameters and hepatocellular vacuolation at 2500 ppm (equal to 407 mg/kg bw per day for males and 473 mg/kg bw per day for females) (Odin-Feurtet, 2009a).

Rats

In a range-finding study by Capt (2007), flupyradifurone (98.3% purity) in corn oil supplemented with 10% ethanol and 10% water was administered by gavage to groups of five Wistar Rj:WI(IOPS HAN) rats of each sex at a dose of 0, 75, 200 or 350 mg/kg bw per day for 30 or 31 days. Rats were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly. Blood was sampled on day 30 or 31 for the analysis of haematology and clinical chemistry parameters. Following termination on day 30 or 31, rats were necropsied, organs were weighed and histopathology was performed. Liver microsomal preparations were analysed for cytochrome P450 (CYP) isoenzymes.

At 200 mg/kg bw per day, one female was found dead on day 2, whereas at 350 mg/kg bw per day, two females were found dead on day 6. Salivation was observed in all treated rats from day 2 at 200 and 350 mg/kg bw per day and from day 13 at 75 mg/kg bw per day, but did not occur in the control group. There were no significant effects on body weight or body weight gain. During the first week of treatment, mean feed consumption was 16% and 29% lower than the control values ($P < 0.01$) in females at 200 and 350 mg/kg bw per day, respectively. Over this same period, the mean feed consumption of males was 17% lower than the control value ($P < 0.01$) at 350 mg/kg bw per day. There were no treatment-related haematological findings. Perturbations in clinical chemistry parameters occurred consistently at 200 and 350 mg/kg bw per day and included significantly reduced total bilirubin (both sexes) and glucose levels (males), increased triglyceride levels (both sexes) and

increased creatinine levels and ALAT and ALP activities (females) (Table 14). Reductions in bilirubin levels are not considered toxicological relevant.

Table 14. Effects in rats exposed to flupyradifurone for 30 or 31 days

	Males				Females			
Parameter	**0 mg/kg bw per day**	**75 mg/kg bw per day**	**200 mg/kg bw per day**	**350 mg/kg bw per day**	**0 mg/kg bw per day**	**75 mg/kg bw per day**	**200 mg/kg bw per day**	**350 mg/kg bw per day**
Clinical chemistry parameters[a]								
Total bilirubin (µmol/L)	1.2	0.7* (−42%)	0.6* (−50%)	0.4** (−67%)	1.6	0.9 (−44%)	0.7* (56%)	0.5** (−69%)
Glucose (mmol/L)	5.42	4.60 (−15%)	3.92** (−28%)	2.87** (−47%)	4.80	5.07 (+6%)	5.12 (+7%)	3.57 (−26%)
Triglycerides (mmol/L)	1.14	1.50 (+32%)	1.32 (+16%)	1.89 (+66%)	0.45	0.46 (+2%)	0.87 (+93%)	1.51* (+236%)
Creatinine (µmol/L)	50	51 (+2%)	54* (+8%)	53 (+6%)	51	56 (+10%)	63** (+24%)	63* (+24%)
ALAT (IU/L)	39	43 (+10%)	38 (−3%)	46 (+18%)	33	35 (+6%)	43** (+30%)	51** (+55%)
ALP (IU/L)	165	163 (−1%)	166 (+1%)	165	95	110 (+16%)	96 (+1%)	129* (+36%)
Organ weights[a]								
Absolute liver weight (g)	10.9	10.0 (−8%)	12.0 (+10%)	12.4 (+14%)	5.86	6.37 (+9%)	7.32 (+25%)	8.74** (+49%)
Relative liver weight	3.10	2.91 (−6%)	3.44 (+11%)	3.81* (+23%)	2.55	2.85 (+12%)	3.16* (+24%)	3.70** (+45%)
Liver to brain weight ratio	542.7	482.5 (−11%)	602.6 (+11%)	625.7 (+15%)	315.1	328.8 (+4%)	382.3 (+21%)	460.6** (+46%)
Macroscopic findings in the liver								
Enlargement	0/5	0/5	1/5	3/5	0/5	0/5	0/4	3/3
Prominent lobules	0/5	1/5	2/5	5/5	0/5	0/5	0/4	2/3
Histopathology								
Centrilobular or diffuse hypertrophy of hepatocytes	0/5	0/5	5/5	5/5	0/5	0/5	3/4	3/3
Diffuse follicular cell hypertrophy in the thyroid	0/5	0/5	4/5	5/5	0/5	0/5	0/4	3/3

ALAT: alanine aminotransferase; ALP: alkaline phosphatase; bw: body weight; IU: International Units; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Capt (2007)

In females, mean absolute liver weight was increased by 49% compared with the control value ($P < 0.01$) at 350 mg/kg bw per day, with mean relative liver weight increased by 24% ($P < 0.05$) and 45% ($P < 0.01$) at 200 and 350 mg/kg bw per day, respectively. In males, relative liver weight was increased by 23% ($P < 0.05$) at 350 mg/kg bw per day. The liver to brain weight ratio was increased by 46% ($P < 0.01$) in females at 350 mg/kg bw per day. At necropsy, liver enlargement was noted at 200 mg/kg bw per day (males) and 350 mg/kg bw per day (both sexes), whereas prominent lobulation was observed at every dose in males and at 350 mg/kg bw per day in females. Microscopically, centrilobular or diffuse hypertrophy of hepatocytes was observed at 200 and 350 mg/kg bw per day in both sexes, which was graded as minimal to marked and exhibited a dose–response relationship. In the thyroid, minimal or slight follicular cell hypertrophy was revealed at 200 mg/kg bw per day (males) and 350 mg/kg bw per day (both sexes).

CYP3A was induced, as shown by the increased benzoxyresorufin *O*-dealkylase (BROD) activity at and above 75 mg/kg bw per day in males (8-, 25- and 25-fold increases at 75, 200 and 350 mg/kg bw per day, respectively) and at 200 and 350 mg/kg bw per day in females (8- and 28-fold increases, respectively). There was no treatment-related increase in ethoxyresorufin *O*-deethylase (EROD) or pentoxyresorufin *O*-dealkylase (PROD) activities.

In a second range-finding study, groups of five male Wistar Rj:WI(IOPS HAN) rats were exposed to flupyradifurone (99.7% purity) in the diet at a concentration of 0, 410 or 5000 ppm (equal to doses of 0, 33.6 and 385 mg/kg bw per day, respectively) for 30 days. Observations for deaths and clinical signs were made twice daily. Body weight and feed consumption were recorded weekly. Blood was sampled prior to termination on day 30 for the analysis of clinical chemistry parameters; thyroxine (T_4) and thyroid stimulating hormone (TSH) were also analysed. Following termination on day 30, rats were necropsied, organs were weighed and histopathology was performed. Liver microsomal preparations were analysed for CYP isoenzymes.

There were no deaths or treatment-related clinical signs. At 410 ppm, there was no effect on body weight or feed parameters. At 5000 ppm, mean body weight and body weight gain were significantly lower ($P < 0.01$) than the control values (17–19% lower body weight; 38% lower body weight gain from days 1 to 29), whereas feed consumption was 12–39% lower ($P < 0.01$). There was no effect on clinical chemistry parameters at 410 ppm. At 5000 ppm, there were reductions in glucose (−46%, $P < 0.01$) and total bilirubin levels (−73%, $P < 0.05$) and increases in urea (+37%, $P < 0.01$) and total cholesterol levels (+41%, $P < 0.01$). At 5000 ppm, there was a treatment-related increase in TSH (+81%), which was not statistically significant; T_4 was unaffected by treatment. Treatment-related organ weight findings occurred only at 5000 ppm and included increased relative liver weight (+42%, $P < 0.01$) and relative thyroid weight (+32%, $P < 0.01$).

Necropsy revealed prominent lobules in the liver in two and four rats at 410 and 5000 ppm, respectively. Microscopically, centrilobular or diffuse hepatocellular hypertrophy with slight (2/5 rats) to moderate (3/5 rats) intensity was found at 5000 ppm. At the same dose, diffuse follicular cell hypertrophy was observed in the thyroid, which was graded as minimal (2/5 rats) to slight (3/5 rats). CYP3A was slightly induced (by approximately 2-fold above the control), as shown by the increase in BROD activity at 5000 ppm. However, technical issues limit the reliability of this result. There was no treatment-related increase in EROD or PROD activity (Blanck, 2008).

Flupyradifurone (99.5% weight per weight [w/w] purity) was admixed in the diet at a concentration of 0, 100, 500 or 2500 ppm and fed ad libitum to groups of 10 Wistar Rj:WI (IOPS HAN) rats of each sex for at least 90 days. The achieved doses were 0, 6.0, 30.2 and 156 mg/kg bw per day, respectively, for males and 0, 7.6, 38.3 and 186 mg/kg bw per day, respectively, for females. To investigate the reversibility of effects, an additional 10 rats of each sex were fed control or high-dose diet for at least 90 days and subsequently fed control diet for at least 28 days. Rats were examined daily for deaths and clinical signs. A neurotoxicity assessment was conducted on all rats during weeks 12–13, consisting of exploratory locomotor activity, open-field observations, sensory

reactivity and grip strength. Body weight was recorded weekly. Feed consumption was recorded twice weekly until week 6 and weekly thereafter. An ophthalmological examination was performed pretreatment and during week 13. Blood was sampled prior to termination (days 95–97 for the main phase and day 30 or 31 for the recovery phase) for the analysis of haematology and clinical chemistry parameters; an additional blood sample was collected from five rats of each sex per group for the analysis of flupyradifurone and its metabolites in blood. Urine was collected on day 90 or 91 for urine analysis. Following termination, all rats were necropsied, organs were weighed and histopathology was performed.

There were no deaths, treatment-related clinical signs or evidence of neurotoxicity. Mean body weight and body weight gain were significantly lower ($P < 0.01$) than the control values at 2500 ppm in both sexes (Table 15). In the absence of any other treatment-related effects at 500 ppm, including effects on absolute body weight or feed consumption, the significantly lower ($P < 0.05$) body weight gain in females over the later weeks of the study was not considered toxicologically significant. After cessation of exposure, the mean body weight of high-dose rats was comparable with the control value after a 28-day recovery phase. At 2500 ppm, there was an initial reduction in feed consumption in males during the first week of treatment (17%, $P < 0.01$), whereas in females, feed consumption was 9–29% lower than control values ($P < 0.05$ or 0.01) until week 7. There were no treatment-related ophthalmological findings.

Table 15. Effects in rats exposed to flupyradifurone for 90 days

	Males				Females			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**
Body weight (% of control)								
Week 1	0	100	98	96**	0	10	97	90**
Week 4	0	102	99	94**	0	102	98	94**
Week 8	0	103	99	93**	0	101	96	90**
Body weight gain (% of control)								
Weeks 1–4	0	103	98	86**	0	107	91	80**
Weeks 1–8	0	106	98	89**	0	105	89*	84**
Days 1–92	0	105	98	88**	0	100	88*	85**
Clinical chemistry parameters[a]								
Total bilirubin (µmol/L)	1.3	1.1 (−15%)	1.1 (−15%)	0.8** (−38%)	2.0	1.91 (−4%)	1.8 (−10%)	1.1** (−45%)
Glucose (mmol/L)	6.59	6.56	6.19 (−6%)	5.23** (−21%)	6.03	5.82 (−3%)	5.58 (−7%)	4.69** (−22%)
Triglycerides (mmol/L)	1.54	1.78 (+16%)	1.70 (+10%)	1.97 (+28%)	1.55	1.68 (+8%)	1.86 (+20%)	2.26** (+46%)
Organ weights[a]								
Absolute liver weight (g)	10.92	11.37 (+4%)	10.92	11.78 (+8%)	6.18	5.91 (−4%)	5.93 (−4%)	6.65 (+8%)
Relative liver weight (%)	2.19	2.23 (+2%)	2.26 (+3%)	2.55** (+16%)	2.29	2.17 (−5%)	2.28	2.62** (+14%)

Table 15 (continued)

Parameter	Males				Females			
	0 ppm	100 ppm	500 ppm	2 500 ppm	0 ppm	100 ppm	500 ppm	2 500 ppm
Liver to brain weight ratio (%)	504.82	527.86 (+5%)	494.46 (−2%)	546.07 (+8%)	303.73	290.22 (−4%)	294.53 (−3%)	333.35 (+10%)
Absolute thyroid weight (g)	0.019 5	0.022 2 (+14%)	0.022 8 (+17%)	0.023 4 (+20%)	0.016 2	0.016 2	0.016 5 (+2%)	0.017 1 (+6%)
Relative thyroid weight (%)	0.0039	0.004 4 (+13%)	0.004 7** (+21%)	0.004 9** (+26%)	0.006 0	0.006 0	0.006 3 (+5%)	0.006 8 (+13%)
Thyroid to brain weight ratio (%)	0.903	1.028 (+14%)	1.030 (+14%)	1.083 (+20%)	0.799	0.794 (−1%)	0.816 (+2%)	0.860 (+8%)
Histopathology (*n* = 10)								
Centrilobular or diffuse hypertrophy of hepatocytes								
- Minimal	0	0	0	6	0	0	0	3
- Slight	0	0	0	4	0	0	0	0
- Total	0	0	0	10	0	0	0	3
Diffuse follicular cell hypertrophy in the thyroid								
- Minimal	0	0	0	3	0	0	0	0
- Total	0	0	0	3	0	0	0	0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Odin-Feurtet (2009b)

Haematological findings were generally unremarkable; there was a slight increase in mean platelet counts (+15%, $P < 0.05$) in high-dose females, which was reversible during the 28-day recovery phase. Effects on clinical chemistry parameters were confined to the highest dose and included reduced bilirubin and glucose levels and increased cholesterol and triglyceride levels (Table 15). With the exception of total bilirubin in high-dose females, which remained significantly lower than the control value at the end of the 28-day recovery phase (−25%, $P < 0.05$), all these effects were reversible. At 2500 ppm, mean relative liver weights were 16% and 15% higher than the control values ($P < 0.01$) in males and females, respectively. In males, relative thyroid weights were significantly higher than the control values at 500 ($P < 0.05$) and 2500 ppm ($P < 0.01$) although the effect at 500 ppm was not considered toxicologically significant, as there were no corresponding histopathological changes.

At necropsy, an enlarged liver was observed in four males and one female at 2500 ppm. Also at 2500 ppm, a dark thyroid gland was observed in one male, which was coincident with follicular cell hypertrophy. Treatment-related histopathological changes were centrilobular or diffuse hypertrophy of hepatocytes (males and females at 2500 ppm) and diffuse follicular cell hypertrophy in the thyroid (males at 2500 ppm) (Table 15). After the recovery period, no treatment-related liver or thyroid changes were observed.

The NOAEL was 500 ppm (equal to 30.2 mg/kg bw per day for males and 38.3 mg/kg bw per day for females), for reduced body weight and body weight gain (both sexes) and thyroid follicular cell hypertrophy at 2500 ppm (equal to 156 mg/kg bw per day for males and 186 mg/kg bw per day for females) (Odin-Feurtet, 2009b).

Dogs

In a preliminary study by Odin-Feurtet (2008), flupyradifurone (99.5% purity) was admixed in the diet at a concentration of 0, 500, 2000 or 4000 ppm, and 400 g was offered to groups of two Beagle dogs of each sex for at least 28 days. The achieved doses were 0, 16, 62 and 118 mg/kg bw per day for males and 0, 18, 77 and 131 mg/kg bw per day for females, respectively. Observations for mortality and clinical signs were made at least daily. Body weight and feed consumption were recorded weekly. Ophthalmoscopy was performed pretreatment and at study termination (day 29 or 30). Blood was sampled pretreatment, on day 13 or 14 and on day 27 for the analysis of clinical chemistry parameters. Urine was collected pretreatment and on days 14 and 28 for urine analysis. Following termination on day 29 or 30, dogs were necropsied, organs weighed and tissues examined histopathologically.

There were no deaths or treatment-related clinical signs. Whereas control dogs gained 0.5–1.1 kg bw over the exposure period, one 4000 ppm male lost 0.2 kg bw and the other male gained no weight. One 4000 ppm female gained 0.7 kg bw, whereas the other female gained none. There was no effect on body weight at 500 or 2000 ppm. Although highly variable, feed consumption tended to be lower than the control values at 4000 ppm. At 4000 ppm and relative to pretreatment values, mean platelet counts were increased in both females (+36% or +43%) and one male (+30%). An increase in serum creatinine level in one 4000 ppm female (+31% relative to pretreatment value) was considered an incidental finding owing to the absence of any corroborating findings. Urine analysis and organ weights were unremarkable. At necropsy, both 4000 ppm females showed enlarged thyroids, but there were no accompanying organ weight or histopathological changes. Glycogen accumulation in hepatocytes was reduced in its incidence and/or severity at 2000 ppm (males) and 4000 ppm (both sexes), but was not a toxicologically significant finding.

In a 90-day toxicity study by Eigenberg (2010), groups of four Beagle dogs of each sex were offered 400 g diet a day, which contained 0, 400, 1200 or 3600 ppm flupyradifurone (99.5% purity). The high dose was reduced to 2400 ppm from week 9 due to clinical signs in two dogs and continued weight loss in the high-dose group. The achieved doses were 0, 12, 33 and 102/85 mg/kg bw per day for males and 0, 12, 41 and 107/78 mg/kg bw per day for females, respectively. Observations for mortalities and clinical signs were made daily, with a more comprehensive clinical assessment performed weekly. Body weight and feed consumption were recorded weekly. Blood and urine were collected pretreatment and during weeks 5, 9 and 13 for the analysis of haematology, clinical chemistry or urine analysis parameters. Ophthalmoscopy was performed pretreatment and prior to termination during week 13. Following scheduled termination on day 91, 92 or 93, dogs were necropsied and their organs weighed and examined histopathologically.

There were no deaths. Treatment-related clinical signs occurred only at the highest dose (3600 ppm) and included unsteady and stiff back legs in one male (days 44, 53 and 54) and one female (day 44). As a consequence of these clinical signs, the highest dose was reduced to 2400 ppm. Mean body weight and body weight gain were lower than the control values at the middle and high doses (Table 16). There was a trend of reduced feed consumption at the middle and high doses, which was most evident during the first 4 weeks of exposure (up to 35% lower in males and 52% in females); there were only a few instances where these differences were statistically significant. There were no treatment-related ophthalmological findings.

Table 16. Effects in dogs exposed to flupyradifurone for 90 days

	Males				Females			
Parameter	**0 ppm**	**400 ppm**	**1 200 ppm**	**3 600/2 400 ppm**	**0 ppm**	**400 ppm**	**1 200 ppm**	**3 600/ 2 400 ppm**
Body weight (% of control)								
Week 1	0	102	96	97	0	98	95	94
Week 8	0	105	92	86*	0	98	96	84
Week 9	0	105	93	88*	0	100	96	87
Final	0	105	91	89	0	95	93	87
Body weight gain (g)								
Weeks 0–1	188.7	364.0	50.3	−252	500.7	140.5	96.8	−205.3
Weeks 0–9	809.7	1 324.5	265.5	−538	1 241	1 058	810.3	−69.3
Weeks 9–12	−33.2	−42.7	−20.5	117	84	−294.5	−139.5	70.0
Overall	777	1 282	60*	−421*	1 325	774.8	670.8	0.8*
Clinical chemistry parameters[a]								
CPK (U/L)	242	248 (+2%)	2 834[§] (+1 070%)	2 944[§] (+1 117%)	282	175* (−38%)	1 538[§] (+445%)	4 836* (+1 615%)
ASAT (U/L)	38	36 (−5%)	241 (+534%)	266[§] (+600%)	42	31* (−26%)	130* (+210%)	370* (+781%)
ALAT (U/L)	30	36 (+20%)	221 (+637%)	311 (+937%)	28	27 (−4%)	151* (+439%)	552* (+1 871%)
Organ weights[a]								
Absolute liver weight (g)	269	260 (−3%)	261 (−3%)	307* (+14%)	226	201 (−11%)	225	252 (+12%)
Relative liver weight (%)	2.8	2.5 (−10%)	2.9 (+4%)	3.5* (+25%)	2.9	2.6 (−10%)	3.0 (+3%)	3.6* (+24%)
Absolute kidney weight (g)	47	55 (+17%)	51 (+9%)	55 (+17%)	38	34 (−11%)	36 (−5%)	38
Relative kidney weight (%)	0.49	0.54 (+10%)	0.57* (+16%)	0.63* (+29%)	0.47	0.44 (−6%)	0.49 (+4%)	0.55 (+17%)
Histopathology – focal myofibril degeneration/atrophy in the skeletal muscle (n = 4)								
Minimal	0	0	2	1	0	0	3	4
Slight	0	0	0	1	0	0	1	0
Total	0	0	2	2	0	0	4	4

ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; CPK: creatinine phosphokinase; ppm: parts per million; U: Units; *: $P < 0.05$; **: $P < 0.01$: [§]: $P > 0.05$ due to high intragroup variation

[a] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Eigenberg (2010)

Adverse effects on haematology parameters occurred only at the highest dose and included significantly reduced ($P < 0.05$) haemoglobin (males at day 28, −9%; both sexes at day 56, −13% in male and −21% in females), haematocrit (males at days 28, 56 and 84, −9% to −14%; females at day 56, −18%) and erythrocytes (females at day 56, −9%). Creatinine phosphokinase (CPK), ASAT and ALAT activities were elevated in the mid- and high-dose groups at day 56 by up to an order of magnitude above the control values (Table 16), with no treatment-related elevations determined at other sampling points. There were no treatment-related urine analysis findings.

There were no treatment-related macroscopic abnormalities. At the highest dose, absolute (males) and relative (both sexes) liver weights were significantly higher ($P < 0.05$) than control values. Histopathological examination revealed brown pigment in Kupffer cells in the liver of two high-dose females. In males, relative kidney weight was significantly higher than the control values at the middle and high doses, but was not accompanied by any histopathological abnormalities. Microscopically, myofibril degeneration/atrophy of the skeletal muscle was detected at the middle and high doses in both sexes. Relative kidney weight was increased in males at the middle and high doses, but there were no accompanying histopathological findings (Table 16).

The NOAEL was 400 ppm (equal to 12 mg/kg bw per day for males and females), for skeletal muscle degeneration and increases in liver enzymes at 1200 ppm (equal to 33 mg/kg bw per day for males and 41 mg/kg bw per day for females).

Flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 150, 300 or 1000 ppm, and 400 g/day was offered to groups of four Beagle dogs of each sex for at least 1 year. The achieved doses were 0, 4.6, 7.8 and 28.1 mg/kg bw per day, respectively, for males and 0, 4.1, 7.8 and 28.2 mg/kg bw per day, respectively, for females. Observations for mortalities and clinical signs were made at least daily, with a more comprehensive clinical assessment performed weekly covering posture, gait and motor function, muscle tone, mental state, level of consciousness, behavioural change and a general examination of the head. Body weight and feed consumption were recorded weekly. Ophthalmoscopy was performed pretreatment and prior to termination. Blood and urine were collected pretreatment and at 3, 6, 9 and 12 months for the analysis of haematology, clinical chemistry or urine analysis parameters. On day 141, blood was collected from one male and one female from the control and 300 ppm groups at 1, 3 and 8 hours after feeding for the analysis of flupyradifurone in plasma. Following scheduled termination on days 365–368, dogs were necropsied, their organs were weighed and histopathology was performed.

Plasma concentrations of flupyradifurone were less than or equal to 3.00 µg/mL and were maximal at the 3-hour time point. Treatment-related effects were limited to decreased body weight gain in females at 1000 ppm (approximately 60% lower than the control values over weeks 13–26, 26–52 and 1–52, with terminal body weight 9% lower than the control value, $P = 0.05$). Microscopically, degeneration of the skeletal muscle (gastrocnemius and biceps femoris) was observed in both sexes at 1000 ppm (Table 17). The degeneration was graded as minimal to slight and found as focal to multifocal areas accompanied by one or more of the following changes: atrophy or necrosis of skeletal muscle and/or inflammatory cells surrounding the affected myofibre.

The NOAEL was 300 ppm (equal to 7.8 mg/kg bw per day for both sexes), based on reduced body weight gain (females) and skeletal muscle degeneration (both sexes) at 1000 ppm (equal to 28.1 mg/kg bw per day for males and 28.2 mg/kg bw per day for females) (Cada, 2011).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a combined chronic toxicity and carcinogenicity study by Kennel (2012a), flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 70, 300 or 1500 ppm and fed ad libitum to groups of 60 C57BL/6J mice of each sex. After 52 weeks, 10 mice of each sex per dose allocated to the chronic (12 months) phase were necropsied at the scheduled interim termination. The

Table 17. Histopathology of skeletal muscle in dogs following 52 weeks of dietary administration of flupyradifurone

Observation	Incidence (n = 4)							
	Males				Females			
	0 ppm	150 ppm	300 ppm	1 000 ppm	0 ppm	150 ppm	300 ppm	1 000 ppm
Skeletal muscle (gastrocnemius)								
Degeneration, myofibre	0	0	0	2	0	0	0	2
Muscle, other (biceps femoris)								
Degeneration, myofibre	0	0	0	3	0	0	0	3

ppm: parts per million
Source: Cada (2011)

remaining 50 mice of each sex per dose, allocated to the carcinogenicity (24 months) phase of the study, continued treatment for at least 78 weeks. The mean intake of flupyradifurone over 18 months was 0, 10, 43 and 224 mg/kg bw per day for males and 0, 12, 53 and 263 mg/kg bw per day for females at 0, 70, 300 and 1500 ppm, respectively. Observations for mortality and clinical signs were made daily. Detailed physical examinations, including palpation for masses, were performed at least weekly. Body weight and feed consumption were recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Blood was sampled at approximately 52 and 78 weeks for the analysis of haematology parameters and for the analysis of flupyradifurone in plasma. Following scheduled termination, rats were necropsied, their organs were weighed and histopathology was performed.

There were no treatment-related deaths, clinical signs or effects on haematology parameters. At 1500 ppm, mean body weight was up to 7% lower than the control values in males ($P < 0.01$ on weeks 38, 58, 66 and 70) and 8% lower in females ($P < 0.01$ on week 66). At 1500 ppm, mean body weight gain was significantly lower ($P < 0.01$ or 0.05) than control values throughout most study intervals in males (weeks 1–2, 14–26 and 54–78) and from week 14 onwards in females (weeks 14–26, 26–54 and 54–78). Overall body weight gain was 19% lower than the control value in males and 13% lower than the control value in females at 1500 ppm. Small reductions in feed consumption (< 5%) were not statistically significant. Slightly lower body weight (up to 5%) and body weight gain (up to 10%) at 300 ppm were not considered toxicologically significant because of their small magnitude and/or the lack of statistical significance.

There was a dose-related increase in mean plasma concentrations of flupyradifurone during weeks 52 and 78 (Table 18).

In male mice killed after 12 months (chronic phase), lower terminal body weight (6% lower than the control value), lower absolute kidney weight (−13%, $P < 0.05$), lower kidney to brain weight (−15%, $P < 0.01$) and increased relative brain weight (+9%, $P < 0.05$) were noted at 1500 ppm; the increase in relative brain weight is attributable to the lower terminal body weight. Similar changes did not occur in females at the same dose or in both sexes at lower doses.

At 1500 ppm, the terminal body weight of rats killed after 18 months (carcinogenicity phase) was slightly lower than the control values (−4% in males, $P < 0.05$; −6% in females, not statistically significant). Selected organ weight findings in mice killed after 18 months are summarized in Table 18. Increased absolute liver, relative liver and liver to brain weights occurred in males at 1500 ppm; relative liver weight also increased in females at the same dose. Also in males at 1500 ppm, decreases in absolute kidney, relative kidney and kidney to brain weights occurred.

No treatment-related macroscopic abnormalities were observed in mice killed after 12 months. In mice killed after 18 months, atrophic/small kidneys were observed in males at 1500 ppm (5/42 mice versus 0/38 in the controls, $P < 0.05$). Treatment-related histopathological

Table 18. Findings in mice exposed to flupyradifurone in the diet for up to 78 weeks

	Males				Females			
Parameter	**0 ppm**	**70 ppm**	**300 ppm**	**1 500 ppm**	**0 ppm**	**70 ppm**	**300 ppm**	**1 500 ppm**
Plasma levels (mg/mL)[a]								
Week 52	< 0.025	1.29 ± 0.16	6.05 ± 0.92	27.4 ± 4.42	< 0.025	0.94 ± 0.39	4.01 ± 1.08	24.8 ± 4.39
Week 78	< 0.025	1.45 ± 0.13	6.58 ± 1.28	30.3 ± 1.68	< 0.025	1.03 ± 0.22	3.90 ± 0.97	28.7 ± 3.56
Organ weights[b]								
Absolute liver (g)	1.13	1.13	1.14 (+1%)	1.23** (+9%)	1.29	1.29	1.22 (−5%)	1.29
Relative liver (%)	4.17	4.17	4.17	4.76** (+14%)	5.11	5.17 (+1%)	4.99 (−2%)	5.51** (+8%)
Liver to brain (%)	249.2	248.4	251.2 (+1%)	270.8** (+9%)	273.0	270.0 (−1%)	257.3 (−6%)	273.9
Absolute kidney (g)	0.50	0.51 (+2%)	0.49 (−2%)	0.43** (−14%)	0.44	0.45 (+2%)	0.44	0.42 (−5%)
Relative kidney (%)	1.83	1.90 (+4%)	1.81 (−1%)	1.65*** (−10%)	1.77	1.82 (+3%)	1.80 (+2%)	1.78 (+1%)
Liver to kidney (%)	109.5	113.2 (+3%)	108.8 (−1%)	94.2** (−14%)	93.9	95.1 (+1%)	92.2 (−2%)	88.6 (−6%)
Microscopic findings – carcinogenicity phase (*n* = 50)								
Centrilobular or diffuse vacuolation of hepatocytes								
- Minimal	10	16	2	0	0	0	0	0
- Slight	16	16	22	5	0	1	0	1
- Moderate	2	2	12	31	0	1	0	3
- Marked	0	0	0	5	1	0	0	0
- Total	28	34	36	41**	1	2	0	4

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean ± 1 standard deviation.

[b] Eighteen-month organ weights expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Kennel (2012a)

findings were observed in the liver only at the highest dose and included increased centrilobular or diffuse vacuolation of hepatocytes in males (Table 18).

No treatment-related neoplastic changes were observed.

The NOAEL for chronic toxicity was 300 ppm (equal to 43 mg/kg bw per day for males and 53 mg/kg bw per day for females), for reduced body weight and body weight gain at 1500 ppm (equal to 224 mg/kg bw per day for males and 263 mg/kg bw per day for females). The NOAEL for carcinogenicity was 1500 ppm (equal to 224 mg/kg bw per day for males and 263 mg/kg bw per day for females), the highest dose tested (Kennel, 2012a).

Rats

In a combined chronic toxicity and carcinogenicity study by Garcin (2012), flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 80, 400 or 2000 ppm and fed ad libitum to groups of 70 Wistar Rj:WI (IOPS HAN) rats of each sex. After 52 weeks, 10 rats of each sex per dose allocated to the chronic (12 months) phase were necropsied. The remaining 60 rats of each sex per dose, allocated to the carcinogenicity (24 months) phase of the study, continued treatment for at least 104 weeks. The intake of flupyradifurone from weeks 1 to 105 was 0, 3.16, 15.8 and 80.8 mg/kg bw per day for males and 0, 4.48, 22.5 and 120 mg/kg bw per day for females at 0, 80, 400 and 2000 ppm, respectively.

Observations for mortality and clinical signs were made daily. Detailed physical examinations, including palpation for masses, were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Feed consumption was recorded twice weekly for the first 6 weeks of the study, then approximately weekly up to week 13, then every 4 weeks thereafter. Ophthalmology examinations were performed prior to the commencement of dosing and after approximately 24 months. In addition, ophthalmology examinations were performed on all animals from the control and high-dose groups after approximately 12 months. Blood and urine samples were collected at 4, 6, 12, 18 and 24 months for the analysis of haematology, clinical chemistry or urine analysis parameters. Following scheduled termination, rats were necropsied, their organs were weighed and histopathology was performed.

There were no treatment-related deaths. Slight increases in the incidence of clinical signs were noted at 2000 ppm. During the first year of exposure, these included soiled fur (12.9% versus 7.1% in the controls), hyper-reactivity to external stimuli (7.1% versus 1.4% in the controls) and resistance to handling (15.8% versus 5.7% in the controls) in males and hair loss in females (31.4% versus 11.4% in the controls). During the second year of exposure, the incidences of hair loss (6.7% versus 1.7% in the controls) and soiled fur (31.7% versus 20.7% in the controls) were slightly increased in males.

Toxicologically significant reductions in body weight and body weight gain occurred in males and females at 2000 ppm. In males, mean body weight was 5–7% ($P < 0.01$ or 0.05) lower than the control values from week 1 to week 54, with mean body weight gain significantly lower than the control values during the first week of exposure (−27%, $P < 0.01$) and over the first 3 months of exposure (−13%, $P < 0.05$). In females, mean body weight was 5–17% lower than the control values ($P < 0.05$–0.001) throughout the study, whereas body weight gain was 18–62% lower than the control values ($P < 0.05$–0.001). There was no adverse effect on feed consumption.

Ophthalmological examination revealed an increased incidence of cataracts in 2000 ppm females after 1 year (14.3% versus 4.3% in the controls). After 2 years, the incidences of lens opacity (96% versus 50% in the controls), iris mydriasis (8.3% versus 0% in the controls) and pale retinal fundus (6.3% versus 0% in the controls) were increased in 2000 ppm females. As cataract is a common age-related finding in this particular rat strain and as the historical control range is 81.4–100%, this finding was not considered treatment related.

No toxicologically significant effects on haematological or urinary parameters occurred. Total cholesterol level was significantly elevated ($P < 0.01$) in females at 2000 ppm during the first 12 months of exposure (29%, 35% and 24% higher than the control values at 4, 6 and 12 months, respectively). At 2000 ppm, significantly reduced ($P < 0.01$) glucose levels occurred in both sexes at 4 months (−23% and −18%, respectively) and in males at 6 months (−16%, $P < 0.05$). Similar to other studies, non-toxicologically relevant reductions in total bilirubin occurred at the highest dose.

The analysis of plasma concentrations of flupyradifurone in blood collected at 12 and 24 months is summarized in Table 19 and demonstrated a dose-related increase, with concentrations higher in females than in males.

Table 19. Findings in rats exposed to flupyradifurone in the diet for up to 2 years

	Males				Females			
Parameter	**0 ppm**	**80 ppm**	**400 ppm**	**2 000 ppm**	**0 ppm**	**80 ppm**	**400 ppm**	**2 000 ppm**
Plasma levels (mg/mL)[a]								
12 months	< 0.025	1.29 ± 0.24	5.17 ± 0.38	18.8 ± 0.98	< 0.025	1.36 ± 0.18	7.84 ± 1.86	33.7 ± 3.47
24 months	< 0.025	1.51 ± 0.10	7.19 ± 1.18	21.4 ± 4.47	< 0.025	1.06 ± 0.50	8.20 ± 1.35	30.3 ± 3.80
Organ weights (12 months)[b]								
Absolute liver (g)	13.42	13.22 (−1%)	13.14 (−2%)	14.86 (+11%)	7.85	7.18 (−9%)	7.25 (−8%)	7.22 (−8%)
Relative liver (%)	1.99	1.97 (−1%)	1.95 (−2%)	2.29** (+15%)	2.14	2.19 (+3%)	2.10 (−2%)	2.44** (+14%)
Organ weights (24 months)[b]								
Absolute liver (g)	12.40	12.07 (−3%)	11.97 (−3%)	12.92 (+4%)	8.88	9.25 (+4%)	9.41 (+6%)	8.31 (−6%)
Relative liver (%)	1.97	2.01 (+2%)	1.94 (−2%)	2.22** (+13%)	2.24	2.29 (+2%)	2.28 (+2%)	2.46** (+10%)
Macroscopic findings (12 months)								
Enlarged liver	0/10	0/9	0/9	3/10	1/10	1/9	0/8	0/10
Pale liver	0/10	1/9	0/9	3/10	1/10	1/9	1/8	0/10
Macroscopic findings (24 months)								
Lungs – white foci (moribund and found dead)	4/35	8/39	5/30	7/33	2/31	2/30	2/28	6/14
Lungs – white foci (scheduled termination)	5/25	4/21	4/30	5/27	2/29	10/30	5/32	21/46
Microscopic findings (12 months; n = 10)								
Eosinophilic focus of hepatocellular alteration								
- Minimal	2	6	3	8	0	0	1	1
Tigroid focus of hepatocellular alteration								
- Minimal	1	1	0	3	3	2	1	1

Table 19 (continued)

Parameter	Males				Females			
	0 ppm	80 ppm	400 ppm	2 000 ppm	0 ppm	80 ppm	400 ppm	2 000 ppm
Centrilobular hepatocellular hypertrophy								
- Minimal	0	0	2	7	0	0	0	5
- Slight	0	0	0	3	0	0	0	0
- Total	0	0	2	10	0	0	0	5
Centrilobular or diffuse macrovacuolation of hepatocyte								
- Minimal	0	2	1	6	0	0	0	2
- Slight	0	0	0	4	0	0	0	0
- Total	0	2	1	10	0	0	0	2
Thyroid – colloid alteration								
- Minimal	2	1	6	3	0	0	0	3
- Slight	0	1	1	3	0	0	0	0
- Moderate	0	1	0	0	0	0	0	0
- Marked	0	1	0	0	0	0	0	0
- Total	2	4	7	6	0	0	0	3
Microscopic findings – 24 months (n = 60)								
Eosinophilic focus of hepatocellular alteration								
- Minimal	22	21	19	28	14	9	8	16
- Slight	4	6	9	9	2	3	1	6
- Moderate	1	0	2	7	0	0	1	1
- Marked	0	0	0	1	0	0	0	0
- Total	27	27	30	45**	16	12	10	23
Tigroid focus of hepatocellular alteration								
- Minimal	29	28	32	42	30	30	24	36
- Slight	5	8	7	3	5	7	5	5
- Moderate	0	0	0	0	1	0	1	0
- Total	34	36	39	45	36	37	30	41
Mixed focus of hepatocellular alteration								
- Minimal	0	0	0	0	0	0	0	0
- Total	0	0	0	3	0	0	0	0

	Males				Females			
Parameter	**0 ppm**	**80 ppm**	**400 ppm**	**2 000 ppm**	**0 ppm**	**80 ppm**	**400 ppm**	**2 000 ppm**
Centrilobular hepatocellular hypertrophy								
- Minimal	0	0	6	23	0	0	0	27
- Slight	0	0	0	2	0	0	0	1
- Total	0	0	6*	25**	0	0	0	28**
Centrilobular or diffuse macrovacuolation of hepatocyte								
- Minimal	3	4	3	18	1	0	0	19
- Slight	0	0	1	6	0	1	0	5
- Moderate	0	1	0	0	1	0	0	0
- Total	3	5	4	24**	2	1	0	24**
Focal hepatocellular brown pigment								
- Minimal	0	0	0	0	1	0	1	13
- Slight	0	0	0	0	0	0	0	3
- Total	0	0	0	0	1	0	1	16**
Accumulation of brown pigment in Kupffer cells								
- Minimal	8	11	10	9	10	9	14	18
- Slight	2	5	8	4	4	4	1	8
- Moderate	0	0	0	1	1	1	1	1
- Total	10	16	18	14	15	14	16	27*
Focal infiltration of mononuclear cells in the liver								
- Minimal	20	19	21	24	22	28	29	35
- Slight	1	0	1	2	1	4	0	1
- Moderate	0	0	0	0	1	0	0	0
- Total	21	19	22	26	24	32	29	36*
Focal alveolar macrophages								
- Minimal	18	24	22	25	27	18	11	25
- Slight	10	12	8	7	2	11	16	18
- Moderate	2	4	4	3	1	0	2	6
- Marked	0	0	0	0	0	0	0	1
- Total	30	40*	34	35	30	29	19*	50**
Focal chronic interstitial inflammation in the lungs								

Table 19 (continued)

Parameter	Males				Females			
	0 ppm	80 ppm	400 ppm	2 000 ppm	0 ppm	80 ppm	400 ppm	2 000 ppm
- Minimal	1	4	4	3	2	3	5	10
- Slight	0	1	0	0	0	0	0	0
- Moderate	0	0	1	0	0	0	0	0
- Total	1	5	5	3	2	3	5	10*
Focal perivascular inflammation in the lungs								
- Minimal	23	20	30	20	17	22	17	35
- Slight	1	3	2	1	0	1	0	1
- Total	24	23	32	21	17	23	17	36**
Thyroid – colloid alteration								
- Minimal	14	12	28	18	11	10	10	11
- Slight	7	9	9	18	1	3	0	4
- Moderate	0	1	1	4	0	1	0	0
- Marked	0	0	0	0	1	0	0	0
- Total	21	22	38**	40**	13	14	10	15
Diffuse follicular cell hypertrophy								
- Minimal	1	0	1	3	0	1	0	3
- Total	1	0	1	3	0	1	0	3
Brown pigments in follicular cells – thyroid								
- Minimal	16	10	18	22	8	7	5	17
- Slight	1	0	1	1	0	0	0	0
- Moderate	0	0	1	0	0	0	0	0
- Total	17	10	20	23	8	7	5	17

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean ± 1 standard deviation.

[b] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

Source: Garcin (2012)

At 2000 ppm, the terminal body weight (24 months) of females was 13% lower ($P < 0.01$) than the control value. Results of organ weight, macroscopic and microscopic findings are summarized in Table 19. In rats killed after 12 and 24 months, relative liver weight was significantly elevated ($P < 0.01$), whereas absolute liver weight was unaffected. In rats killed after 12 months, an enlarged and pale liver was observed macroscopically in three males at 2000 ppm. Histopathological examination revealed a higher incidence of eosinophilic and tigroid foci of altered hepatocytes in males at 2000 ppm, in addition to an increase in centrilobular hepatocellular hypertrophy and centrilobular or diffuse macrovacuolation of hepatocytes. Centrilobular hypertrophy of hepatocytes occurred in both sexes at 2000 ppm. In the thyroid, the incidence and severity of colloid alteration were increased in both sexes at 2000 ppm and in males at 400 ppm.

In rats killed after 24 months, an increased incidence of white foci in the lungs was observed macroscopically at the highest dose. Adverse histopathological abnormalities occurred only at 2000 ppm and included increased incidences of eosinophilic, mixed and tigroid foci of altered hepatocytes in males, centrilobular hypertrophy and centrilobular macrovacuolation of hepatocytes in both sexes, brown pigmentation in hepatocytes and Kupffer cells, and mononuclear cell infiltration. In the lung, higher incidences of foamy macrophages, chronic interstitial inflammation and perivascular inflammation occurred in females at 2000 ppm. In the thyroid gland, colloid alteration was increased in males at 400 and 2000 ppm, with the increase at 400 ppm only minor and therefore not considered to be toxicologically significant. Follicular cell hypertrophy and brown pigments in follicular cells occurred in both sexes at 2000 ppm.

No treatment-related neoplastic changes were observed.

The NOAEL for chronic toxicity was 400 ppm (equal to 15.8 mg/kg bw per day for males and 22.5 mg/kg bw per day for females), for reduced body weight and body weight gain, liver enlargement with accompanying histopathological changes, and histopathological changes in the lungs and thyroid at 2000 ppm (equal to 80.8 mg/kg bw per day for males and 120 mg/kg bw per day for females). The NOAEL for carcinogenicity was 2000 ppm (equal to 80.8 mg/kg bw per day for males and 120 mg/kg bw per day for females), the highest dose tested (Garcin, 2012).

2.4 Genotoxicity

The results of genotoxicity studies on flupyradifurone are summarized in Table 20.

Table 20. Results of genotoxicity assays on flupyradifurone

End-point	Test object	Concentration or dose	Purity (%)	Results	Reference
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	16–5 000 µg/plate (±S9) DMSO vehicle	96.2	Negative	Herbold (2009a)
Gene mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate (±S9) DMSO vehicle	97.2	Negative	Sokolowski (2011a)
Gene mutation	CHO V79 cells (*HPRT* locus)	15.6–3 000 µg/mL (±S9) DMSO vehicle	96.2	Negative	Entian (2009)
Cytogenetic test	CHO V79 cells	200–3 000 µg/mL (±S9) DMSO vehicle	96.2	Negative[a]	Thum (2009)
In vivo micronucleus	Crl:NMRI BR male mice (bone marrow)	10, 20 or 40 mg/kg bw, intraperitoneally Corn oil vehicle	96.2	Negative[b]	Herbold (2009b)
In vivo micronucleus	NMRI female mice (bone marrow)	12.5, 25 or 50 mg/kg bw, intraperitoneally DMSO/corn oil (10%/90%) vehicle	96.2	Negative[c]	Wieland (2011)

bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; *HPRT*: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Cytotoxicity at and above 400 µg/mL (−S9) and at and above 2000 µg/mL (+S9).

[b] Clinical signs observed at every dose and lasting until termination included apathy, roughened fur, weight loss, sternal recumbency, spasm, periodic stretching of body and difficulty breathing.

[c] Clinical signs observed at 25 and 50 mg/kg bw included reduced motor activity, ruffled fur, abdominal position and eyelid closure. At 25 mg/kg bw, these occurred only at 2–4 hours after the first dose; at 50 mg/kg bw, these occurred after each dose.

2.5 Reproductive and developmental toxicity

(a) Single-generation or multigeneration studies

Rats

In a range-finding study by Milius (2010), flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 200, 700 or 2000 ppm and fed ad libitum to one parental generation of Wistar Han CRL:WI(HAN) rats (10 of each sex per dose) and their offspring through premating, mating, gestation and lactation. The mean intake of flupyradifurone during the premating phase was 9, 14.5, 50.1 and 147.5 mg/kg bw per day for males and 0, 17.5, 60.0 and 168.9 mg/kg bw per day for females at 0, 200, 700 and 2000 ppm, respectively. During gestation, the mean intake in dams was 0, 15.8, 48.8 and 164.4 mg/kg bw per day, respectively, whereas it was 0, 17.5, 60.9 and 182.3 mg/kg bw per day, respectively, during lactation.

Observations for mortalities and clinical signs were made daily, with body weight and feed consumption recorded weekly. Standard reproduction, offspring and litter parameters were recorded or calculated. Following scheduled termination, all rats were necropsied and organs weighed.

There were no treatment-related deaths or clinical signs. During premating, gestation and lactation, the mean body weights of high-dose dams were up to 12% lower than the control values ($P < 0.01$). During lactation days 14 and 21, the mean body weights of dams at 700 ppm were also significantly lower ($P < 0.05$; 6–7%) than the control values. No adverse effects on body weight parameters occurred in parental males, and there was no adverse effect on feed consumption. There was no treatment-related effect on reproduction or litter parameters. From postnatal day 14, mean combined male and female pup weights were significantly lower ($P < 0.05$) than the control values at 700 and 2000 ppm (approximately 10%), with overall body weight gain during lactation approximately 10% ($P < 0.05$) lower than the control values. Similar effects on body weight were also noted in female pups only. There were no treatment-related macroscopic findings. In high-dose dams, absolute spleen weight was 14% lower ($P < 0.05$) than the control value.

The NOAEL for reproductive toxicity was 2000 ppm (equal to 147.5 mg/kg bw per day for males and 168.9 mg/kg bw per day for females), the highest dose tested. The NOAEL for both parental toxicity and offspring toxicity was 700 ppm (equal to 50.1 mg/kg bw per day for males and 60.0 mg/kg bw per day for females), based on reduced body weight at 2000 ppm (equal to 147.5 mg/kg bw per day for males and 168.9 mg/kg bw per day for females) (Milius, 2010).

In the main reproductive toxicity study by Milius (2011), flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 100, 500 or 1800 ppm and fed ad libitum to two parental generations of Wistar Han CRL:WI(HAN) rats (30 of each sex per dose) and their offspring through premating, mating, gestation and lactation. The achieved doses during premating, gestation and lactation are summarized in Table 21. Observations for mortalities and clinical signs were made daily, with a more detailed clinical examination performed on each rat weekly. Body weight and feed consumption were recorded weekly. Standard reproduction, offspring and litter parameters were recorded or calculated, in addition to the determination of estrous cycle, sperm counts, sperm motility and sperm morphology. At scheduled termination, rats were necropsied, organs weighed and tissues collected for histopathological examination.

There were no treatment-related deaths or clinical signs. Effects on body weight and feed parameters are summarized in Table 21. There were no treatment-related effects on body weight, body weight gain or feed consumption in the first parental generation of males, whereas in the second generation, mean body weight was significantly lower ($P < 0.01$) than the control value at 1800 ppm during the premating period (10% lower during week 14). In the first generation of dams, reductions in body weight (−10%; $P < 0.01$), body weight gain (up to approximately 40% lower than the control) and feed consumption (−10%; $P < 0.01$) occurred only at 1800 ppm during premating, gestation and lactation. In the second parental generation of dams, dose-related reductions in body weight, body weight gain and feed consumption occurred at 500 and 1800 ppm.

Table 21. Effects in rats exposed to flupyradifurone over two generations

	Males				Females			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**1 800 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**1 800 ppm**
Mean achieved doses (mg/kg bw per day)[a]								
Premating ($P + F_1$)	–	6.5	32.3	119.8	0	7.8	39.2	140.2
Gestation ($P + F_1$)	–	–	–	–	0	7.0	35.5	151.4
Lactation ($P + F_1$)	–	–	–	–	0	7.8	39.8	150.4
Body weight (g)[b]								
Premating – P (week 10)	436	436	441	438	237.4	235.5	229.0	213.3**
Premating – F_1 (week 14)	454.5	456.0	452.3	410.8** (−10%)	242.1	237.3	224.2**	203.5** (−10%)
Gestation – P								
- Day 0	–	–	–	–	238.1	235.2 (−1%)	232.2 (−2%)	214.8** (−10%)
- Day 6	–	–	–	–	255.0	250.2 (−2%)	248.6 (−3%)	228.1** (−11%)
- Day 13	–	–	–	–	276.0	272.3 (−1%)	267.9 (−3%)	247.1** (−10%)
- Day 20	–	–	–	–	333.6	326.5 (−2%)	322.1 (−4%)	301.1** (−10%)
Gestation – F_1								
- Day 0	–	–	–	–	243.4	238.3 (−2%)	224.4** (−8%)	202.6** (−17%)
- Day 6	–	–	–	–	257.6	251.0 (−3%)	238.7** (−7%)	214.0** (−17%)
- Day 13	–	–	–	–	277.8	270.0 (−3%)	258.3** (−7%)	230.8** (−17%)
- Day 20	–	–	–	–	335.1	327.4 (−2%)	313.8** (−6%)	277.2** (−17%)
Lactation – P								
- Day 0	–	–	–	–	262.8	256.1 (−3%)	250.8 (−5%)	233.3** (−11%)
- Day 4	–	–	–	–	271.3	271.6	265.8 (−2%)	245.7** (−9%)
- Day 7	–	–	–	–	277.4	276.7	271.2 (−2%)	251.6** (−9%)
- Day 14	–	–	–	–	292.6	292.3	287.2	267.1**

Table 21 (continued)

Parameter	Males				Females			
	0 ppm	**100 ppm**	**500 ppm**	**1 800 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**1 800 ppm**
							(−6%)	(−9%)
- Day 21	–	–	–	–	285.8	281.4 (−2%)	280.1 (−2%)	264.3** (−8%)
Lactation – F_1								
- Day 0	–	–	–	–	263.0	254.5 (−3%)	242.5** (−8%)	218.4** (−17%)
- Day 4	–	–	–	–	277.3	265.9 (−4%)	256.0** (−8%)	225.1** (−19%)
- Day 7	–	–	–	–	278.7	268.5 (−4%)	260.9** (−6%)	231.3** (−17%)
- Day 14	–	–	–	–	294.8	282.8 (−4%)	272.2** (−8%)	244.3** (−17%)
- Day 21	–	–	–	–	283.8	278.5 (−2%)	261.6** (−8%)	247.1** (−13%)
Body weight gain (g)[a]								
Premating – P (weeks 1–14)	436.0	436.0	441.3	438.4	63.4	61.8 (−3%)	50.4 (−31%)	36.3 (−43%)
Premating – F_1 (weeks 1–14)	152.3	152.0	166.2	145.1	52.2	51.5	43.7 (−16%)	41.2 (−21%)
Gestation – P (days 0–20)	–	–	–	-	95.6	91.3 (−4%)	89.9 (−6%)	86.4 (−10%)
Gestation – F_1 (days 0–20)	–	–	–	–	91.8	89.1 (−3%)	89.4 (−3%)	74.7** (−19%)
Feed consumption (g/rat/day)[a]								
Premating – P (weeks 1–14)	21.4	21.9	21.9	21.7	16.3	16.0 (−2%)	16.0 (−2%)	14.7** (−10%)
Premating – F_1 (weeks 1–10)	23.4	23.8	23.4	22.8	16.8	16.3 (−3%)	16.3 (−3%)	14.8** (−12%)
Reproductive parameters[a]								
No. of estrous cycles								
- P	–	–	–	–	3.5	3.7	3.4	3.4
- F_1	–	–	–	–	3.5	3.3	3.3	2.9*
Estrous cycle length								
- P	–	–	–	–	4.4	4.3	4.3	4.3

	Males				Females			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**1 800 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**1 800 ppm**
- F_1	–	–	–	–	4.0	4.1	4.4	4.4
Total no. of implantation sites (median)								
- F_1 offspring	–	–	–	–	311	285	298	289
- F_2 offspring	–	–	–	–	305	314	323	281**
Litter size[a]								
- Mean – F_1	–	–	–	–	10.2	10.3	10.1	10.0
- Median – F_1	–	–	–	–	11.0	11.0	10.0	10.0
- Mean – F_2	–	–	–	–	10.8	10.7	11.1	9.2
- Median – F_2	–	–	–	–	11.0	11.0	10.5	10.0*
Pup weights[a]								
F_1 litters								
- Day 0	6.2	6.2	6.0	5.7**	6.2	6.0	6.0	6.0
- Day 4	10.4	10.4	9.9	9.4*	10.3	9.7	9.8	9.8
- Day 7	10.4	10.4	9.9	9.4*	10.3	9.7	9.8	9.8
- Day 14	16.2	16.3	15.5	14.4**	16.3	15.8	15.5	15.0
- Day 21	32.5	32.1	30.8	28.2**	32.4	31.5	30.3*	28.4**
- Body weight gain	49.8	49.3	48.0	43.5**	49.6	48.1	45.9**	43.8**
F_2 litters								
- Day 0	5.9	5.9	5.7	5.4*	5.9	5.8	5.7	5.6
- Day 4	10.0	10.1	9.7	9.1	9.9	9.6	9.4	9.5
- Day 7	10.0	10.1	9.7	9.1	9.9	9.6	9.4	9.5
- Day 14	15.8	15.8	15.1	14.0**	15.7	15.4	15.0	14.4*
- Day 21	31.9	31.3	30.0	27.7**	31.8	30.9	29.6*	27.5**
- Body weight gain	48.4	47.8	46.1	42.2**	47.8	46.8	44.4**	41.8**
Sexual maturation – F_1 litters[a]								
Preputial separation (days)	42.6	42.5	43.3	46.5**	–	–	–	–
Vaginal opening (days)	–	–	–	–	34.3	34.0	34.1	36.1

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean.

[b] Results expressed as the mean, with the % increase (+) or decrease (−) relative to the controls contained in parentheses.

[c] Results expressed as the absolute number of rats per group.

Source: Milius (2011)

Selected reproduction and litter parameters are summarized in Table 21. At 1800 ppm, there was a significant decrease ($P < 0.05$) in the number of estrous cycles in F_1 females (2.9 versus 3.5 in the controls) and a concomitant increase in cycle length (not significant). There was no treatment-related effect on sperm parameters. At 1800 ppm in F_1 dams, the total number of implantation sites was significantly lower ($P < 0.01$) than the control value, whereas overall reproductive performance was unaffected by treatment. Mean and median litter sizes of the F_2 generation were lower than the control values, but only the difference in median litter size was statistically significant and outside the performing laboratory's historical control range of 9.8–11.8.

Following scheduled termination of parental rats, no treatment-related macroscopic findings were evident. There was no consistent treatment-related effect on organ weights, although there was a small (9%) increase in absolute and relative liver weights at 1800 ppm in the first parental generation of males, which was coincident with minimal centrilobular hypertrophy of hepatocytes. Also in this group, there were 13% and 21% increases in the weights of the left and right thyroid, respectively, with no accompanying histopathological changes.

In F_1 and F_2 litters, mean pup weight and weight gain during lactation were significantly lower ($P < 0.01$ or 0.05; up to approximately 14% lower than the control) than the control values at 500 ppm (females only) and 1800 ppm (both sexes). At 1800 ppm, there was a significant delay ($P < 0.01$) in preputial separation and a slight, non-statistically significant delay in vaginal opening; these findings are most likely a secondary effect of lower body weight gain rather than a direct endocrine-disrupting effect. A mode of action analysis undertaken by Lasserre (2014) confirmed this conclusion. There were no treatment-related macroscopic abnormalities or histopathological findings in pups.

Variations in pup organ weights were noted at the highest dose, but these were generally of a low magnitude and/or were not consistent between generations or sexes. In F_2 pups, relative brain weight was significantly increased ($P < 0.05$; +11.5%) at the highest dose, which was attributable to the lower body weight of this group. Absolute thymus weight was significantly lower ($P < 0.05$) than the control value in F_1 males (−11.3%) and F_2 females (−10.96%), with relative thymus weight significantly lower ($P < 0.05$) in F_1 females (−11.3%). Absolute spleen weight was significantly lower ($P < 0.05$) than the control values in F_1 males (−17.98%) and in F_2 males (−14.98%) and females (−15.48%).

The NOAEL for reproductive toxicity was 500 ppm (equal to 32.3 mg/kg bw per day for males and 39.2 mg/kg bw per day for females), based on a decrease in estrous cycle length, in the number of implantation sites and in litter size at 1800 ppm (equal to 119.8 mg/kg bw per day for males and 140.2 mg/kg bw per day for females). The NOAEL for parental toxicity in females was 100 ppm (equal to 7.8 mg/kg bw per day), for decreased body weight at 500 ppm (equal to 39.2 mg/kg bw per day); the NOAEL for the same end-point in males was 500 ppm (equal to 32.3 mg/kg bw per day). The NOAEL for offspring toxicity in females was 100 ppm (equal to 7.8 mg/kg bw per day), for decreased pup weight and weight gain at 500 ppm (equal to 39.2 mg/kg bw per day); the NOAEL for the same end-point in males was 500 ppm in males (equal to 32.3 mg/kg bw per day) (Milius, 2011).

(b) Developmental toxicity

Rats

Flupyradifurone (96.2% purity) in 0.5% (weight per volume [w/v]) methyl cellulose was administered by gavage to groups of 25 pregnant Crl:CD(SD) rats at a dose of 0, 15, 50 or 150 mg/kg bw per day from day 6 to day 20 of gestation. Dams were observed daily for clinical signs, with body weight and feed consumption recorded throughout this period. On day 21 of gestation, surviving dams were killed and necropsied, and the following parameters were recorded or calculated: liver weight, gravid uterine weight, corpora lutea counts, total resorptions, number of implantations, live fetuses, dead fetuses and pup sex ratio. Fetuses were examined for external, visceral and skeletal abnormalities.

There were no treatment-related deaths. At 150 mg/kg bw per day, salivation occurred in 20 dams on at least one occasion between gestation days 11 and 21. Also at 150 mg/kg bw per day, mean body weight gain on gestation days 6–8 and 8–10 was significantly lower ($P < 0.01$) than the control values; dams lost 5.7 g body weight between gestation days 6 and 8, while controls gained a mean of 5.9 g over this same period. Mean feed consumption was 9–27% lower than the control values ($P < 0.01$ or 0.05) at 150 mg/kg bw per day between gestation days 6 and 12. In high-dose dams, mean absolute liver weight was 13% higher than the control value ($P < 0.05$).

There were no treatment-related effects on any litter parameters; a 2–3% reduction in fetal weight at 150 mg/kg bw per day was not statistically significant. There were no treatment-related external or visceral malformations or variations in fetuses. Similarly, there were no treatment-related skeletal malformations. There was a slight delay in skeletal development at 150 mg/kg bw per day, evidenced as increased litter and fetal incidences of incomplete ossification (parietal [uni/bi]: incomplete ossification; hyoid centrum: incomplete ossification) that were outside the performing laboratory's historical control range (Table 22).

Table 22. Skeletal variations of fetuses exposed to flupyradifurone

Parameter	0 mg/kg bw per day	15 mg/kg bw per day	50 mg/kg bw per day	150 mg/kg bw per day	Historical control range
No. of litters	22	22	22	23	–
No. of fetuses	158	165	161	162	–
Parietal (uni/bi): incomplete ossification					
Litter	0 (0)	2 (9.1)	0 (0.0)	4 (17.4)	0–9.1%
Fetal	0 (0)	6 (3.6)*	0 (0)	9 (5.6)**	0–1.3%
Hyoid centrum: incomplete ossification					
Litter	0 (0)	2 (9.1)	1 (4.5)	4 (17.4)	0–12.5%
Fetal	0 (0)	2 (1.2)	1 (0.6)	9 (5.6)**	0–1.9%
7th cervical centrum: unossified					
Litter	2 (9.1)	2 (9.1)	4 (18.2)	7 (30.4)	0–41.7%
Fetal	6 (3.8)	7 (4.2)	4 (2.5)	1 (6.8)	0–12.2%
5th sternebra: incomplete ossification					
Litter	10 (45.5)	11 (50)	7 (31.8)	12 (52.2)	19–70.8%
Fetal	17 (10.8)	21 (12.7)	8 (5.0)*	24 (14.8)	3.1–19.9%
13th costal cartilage (uni): short					
Litter	0 (0)	0 (0)	0 (0)	2 (8.7)	0%
Fetal	0 (0)	0 (0)	0 (0)	2 (1.2)	0%

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the number of litters or fetuses affected, with the % difference relative to the control values contained in parentheses.

Source: Langrand-Lerche (2010)

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on clinical signs and body weight loss at 150 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on slightly delayed ossification at 150 mg/kg bw per day (Langrand-Lerche, 2010).

In a follow-up study by Langrand-Lerche (2012), flupyradifurone (96.2% purity) in 0.5% (w/v) methyl cellulose was administered by gavage to groups of 23 pregnant Crl:CD(SD) rats at a dose of 0, 20 or 30 mg/kg bw per day from day 6 to day 20 of gestation. Toxicological end-points were consistent with the preceding study. The NOAEL for maternal toxicity and embryo and fetal toxicity was 30 mg/kg bw per day, the highest dose tested.

Rabbits

In a developmental toxicity study by Kennel (2012b), flupyradifurone (96.2% purity) in 0.5% (w/v) methyl cellulose was administered by gavage to groups of 23 pregnant New Zealand White rabbits at a dose of 0, 7.5, 15 or 40 mg/kg bw per day from day 6 to day 28 of gestation. Dams were observed daily for clinical signs, with body weight and feed consumption recorded from gestational days 3 to 29. On day 29 of gestation, surviving dams were killed and necropsied, and the following parameters were recorded or calculated: gravid uterine weight, corpora lutea counts, total resorptions, number of implantations, live fetuses, dead fetuses and pup sex ratio. Fetuses were examined for external, visceral and skeletal abnormalities.

There were no deaths or treatment-related clinical signs. At 40 mg/kg bw per day, dams lost a mean of 0.01 g body weight between gestation days 6 and 8, compared with a gain of 0.02 g in the controls; this difference was not statistically significant. At this same dose, mean maternal body weight gain between gestation days 6 and 10 was 67% lower ($P < 0.01$) than the control value, whereas feed consumption was 20% lower than the control value between gestation days 6 and 8 ($P < 0.01$) and 10% lower than the control value between gestation days 8 and 10 (not statistically significant). Mean maternal body weight gain corrected for gravid uterine weight from gestation days 6 to 29 was 12% lower than the control value, a result that was not statistically significant. There were no treatment-related effects at lower doses and no effect on litter parameters or on the incidence of external, visceral or skeletal abnormalities.

The NOAEL for maternal toxicity was 15 mg/kg bw per day, based on body weight loss and reduced feed consumption over the first few days of dosing at 40 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, the highest dose tested.

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study by Garcin (2011a), groups of 12 Wistar Rj:WI (IOPS HAN) rats of each sex received a single gavage dose of flupyradifurone (96.2% purity) in 0.5% (w/v) aqueous methyl cellulose at 0, 50, 200 or 800 mg/kg bw. Observations were made twice daily for deaths and clinical signs. Body weights were recorded on day 1 and weekly thereafter. A functional observational battery and motor activity assessment were performed pretreatment, at 2 hours after dosing (i.e. the approximate time to peak effect) and at days 7 and 14. On day 15, 16 or 17, survivors were killed and necropsied. The brain from six rats of each sex per group was weighed, and these same rats were examined neurohistopathologically.

There were no deaths or treatment-related clinical signs in males. At 800 mg/kg bw, one female died during the neurobehavioural test at the time of peak effect, with another female found dead 5 days after dosing. Clinical signs recorded in the former female included piloerection, low muscle tone and arousal, rapid respiration, tremors, myoclonic jerks, convulsions, dilated pupils, absence of pupil and flexor reflexes, and uncoordinated or slow surface righting reflex. There were no intergroup differences in mean body weight. At 800 mg/kg bw, mean body weight gains from days 1 to 7 after dosing were 40% and 46% lower than the control values ($P < 0.01$) in males and females, respectively.

Treatment-related functional observational battery and locomotor findings were confined to the time of peak effect (approximately 2 hours after dosing) and are summarized in Table 23. Observations included increased piloerection at and above 50 mg/kg bw, low muscle tone, rats feeling cold to touch, rapid respiration, tremors, gait incoordination, reduced rearing, low arousal, flattened or

Table 23. Results of functional observational battery and locomotor activity assessment in rats following a single oral dose of flupyradifurone

	Males				Females			
Parameter	**0 mg/kg bw**	**50 mg/kg bw**	**200 mg/kg bw**	**800 mg/kg bw**	**0 mg/kg bw**	**50 mg/kg bw**	**200 mg/kg bw**	**800 mg/kg bw**
Home cage observations[a]								
Piloerection	4/12	3/12	7/12	9/12	2/12	6/12	3/12	8/12
Handling observations								
Cold-to-touch	0/12	0/12	2/12	10/12	0/12	0/12	0/12	9/12
Low muscle tone	1/12	0/12	3/12	5/12	0/12	0/12	1/12	8/12
Soiled fur	0/12	0/12	1/12	1/12	0/12	0/12	0/12	2/12
Open-field observations[a]								
Piloerection	4/12	8/12	12/12	12/12	4/12	7/12	11/12	12/12
Rapid respiration	1/12	0/12	9/12	12/12	0/12	0/12	6/12	12/12
Tremors	0/12	0/12	4/12	8/12	0/12	0/12	4/12	10/12
Myoclonic jerks	0/12	0/12	1/12	5/12	0/12	0/12	0/12	7/12
Chewing	0/12	0/12	0/12	5/12	0/12	0/12	1/12	4/12
Convulsions	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12
Gait incoordination	0/12	0/12	3/12	7/12	0/12	0/12	5/12	5/12
Rearing[b]	7.2	7.8	4.3	2.7	12.3	10.7	9.9	2.8
Low arousal	1/12	1/12	6/12	11/12	0/12	0/12	1/12	8/12
Repetitive licking of lips	1/12	0/12	0/12	2/12	0/12	1/12	3/12	8/12
Flattened or hunched posture	3/12	2/12	8/12	11/12	0/12	0/12	3/12	11/12
Sensory reactivity								
Right pupil: dilated	0/12	1/12	4/12	6/12	1/12	6/12	7/12	10/12
Left pupil: dilated	0/12	1/12	4/12	6/12	1/12	6/12	7/12	10/12
Uncoordinated or slow surface righting reflex	0/12	0/12	4/12	6/12	0/12	1/12	2/12	5/12
Abnormal tail pinch response	1/12	4/12	2/12	4/12	3/12	2/12	4/12	8/11
Reflex and physiological observations								
Rectal temperature (°C)	36.8	36.9	36.3	35.3*	37.5	37.2	37.2	35.0*
Exploratory locomotor activity[b]								
First interval (10 min)	107	120	64*	31*	136	111	72*	37*
Total activity (60 min)	194	254	163	97*	293	272	199	115*

bw: body weight; *: $P < 0.05$

[a] Results expressed as the absolute number of rats per group.

[b] Results expressed as the mean.

Source: Garcin (2011a)

hunched posture, uncoordinated or slow righting reflex, and reduced locomotor activity at 200 and 800 mg/kg bw and myoclonic jerks, chewing, dilated pupils, decreased rectal temperature and abnormal tail pinch response at 800 mg/kg bw.

There were no treatment-related effects on terminal body weight or on the occurrence of macroscopic or histopathological changes in the nervous system (Garcin, 2011a).

In a follow-up study by the same author (Garcin, 2011a), groups of 12 rats of each sex per group received a single gavage dose of flupyradifurone at 0, 20 or 35 mg/kg bw under experimental conditions similar to those of the preceding study.

No treatment-related systemic toxicity or neurotoxicity was observed (Garcin, 2011a).

On this basis, the overall NOAEL for systemic toxicity from the two studies was 35 mg/kg bw per day, based on piloerection observed during the functional observational battery 2 hours after dosing at 50 mg/kg bw per day.

In a 90-day neurotoxicity study by Garcin (2011b), groups of 12 Wistar Rj:WI (IOPS HAN) rats of each sex were fed diets containing 0, 100, 500 or 2500 ppm flupyradifurone (96.2% purity) ad libitum. The achieved doses were 0, 5.7, 29.4 and 143 mg/kg bw per day for males and 0, 6.9, 34.8 and 173 mg/kg bw per day for females at 0, 100, 500 and 2500 ppm, respectively. Observations were made daily for deaths and clinical signs, with body weight and feed consumption recorded weekly. A detailed physical examination was performed pretreatment and weekly thereafter. A functional observational battery and motor activity assessment were performed pretreatment and during weeks 2, 4, 8 and 13–14. Ophthalmology was performed on all rats pretreatment and during week 13. Following scheduled termination, all rats were necropsied, and brain weights were recorded. Nervous tissue was examined histopathologically from at least six rats of each sex per group.

There were no treatment-related deaths, clinical signs, ophthalmological findings, functional observational battery or motor activity changes, effects on brain weight, or macroscopic or histopathological abnormalities.

Adverse effects on body weight parameters and feed consumption were confined to the highest dose. In males, mean absolute body weight was significantly lower than the control values ($P < 0.05$) during weeks 3 and 4 (10% and 8% lower, respectively), whereas in females, it was significantly lower than the control values ($P < 0.05$) over the majority of the dosing period (7–9% lower). In males, mean body weight gain was 26% and 21% lower than the control values from weeks 1 to 4 ($P < 0.01$) and 1 to 8 ($P < 0.05$), respectively. In females, mean body weight gain was 29%, 27% and 21% lower than the control values during weeks 1–4, 1–8 and 1–13, respectively. During the first week of treatment, mean feed consumption was 18% and 29% lower than the control values ($P < 0.01$) in males and females, respectively. Thereafter, feed consumption remained lower than the control values (3–14% in males and 4–13% in females), reaching statistical significance ($P < 0.05$) during weeks 7, 11 and 12 in males and weeks 1, 2, 5, 6 and 7 in females.

The NOAEL for systemic toxicity was 500 ppm (equal to 29.4 mg/kg bw per day for males and 34.8 mg/kg bw per day for females), for reduced body weight, body weight gain and feed consumption at 2500 ppm (equal to 143 mg/kg bw per day for males and 173 mg/kg bw per day for females). The NOAEL for neurotoxicity was 2500 ppm (equal to 143 mg/kg bw per day for males and 173 mg/kg bw per day for females), the highest dose tested (Garcin, 2011b).

In a developmental neurotoxicity study, groups of 30 mated female rats were fed diets containing 0, 120, 500 or 1200 ppm flupyradifurone (96.2% purity) ad libitum from gestation day 6 to lactation day 21. The mean intakes of flupyradifurone during gestation and lactation were 0, 10.3, 42.4 and 102 mg/kg bw per day at 0, 120, 500 and 1200 ppm, respectively. Observations for mortality

and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Dams (10 per group) were subjected to a functional observational battery on gestation days 13 and 20 and lactation days 11 and 21. Following delivery, litter size and pup viability were recorded. On postnatal day 4, litters were culled to yield four pups of each sex. Subsets of surviving offspring, representing 19–20 litters per dose, were subjected to a functional observational battery, motor activity, auditory startle, reflex habituation, learning and memory, passive avoidance and water maze assessments. Ophthalmoscopy was performed on a minimum of 10 rats of each sex per group at approximately 50–60 days of age. Dams were killed on day 21 of gestation, but were not examined further. On postnatal day 21 or 75, 10 offspring of each sex per dose were killed and necropsied. Brain weight was recorded, brain morphometry was analysed and histopathology of neural tissues was performed.

There were no deaths in dams. There was a marginal increase in hair loss during gestation (three rats versus one rat in the control group) and lactation (four rats versus one rat in the control group), but this difference was not considered toxicologically significant. There were no treatment-related functional observational battery findings in dams. Reduced body weight and body weight gain occurred in dams only at the highest dose; mean body weight was significantly lower than the control values on gestation day 20 (−7%, $P < 0.01$), lactation day 0 (−4%, $P < 0.05$) and lactation day 4 (−6%, $P < 0.01$), whereas mean body weight gain was significantly lower than the control value from gestation days 0 to 20 (−21%, $P < 0.01$). There was no treatment-related effect on feed consumption. Litter parameters were comparable between treated and control groups.

During lactation, there was no effect on mean pup weight. Mean weight gain was lower than the control values from day 4 to day 17 (−7%, $P < 0.05$) and from day 11 to day 17 (−10%, $P < 0.01$) in males and from day 11 to day 17 in females (−11%, $P < 0.05$). There was no effect on pup weights or weight gain after weaning. There was no treatment-related effect on developmental landmarks (preputial separation, vaginal opening, surface righting or pupil constriction). There were no treatment-related functional observational battery findings in offspring. An increase in overall motor activity that occurred in high-dose males on postnatal day 13 was not statistically significant and therefore was not considered to be treatment related. The auditory startle response was increased 2-fold ($P < 0.05$) above the control value in high-dose female offspring on postnatal day 60 (Table 24). During the post-weaning period, there were no treatment-related effects on learning or memory in the passive avoidance test. In adult offspring, there were no treatment-related effects on acquisition or retention during the assessment of water maze performance. Ophthalmoscopy was unremarkable.

There were no treatment-related macroscopic or histopathological findings in pups killed on postnatal day 21 or in offspring killed on postnatal day 75. Although the terminal body weight of pups killed on postnatal day 21 was reduced at the highest dose (−8% compared with the controls in both sexes), the difference was not statistically significant. At the highest dose, fresh brain weight (but not perfused brain weight) was significantly lower ($P < 0.05$) than the control value in male offspring killed at postnatal day 75 (−4%). In the absence of a difference in relative brain weight, a similar difference in age- and dose-matched females and any histopathological or morphometric differences, this finding was not considered to be treatment related.

Table 24. Results of behavioural assessments of offspring

	Males				Females			
Parameter	**0 ppm**	**120 ppm**	**500 ppm**	**1 200 ppm**	**0 ppm**	**120 ppm**	**500 ppm**	**1 200 ppm**
Auditory startle response[a]								
PND 23	41	48	40	41	35	36	33	41
PND 60	211	249	181	230	78	104	110	156*

PND: postnatal day; ppm: parts per million; *: $P < 0.05$

[a] Results expressed as the mean.

Source: Gilmore (2012)

The NOAEL for maternal toxicity and offspring toxicity was 500 ppm (equal to 42.4 mg/kg bw per day), based on reduced body weight and body weight gain in dams, reduced body weight gain in pups during lactation and increased auditory startle reflex in pups at 1200 ppm (equal to 102 mg/kg bw per day). The NOAEL for developmental neurotoxicity was 1200 ppm (equal to 102 mg/kg bw per day), the highest dose tested (Gilmore, 2012).

(b) Immunotoxicity

Flupyradifurone (96.2% purity) was admixed in the diet at a concentration of 0, 125, 600 or 3000 ppm and fed ad libitum to groups of 10 female Wistar rats for at least 28 days. The achieved doses were 0, 10, 50 and 230 mg/kg bw per day at 0, 125, 600 and 3000 ppm, respectively. A positive control group of 10 female rats was administered cyclophosphamide at 3.5 mg/kg bw per day by gavage for at least 28 days. Four days before necropsy, all rats were immunized with sheep red blood cell (sRBC) antigen. Rats were observed daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly. A detailed physical examination was performed at least weekly throughout the study. Blood samples were collected on day 30 (prior to necropsy) for specific anti-sRBC immunoglobulin M (IgM) analysis. All rats were necropsied, and spleen and thymus weights were recorded.

There were no deaths or clinical signs. At the highest dose, mean body weight was 6–11% lower than the control values from day 8 to day 29, reaching statistical significance ($P < 0.01$ or 0.05) on days 1–8 and 8–15. Also at the highest dose, cumulative body weight gain was 23% lower than the control value ($P < 0.05$), and mean feed consumption was 34% lower than the control value ($P < 0.01$) on day 8 and 9–13% lower thereafter. Terminal body weight was 6% lower than the control value, which was not statistically significant. There was no treatment-related effect on anti-sRBC IgM concentrations.

The NOAEL for systemic toxicity was 600 ppm (equal to 50 mg/kg bw per day), based on reduced body weight parameters and feed consumption at 3000 ppm (equal to 230 mg/kg bw per day). The NOAEL for immunotoxicity was 3000 ppm (equal to 230 mg/kg bw per day), the highest dose tested (Repetto, 2011).

(c) Studies on metabolites

Toxicity tests were conducted on six flupyradifurone metabolites, the details of which are provided in Table 25.

Acute toxicity

The results of acute oral toxicity tests on flupyradifurone metabolites in rats are summarized in Table 26.

Short-term studies of toxicity

Short-term repeated-dose toxicity studies were conducted on three metabolites of flupyradifurone: DFA, difluoroethyl-amino-furanone and (6-chloro-3-pyridyl)methanol.

Difluoroacetic acid. In a non-GLP preliminary toxicity study, DFA (96.7% purity) was administered ad libitum via the diet to groups of Wistar Rj: WI (IOPS HAN) rats (five of each sex per group) for 14 days at a concentration of 0, 500, 2000 or 8000 ppm (equal to 0, 48, 187 and 745 mg/kg bw per day for males and 0, 51, 201 and 800 mg/kg bw per day for females, respectively). Rats were observed daily for mortality and clinical signs. Detailed physical examinations were performed at least weekly. Body weight and feed consumption were recorded weekly. Blood was collected at the end of the study for the analysis of haematology and clinical chemistry parameters. Following

Table 25. Flupyradifurone metabolites on which additional toxicity tests were conducted

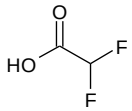
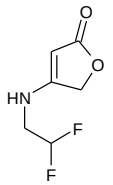
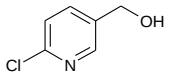
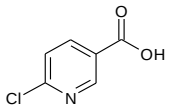
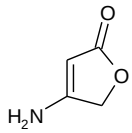
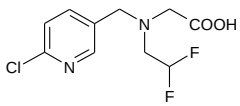
Chemical name	Structure	Description
Difluoroacetic acid (DFA)		Major soil, water, plant and livestock metabolite. Also present in rat urine (approximately 6% of the administered dose).
Difluoroethyl-amino-furanone		Minor plant metabolite. Also present in rat urine (approximately 10% of the administered dose).
(6-Chloro-3-pyridyl)methanol		Plant metabolite. Metabolite common to other pesticides.
6-Chloronicotinic acid		Plant metabolite. Metabolite common to other pesticides.
Amino-furanone		Plant metabolite.
Flupyradifurone-acetic acid		Plant metabolite.

Table 26. Results of studies of acute oral toxicity of flupyradifurone metabolites in rats

Metabolite/purity	Strain/sex	Vehicle	LD_{50} (mg/kg bw)	Signs	Reference
Difluoroacetic acid (DFA) 99.6%	Rj: SD (IOPS HAN) Female	Water	> 300–2 000	Loud breathing, hypoactivity, sedation, lateral recumbency, dyspnoea, piloerection, staggering gait	Gerbeix (2010)
Difluoroethyl-amino-furanone 98.5%	Rj Han: SD Female	Water	> 2 000	Hypoactivity, piloerection, chromorhinorrhoea, lower mean body weight gain	Rokh (2011)
(6-Chloro-3-pyridyl)methanol 98.65%	Crj:CD (SD) Male and female	Water	1 842 (males); 1 483 (females)	Decline of righting reflex, motor activity, hypotonia, prone position, ataxia, gastrorrhagia	Mochizuki & Goto (1997a)
6-Chloronicotinic acid 99.4%	Crj:CD (SD) Male and female	Water	> 5 000 (both sexes)	No signs of toxicity	Mochizuki & Goto (1997b)

bw: body weight; LD_{50}: median lethal dose

scheduled termination, rats were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

There were no treatment-related effects on survival, clinical signs, feed consumption, haematological parameters, or macroscopic or microscopic findings. Body weight gain was reduced, but not significantly, at 8000 ppm in both sexes (13% in males and 12% in females) and at 2000 ppm in females, compared with the controls. Mean glucose concentrations were reduced significantly (34–54% in males, 42–48% in females, $P < 0.01$) at all dose levels. Slightly higher mean urea concentrations were seen in females (23–27%) and males (6–19%) at all dose levels, but were not considered to be treatment related owing to the absence of a dose–response relationship, statistical significance or concomitant effects on creatinine. Total bilirubin concentrations were reduced at all dose levels in both sexes, but did not exhibit a dose–response relationship, and this change is not considered to be toxicologically relevant (Kennel, 2011).

In a 90-day toxicity study, DFA (97.1% purity) was administered to Wistar Rj:WI (IOPS HAN) rats (10 of each sex per group) at a dietary concentration of 0, 200, 1000 or 6000 ppm (equal to 0, 12.7, 66.2 and 380 mg/kg bw per day, respectively, for males and 0, 15.6, 78.7 and 472 mg/kg bw per day, respectively, for females). Clinical signs were recorded daily, and body weights were recorded weekly. Feed consumption was recorded twice weekly during the first 6 weeks of treatment and weekly thereafter. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving rats were subjected to a functional observational battery and motor activity assessment during week 11. Ophthalmological examinations were performed on all rats during the acclimatization phase and on all surviving rats of the control and high-dose groups during week 12. Urine samples were collected overnight during the week before necropsy from all surviving animals. Before necropsy, a blood sample was collected from each rat for haematology and clinical chemistry investigations. Following scheduled termination, all rats were necropsied, selected organs were weighed and a range of tissues was examined microscopically.

There were no treatment-related clinical signs, mortalities, neurotoxicity or ophthalmological changes. Mean body weight was decreased by 7–13% in males and by 6–9% in females (statistically significant on several occasions) at 1000 and 6000 ppm. The effect was progressive throughout the study, and at the end of the treatment (study day 92), the overall mean body weight gain was reduced by 11–20% in males ($P < 0.01$) and by 9–20% ($P < 0.01$) in females at 1000 and 6000 ppm. At 1000 and 6000 ppm, mean feed consumption was decreased, but not significantly (up to 7% and 9%, respectively, for males and up to 10% and 13%, respectively, for females).

Lower haemoglobin concentration and lower mean cell volume were observed at 1000 and 6000 ppm in females only. As a consequence, lower mean corpuscular haemoglobin and lower haematocrit were also noted. These changes were observed with no clear dose–response relationship. No variation was noted in erythrocyte or reticulocyte counts. The statistically significantly lower mean corpuscular haemoglobin observed at 200 ppm was not considered to be biologically relevant in view of its low magnitude (Table 27). No treatment-related changes were noted in males at any dose level.

In both sexes, blood glucose was significantly lower ($P < 0.01$) than the control value at every dose (Table 28). However, the reduction at 200 ppm was not considered adverse because it was not corroborated by any other findings and was within the normal range (3.95–5.65 mmol/L). In contrast, at the middle and high doses, the reduction in blood glucose was of a large magnitude and associated with a large increase in urine volume and ketones. Total bilirubin was reduced at every dose (Table 28), but this finding is not toxicologically relevant. The significant elevation in urea level was not considered to be treatment related owing to the absence of a dose–response relationship and the lack of corroborating findings, such as increased creatinine. Slightly higher mean inorganic phosphorus concentrations were noted at 6000 ppm in males and females (Table 28).

Table 27. Haematological changes[a] in female rats exposed to DFA for 90 days in the diet

Parameter	Control	200 ppm	1 000 ppm	6 000 ppm
Haemoglobin concentration (g/dL)	15.89	15.43 (−3%)	14.43** (−9%)	14.60 ** (−8%)
Mean cell volume (fL)	54.6	53.6 (−2%)	51.3** (−6%)	52.1** (−5%)
Mean corpuscular haemoglobin (pg)	17.76	17.23* (−3%)	16.35** (−8%)	16.58** (−7%)
Haematocrit (L/L)	0.488	0.480 (−2%)	0.453** (−7%)	0.460** (−6%)

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$
[a] Results expressed as the mean, with the % change relative to the concurrent control contained in parentheses.
Source: Kennel (2012c)

Table 28. Clinical chemistry changes and urine analysis findings in rats exposed to DFA in the diet for up to 90 days[a]

	Males				Females			
Parameter	**0 ppm**	**200 ppm**	**1 000 ppm**	**6 000 ppm**	**0 ppm**	**200 ppm**	**1 000 ppm**	**6 000 ppm**
Clinical chemistry								
Glucose (mmol/L)	5.68	4.00** (−30%)	3.14** (−45%)	3.12** (−45%)	6.04	4.42** (−27%)	3.07** (−49%)	2.82** (−53%)
Total bilirubin (µmol/L)	1.47	1.07* (−27%)	0.70** (−52%)	0.65** (−56%)	2.45	2.16 (−12%)	1.55** (−37%)	1.30** (−47%)
Phosphorus (mmol/L)	2.17	2.34 (+7.8%)	2.33 (+7.4%)	2.48** (+14%)	1.84	1.94 (+5.4%)	1.95 (+6.0%)	2.10* (+14%)
Urea (mmol/L)	4.83	5.57* (+15%)	6.08** (+26%)	5.70* (+18%)	5.51	6.25* (+13%)	6.76** (+23%)	6.17 (+12%)
Urine analysis								
Refractive index	1.36	1.35	1.35***	1.35***	1.36	1.36	1.35**	1.35*
Volume (mL)	3.50	7.43	11.91***	12.80***	1.66	2.13	3.41	4.24**
Protein								
N	10	10	10	10	7	8	9	9
0	0	0	0	0	0	0	1	0
1 (trace)	0	0	0	1	1	1	6	5
2 (1+) 0.3 g/L	1	8	9	8	3	3	2	4
3 (2+) 1 g/L	9	2	1	0	3	4	0	0
4 (3+) ≥ 3 g/L	0	0	0	1	0	0	0	0
Crystal								
N	10	10	10	10	8	9	9	9
0	0	0	3	2	4	5	5	7
1	0	0	0	1	1	1	1	1

Table 28 (continued)

Parameter	Males				Females			
	0 ppm	200 ppm	1 000 ppm	6 000 ppm	0 ppm	200 ppm	1 000 ppm	6 000 ppm
2	8	5	2	7	2	3	3	1
3	1	2	4	0	1	0	0	0
4	1	3	1	0	0	0	0	0
Ketones								
N	10	10	10	10	7	8	9	9
0	0	0	0	0	6	6	0	0
1	7	2	0	0	1	2	0	0
2	3	8	0	0	0	0	5	0
3	0	0	0	0	0	0	4	7
4	0	0	10	10	0	0	0	2

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Results expressed as the mean, with the % change relative to the concurrent control contained in parentheses.

Source: Kennel (2012c)

Urine analysis revealed treatment-related changes in males at all dietary levels and in females at 1000 and 6000 ppm, compared with controls. Higher urinary volumes and lower refractive indices were noted in these groups, lower protein levels were seen in females and lower amounts of usually observed crystals were noted in the 1000 and 6000 ppm male groups. Higher ketone levels were noted in the same treated groups, despite higher urinary volumes (Table 28). This change was correlated with the low blood glucose concentration.

There were no treatment-related functional observational battery findings or effects on locomotor activity.

A few black foci were noted in the glandular part of the stomach in both sexes at 1000 and 6000 ppm, and this change was considered to be treatment related and mostly correlated with focal glandular erosion/necrosis observed during the microscopic examination (Table 29).

The NOAEL was 200 ppm (equal to 12.7 mg/kg bw per day for males and 15.6 mg/kg bw per day for females), for reduced body weight, reduced blood glucose levels associated with increased urine volume and ketones, and the presence of black foci and glandular erosion in the stomach at 1000 ppm (equal to 66.2 mg/kg bw per day for males and 78.7 mg/kg bw per day for females) (Kennel, 2012c).

Table 29. Macroscopic and microscopic changes in the stomach in rats exposed to DFA

	Males				Females			
	0 ppm	200 ppm	1 000 ppm	6 000 ppm	0 ppm	200 ppm	1 000 ppm	6 000 ppm
Focus (foci), black	0/10	0/10	1/10	3/10	1/10	0/10	2/10	2/10
Glandular erosion/necrosis: focal								
Minimal	0/10	0/10	1/9	1/10	0/10	0/10	0/10	0/10
Slight	0/10	0/10	0/9	1/10	0/10	0/10	0/10	1/10
Moderate	0/10	0/10	0/9	0/10	0/10	0/10	1/10	0/10
Total	0/10	0/10	1/9	2/10	0/10	0/10	1/10	1/10

ppm: parts per million

Source: Kennel (2012c)

Difluoroethyl-amino-furanone. In a non-GLP range-finding study, difluoroethyl-amino-furanone (98.9% purity) was administered to groups of Wistar Crl:WI rats (five of each sex per group) for 14 days at a dietary concentration of 0, 1280, 3200, 8000 or 20 000 ppm (equal to 0, 135, 339, 736 and 1226 mg/kg bw per day for males and 0, 135, 335, 741 and 2254 mg/kg bw per day for females, respectively). Parameters monitored during this study were clinical observations, body weight and feed consumption. At the end of the 14-day treatment period, rats were killed and necropsied. Blood was collected for assessment of clinical pathology parameters on day 14 prior to necropsy. Selected organs were weighed and fixed. No histopathological examination was performed.

There were no mortalities or clinical signs. At 20 000 ppm, body weight loss occurred (up to 4% in males and 23% in females). At 8000 ppm, significantly lower ($P < 0.01$) body weight (up to 16.5% lower for males and 15.9% for females) and body weight gain (up to 54% lower for males and 64% for females) occurred. At 3200 ppm in males, significantly lower body weight gain ($P < 0.01$; 1.4 g versus 7.4 g in the controls) occurred between days 0 and 1, with overall body weight gain 18% lower than the control value. At 3200 ppm in females, mean body weight was up to 12% lower than the control value after approximately 1 week of exposure.

At 20 000 ppm, mean feed consumption was up to 59% lower than the control values in males and up to 25% lower in females, although the significance of these differences was unclear due to spillage of feed in the high-dose females. Feed conversion efficiency was decreased by 16.5–85.1% in males and by 18.8–85.1% in females at 3200, 8000 and 20 000 ppm. Marginally lower reticulocyte counts were noted at 8000 ppm (females) and 20 000 ppm (both sexes). Blood glucose level was lower than the control values at and above 3200 ppm in males (30–49%) and at all dose levels in females (21–52%). Cholesterol level was increased by up to 65% at 8000 and 20 000 ppm in males and by 49% at 20 000 ppm in females. Albumin level was slightly higher at 8000 and 20 000 ppm, and the albumin to globulin ratio was slightly higher in both sexes at 20 000 ppm and in males at 8000 ppm. Urea level was increased by 32% at 20 000 ppm in males and by 15% and 20% in females at 8000 and 20 000 ppm, respectively.

Potentially treatment-related macroscopic changes were observed at necropsy in the high-dose group. These included small prostate and seminal vesicles in five males and small spleen in two males, were associated with changes in the terminal body weights and were correlated with the organ weight changes; however, a definitive attribution cannot be made without histopathological evaluation.

On the basis of these findings, dietary concentrations of 200, 800 and 3000 ppm were chosen for a subsequent 28-day study (Kubaszky, 2012a).

In a 4-week toxicity study in rats, difluoroethyl-amino-furanone (98.9% purity) was administered ad libitum via the diet to groups of Wistar Crl:WI rats (10 of each sex per group) for 28 days at a concentration of 0, 200, 800 or 3000 ppm (equal to 0, 17, 68 and 243 mg/kg bw per day for males and 0, 19, 76 and 273 mg/kg bw per day for females, respectively). Rats were observed daily for mortality and clinical signs. Physical examinations were performed at least weekly. An evaluation for any potential neurotoxicity or ophthalmic toxicity was conducted on day 26. Body weight and feed consumption were measured at least weekly. Clinical pathology investigations (haematology, coagulation, clinical chemistry and urine analysis) were conducted at the completion of the treatment period, before necropsy on day 28. Prior to necropsy, the estrous cycle of all females was evaluated. Following termination, rats were necropsied. Weights of selected organs were recorded, and representative tissues/organs were sampled and preserved in appropriate fixatives. Histopathology was performed on tissues from the control and high-dose animals.

There were no treatment-related mortalities, clinical signs, signs of neurotoxicity or effects on feed consumption, feed conversion efficiency or urine analysis parameters. Evaluation of vaginal smears prior to necropsy showed the expected distribution of the estrous cycle phases within the normal population. A slight, but not statistically significant, trend towards lower body weights was observed at 3000 ppm in both sexes (5–6% below the control values at termination), corresponding

with findings in the preliminary study, where body weights were 15–20% below the control values at 8000 ppm. There were statistically significantly lower weight gains in both sexes in the first week of the study, but the overall weight gains for the study duration were not statistically significantly different. The variations in haematological parameters and clinical chemistry parameters were not considered toxicologically significant or related to treatment. No test item–related macroscopic or microscopic findings or changes in absolute or relative organ weights (relative to the body or brain weight) were observed at any dose level.

The NOAEL was 3000 ppm (equal to 243 mg/kg bw per day for males and 273 mg/kg bw per day for females), the highest dose tested (Kubaszky, 2012b).

(6-Chloro-3-pyridyl)methanol. In a 13-week toxicity study, (6-chloro-3-pyridyl)methanol (98.94% purity) was administered ad libitum in the diet to groups of Crj: CD™ (SD) rats (10 of each sex per group) at a concentration of 0, 160, 800, 4000 or 20 000 ppm (equal to 0, 9.9, 48.9, 250.1 and 1246.6 mg/kg bw per day for males and 0, 11.1, 55.9, 275.9 and 1173.7 mg/kg bw per day for females, respectively). Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed weekly throughout the study. Ophthalmological examinations were performed on the control and high-dose groups during the acclimatization phase and during week 12. Urine samples were collected overnight during the week before necropsy from all rats for the analysis of urinary parameters. Before necropsy, a blood sample was collected for the analysis of haematology and clinical chemistry parameters. All rats were necropsied, selected organs were weighed and a range of tissues was examined microscopically.

There were no treatment-related effects with regard to survival, clinical signs, ophthalmological examinations, haematological parameters, urine analysis evaluations or macroscopic examinations. Mean body weights for high-dose males and females were significantly less ($P < 0.05$) than those of the respective control groups (77–84% of controls) throughout the study. Mean cumulative (weeks 1–13) body weight gains of the high-dose groups were 67% of control values in males and 57% in females. Feed consumption of these high-dose groups was up to 42% lower than control values at weeks 1–4, 6, 9 and 13 in males and at all weeks in females. Feed efficiency values of the 20 000 ppm animals were significantly decreased ($P < 0.01$) during weeks 1 and 10 in males (50% and 37% of the control values, respectively) and during week 1 in females (91% of the control value). A statistically significant increase ($P < 0.01$; 1.7-fold higher than the controls) in serum ALP activity occurred only in the 20 000 ppm group females at study termination. Decreased absolute weights of lung (−14%, $P < 0.05$) and liver (−20%, $P < 0.05$) (males only) and increased relative weights of brain (males: +25%, $P < 0.01$), lung (females only, +22%, $P < 0.05$), liver (+17%, females only, $P < 0.01$), kidney (approximately +20% in both sexes, $P < 0.01$) and testis (right side only, +21%, $P < 0.05$) were seen only in the 20 000 ppm groups. However, these changes are attributed to the decreased body weights in these groups (21% in males, 22% in females). Eosinophilic intranuclear inclusions were observed in the proximal tubular epithelium of kidneys at 4000 ppm (seven males versus none in the controls; $P < 0.01$) and 20 000 ppm (10 males and nine females versus none in the controls; $P < 0.01$).

The NOAEL in males was 800 ppm (equal to 48.9 mg/kg bw per day), based on the occurrence of eosinophilic intranuclear inclusions in the kidney at 4000 ppm (equal to 250.1 mg/kg bw per day). The NOAEL in females was 4000 ppm (equal to 275.9 mg/kg bw per day), based on decreased body weight gain, decreased feed consumption and increased serum ALP activity at 20 000 ppm (equal to 1173.7 mg/kg bw per day) (Nukui & Ikeyama, 1997).

Genotoxicity

The results of the genotoxicity tests on flupyradifurone metabolites are summarized in Table 30.

Table 30. Results of genotoxicity tests with metabolites of flupyradifurone[a]

Metabolite (purity)	End-point	Test system	Concentration	Result	Reference
Difluoroacetic acid (96.7%)	In vitro reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate (±S9) 33–5 000 µg/plate (±S9)	Negative	Sokolowski (2013a)
	In vitro gene mutation	Chinese hamster V79 lung cells (HPRT test)	7.3–940 µg/mL (±S9) 30–960 µg/mL (±S9)	Negative	Hall (2010a)
	In vitro chromosomal aberration	Chinese hamster V79 lung cells	3.8–960 µg/mL (±S9, 4 h) 60–960 µg/mL (+S9, 4 h) 60–960 µg/mL (−S9, 18 h)	Negative	Hall (2010b)
Difluoroethyl-amino-furanone (98.5%)	In vitro reverse mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate (±S9) 33–5 000 µg/plate (±S9)	Negative	Sokolowski (2011b)
	In vitro chromosomal aberration	Chinese hamster V79 lung cells	6.4–1 636 µg/mL (−S9) 409–636 µg/mL (+S9)	Positive[b] Negative	Hall (2011a)
	In vitro gene mutation	Chinese hamster V79 lung cells (HPRT test)	51.3–1 640 µg/mL (±S9, 4 h) 51.3–1 640 µg/mL (+S9, 4 h) 51.3–1 640 µg/mL (−S9, 24 h)	Negative	Wollny (2011)
	In vivo mouse micronucleus	NMRI mice, males (7/group)	125, 250, 500 mg/kg bw, 2× intraperitoneally at 24 h intervals; sampled at 24 h after last dose	Negative	Hall (2011b)
	In vivo unscheduled DNA synthesis	Wistar rats, males (4/group)	1 000 and 2 000 mg/kg bw, orally (sampled at 4 and 16 h)	Negative	Hall (2011c)
(6-Chloro-3-pyridyl)methanol (99.14%)	In vitro reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	313–5 000 µg/plate (±S9)	Negative	Mochizuki & Kanaguchi (1997a)
6-Chloronicotinic acid (99.4%)	In vitro reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	313–5 000 µg/plate (±S9)	Negative	Mochizuki & Kanaguchi (1997b)
Amino-furanone (97.4%)	In vitro reverse mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate (±S9) 33–5 000 µg/plate (±S9)	Negative	Sokolowski (2014)
	In vitro chromosomal aberration	Human lymphocytes	6.5–1 000 µg/mL (±S9, 4 h) 60.9–1 000 µg/mL (+S9, 4 h) 6.5–1 000 µg/mL (−S9, 20 h)	Negative	Bohnenberger (2014)

Table 30 (continued)

Metabolite (purity)	End-point	Test system	Concentration	Result	Reference
Flupyradifurone-acetic acid (98.9%)	In vitro reverse mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate (±S9) 33–5 000 µg/plate (±S9)	Negative	Sokolowski (2013b)
	In vitro chromosomal aberration	Human lymphocytes	17.2–2 647.0 µg/mL (±S9, 4 h) 161.3–2 647.0 µg/mL (+S9, 4 h) 17.2–2 647.0 µg/mL (−S9, 20 h)	Negative	Bohnenberger (2013)

bw: body weight; DNA: deoxyribonucleic acid; HPRT: hypoxanthine-guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

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

[b] In the absence of S9 mix, aberrations were significantly increased in all dose groups (4.0–8.5%, excluding gaps). Two concentrations, 6.4 and 204.5 µg/mL, showed values of 4.0% that are not considered biologically relevant taking into account the laboratory's historical control values of 0.0–4.0% excluding gaps. All other values clearly exceeded the historical control data range without showing a clear dose dependency. Also, the number of cells carrying exchanges was distinctly increased.

Comparative toxicity of metabolites

As fewer toxicological data were provided for two of the metabolites, amino-furanone and flupyradifurone-acetic acid, supplementary analysis was undertaken using JMPR's Plant and Animal Metabolite Assessment Scheme (WHO, 2015). Both of these metabolites were classified as Cramer class III compounds when evaluated with Toxtree (v2.6.13). However, based on the absence of a mutagenic or clastogenic response in vitro, neither metabolite is likely to be genotoxic in vivo. This conclusion is supported by taking a read-across approach from parent flupyradifurone, which had the same structural alerts as both metabolites (and is classified as a Cramer class III compound), but showed no indication of genotoxicity in both in vitro and in vivo tests. In addition, negligible levels of these metabolites are present in plant commodities. On this basis, dietary exposures are likely to be well below conservative thresholds of toxicological concern.

3. Observations in humans

There were no reports submitted on adverse health effects in workers involved in the manufacture or use of flupyradifurone. No cases of human poisonings have been reported.

Comments

Biochemical aspects

In studies conducted in rats using [^{14}C]flupyradifurone, maximum plasma concentrations of radioactivity were reached at 1 hour after a single oral dose of 2 mg/kg bw and 2–4 hours after a single oral dose of 200 mg/kg bw (Klempner, 2012). Based on the level of radioactivity in urine and tissues following oral dosing, estimates of gastrointestinal absorption ranged from 79% in males to 91% in females (Koester, 2011a,c; Weber, 2011a,b). A comparison of the dose-normalized area under the plasma concentration–time curve following equivalent oral and intravenous doses (2 mg/kg bw) in males indicated that gastrointestinal absorption was 93% (Klempner, 2012). The plasma elimination half-life ranged from 3 to 8 hours (Weber, 2011a; Klempner, 2012). The majority (up to 90%) of radioactivity was excreted in urine within 24 hours (Koester & Weber, 2011; Weber, 2011a; Klempner, 2012). There was no evidence of tissue accumulation (Koester, 2011a,c; Koester & Weber, 2011; Weber, 2011a; Klempner, 2012). Although flupyradifurone was the main compound detected in excreta (up to 50% of the radioactivity in males and 70% of the radioactivity in females), it undergoes

hydroxylation, conjugation and cleavage reactions to generate eight identified metabolites and 19 unidentified metabolites (Koester, 2011b,c; Weber, 2011a; Klempner, 2012).

Toxicological data

In rats, the oral LD_{50} was greater than 300 and less than 2000 mg/kg bw (Gillissen, 2009a), the dermal LD_{50} was greater than 2000 mg/kg bw (Gillissen, 2009b) and the LC_{50} was greater than 4.67 mg/L (Folkerts, 2010). Flupyradifurone was not irritating to the skin or eyes of rabbits or a skin sensitizer in mice (Gmelin, 2009a,b; Vohr, 2009).

In mice, rats and dogs, the liver is the main target organ, with the thyroid an additional target in rats and the skeletal muscle an additional target in dogs. Liver enzyme induction (specifically CYP3A) and liver hypertrophy were noted in short-term repeated-dose studies in rats. A notable feature of this compound (and some of its metabolites) is its ability to reduce blood glucose levels.

In a 30-day range-finding study in mice, which tested dietary concentrations of 0, 300, 600 and 1200 ppm flupyradifurone (equal to 0, 40, 78 and 207 mg/kg bw per day for males and 0, 47, 98 and 192 mg/kg bw per day for females, respectively), reduced body weight gain occurred in males at 1200 ppm (equal to 207 mg/kg bw per day) (Blanck, 2007).

In a 90-day study in mice, which tested dietary concentrations of 0, 100, 500 and 2500 ppm flupyradifurone (equal to 0, 15.6, 80.6 and 407 mg/kg bw per day for males and 0, 18.8, 98.1 and 473 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 80.6 mg/kg bw per day), based on lower body weight and body weight gain, changes in clinical chemistry parameters, increased liver weights and an increase in the severity of hepatocellular vacuolation at 2500 ppm (equal to 407 mg/kg bw per day) (Odin-Feurtet, 2009a).

In a 30-day range-finding study in rats, which tested gavage flupyradifurone doses of 0, 75, 200 and 350 mg/kg bw per day, CYP3A was induced at every dose in males and at 200 and 350 mg/kg bw per day in females. Deaths and clinical signs (females), reduced glucose levels (males), increased triglyceride levels, increased ALAT and ALP activities (females), increased liver weights, liver hypertrophy and thyroid follicular cell hypertrophy occurred at 200 and 350 mg/kg bw per day (Capt, 2007).

In a second 30-day range-finding study conducted only in male rats, which tested dietary flupyradifurone concentrations of 0, 410 and 5000 ppm (equal to 0, 33.6 and 385 mg/kg bw per day, respectively), CYP3A was induced at 5000 ppm. Lower body weight, body weight gain, feed consumption and blood glucose levels, increased plasma urea and cholesterol levels, increased liver weights, liver hypertrophy and thyroid hypertrophy occurred at 5000 ppm (equal to 385 mg/kg bw per day) (Blanck, 2008).

In a 90-day study in rats that incorporated an assessment of neurotoxicity, dietary concentrations of 0, 100, 500 and 2500 ppm flupyradifurone (equal to 0, 6.0, 30.2 and 156 mg/kg bw per day for males and 0, 7.6, 38.3 and 186 mg/kg bw per day for females, respectively) were tested. The NOAEL was 500 ppm (equal to 30.2 mg/kg bw per day), based on lower body weight and body weight gain and thyroid follicular cell hypertrophy at 2500 ppm (equal to 156 mg/kg bw per day) (Odin-Feurtet, 2009b).

In a 28-day range-finding study in dogs, which tested dietary flupyradifurone concentrations of 0, 500, 2000 and 4000 ppm (equal to 0, 16, 62 and 118 mg/kg bw per day for males and 0, 18, 77 and 131 mg/kg bw per day for females, respectively), body weight loss and reduced glycogen accumulation in hepatocytes occurred at 4000 ppm (equal to 118 mg/kg bw per day) (Odin-Feurtet, 2008).

In a 90-day study in dogs, which tested dietary flupyradifurone concentrations of 0, 400, 1200 and 3600/2400 ppm (equal to 0, 12, 33 and 102/85 mg/kg bw per day for males and 0, 12, 41 and 107/78 mg/kg bw per day for females, respectively), the NOAEL was 400 ppm (equal to 12 mg/kg bw

per day), based on increased CPK, ASAT and ALAT activities and skeletal muscle degeneration at 1200 ppm (equal to 33 mg/kg bw per day) (Eigenberg, 2010).

In a 1-year toxicity study in dogs, which tested dietary flupyradifurone concentrations of 0, 150, 300 and 1000 ppm (equal to 0, 4.6, 7.8 and 28.1 mg/kg bw per day for males and 0, 4.1, 7.8 and 28.2 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 7.8 mg/kg bw per day), based on reduced body weight gain (females) and skeletal muscle degeneration (both sexes) at 1000 ppm (equal to 28.1 mg/kg bw per day) (Cada, 2011).

The overall NOAEL in the 90-day and 1-year dog studies was 400 ppm (equal to 12 mg/kg bw per day), with an overall LOAEL of 1000 ppm (equal to 28.1 mg/kg bw per day).

In a 24-month chronic toxicity and carcinogenicity study in mice, which tested dietary flupyradifurone concentrations of 0, 70, 300 and 1500 ppm (equal to 0, 10, 43 and 224 mg/kg bw per day for males and 0, 12, 53 and 263 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 43 mg/kg bw per day), based on reduced body weight and body weight gain at 1500 ppm (equal to 224 mg/kg bw per day). No treatment-related tumours were observed up to the highest dietary concentration of 1500 ppm (equal to 224 mg/kg bw per day) (Kennel, 2012a).

In a 2-year chronic toxicity and carcinogenicity study in rats, which tested dietary flupyradifurone concentrations of 0, 80, 400 and 2000 ppm (equal to 0, 3.16, 15.8 and 80.8 mg/kg bw per day for males and 0, 4.48, 22.5 and 120 mg/kg bw per day for females, respectively), the NOAEL was 400 ppm (equal to 15.8 mg/kg bw per day), based on reduced body weight and body weight gain, liver enlargement with accompanying histopathological changes, and histopathological changes in the lungs and thyroid at 2000 ppm (equal to 80.8 mg/kg bw per day). No treatment-related tumours were observed up to the highest dietary concentration of 2000 ppm (equal to 80.8 mg/kg bw per day) (Garcin, 2012).

The Meeting concluded that flupyradifurone is not carcinogenic in mice or rats.

Flupyradifurone was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found (Entian, 2009; Herbold, 2009a,b; Thum, 2009; Sokolowski, 2011a; Wieland, 2011).

The Meeting concluded that flupyradifurone 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 flupyradifurone is unlikely to pose a carcinogenic risk to humans.

In a one-generation range-finding reproductive toxicity study in rats, which tested dietary flupyradifurone concentrations of 0, 200, 700 and 2000 ppm (equal to 0, 14.5, 50.1 and 147.5 mg/kg bw per day for males and 0, 17.5, 60.0 and 168.9 mg/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 2000 ppm (equal to 147.5 mg/kg bw per day), the highest dose tested. The NOAEL for both parental toxicity and offspring toxicity was 700 ppm (equal to 50.1 mg/kg bw per day), based on reduced body weight at 2000 ppm (equal to 147.5 mg/kg bw per day) (Milius, 2010).

In a two-generation reproductive toxicity study, which tested dietary flupyradifurone concentrations of 0, 100, 500 and 1800 ppm (equal to 0, 6.5, 32.3 and 119.8 mg/kg bw per day for males and 0, 7.8, 39.2 and 140.2 mg/kg bw per day for females, respectively, during premating), the NOAEL for reproductive toxicity was 500 ppm (equal to 39.2 mg/kg bw per day), based on decreases in estrous cycle length, the number of implantation sites and litter size at 1800 ppm (equal to 140.2 mg/kg bw per day). The NOAEL for parental toxicity was 100 ppm (equal to 7.8 mg/kg bw per day), based on decreased body weight in females at 500 ppm (equal to 39.2 mg/kg bw per day). The NOAEL for offspring toxicity was 100 ppm (equal to 7.8 mg/kg bw per day), based on decreased female pup weight and weight gain at 500 ppm (equal to 39.2 mg/kg bw per day) (Milius, 2011).

In a developmental toxicity study in rats, which tested gavage flupyradifurone doses of 0, 15, 50 and 150 mg/kg bw per day from days 6 to 20 of gestation, the NOAEL for maternal toxicity was 50 mg/kg bw per day, for clinical signs (salivation) and body weight loss at 150 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, for slightly delayed ossification at 150 mg/kg bw per day (Langrand-Lerche, 2010).

In a follow-up developmental toxicity study in rats, which tested doses of 0, 20 and 30 mg/kg bw per day, the NOAEL for maternal toxicity and embryo and fetal toxicity was 30 mg/kg bw per day, the highest dose tested (Langrand-Lerche, 2012).

The overall NOAEL for both maternal and embryo/fetal toxicity from both developmental toxicity studies was 50 mg/kg bw per day.

In a developmental toxicity study in rabbits, which tested gavage flupyradifurone doses of 0, 7.5, 15 and 40 mg/kg bw per day, the NOAEL for maternal toxicity was 15 mg/kg bw per day, based on body weight loss and reduced feed consumption over the first few days of dosing at 40 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, the highest dose tested (Kennel, 2012b).

The Meeting concluded that flupyradifurone is not teratogenic.

In an acute neurotoxicity study in rats, which tested flupyradifurone doses of 0, 50, 200 and 800 mg/kg bw per day, piloerection occurred at every dose. In a follow-up study, which tested flupyradifurone doses of 0, 20 and 35 mg/kg bw, no systemic toxicity or neurotoxicity was observed at any dose (Garcin, 2011a).

In a 90-day neurotoxicity study in rats, which tested dietary flupyradifurone concentrations of 0, 100, 500 and 2500 ppm (equal to 0, 5.7, 29.4 and 143 mg/kg bw per day for males and 0, 6.9, 34.8 and 173 mg/kg bw per day for females, respectively), reduced body weight, body weight gain and feed consumption were observed at 2500 ppm (equal to 143 mg/kg bw per day). No neurotoxicity was observed at any dose (Garcin, 2011b).

In a developmental neurotoxicity study in rats, which tested dietary flupyradifurone concentrations of 0, 120, 500 and 1200 ppm (equal to 0, 10.3, 42.4 and 102 mg/kg bw per day, respectively), the NOAEL for maternal toxicity and offspring toxicity was 500 ppm (equal to 42.4 mg/kg bw per day), based on lower body weight and body weight gain in dams, reduced body weight gain in pups during lactation and an increase in the auditory startle reflex in pups at 1200 ppm (equal to 102 mg/kg bw per day). No neurotoxicity was observed at any dose (Gilmore, 2012).

The Meeting concluded that flupyradifurone is not neurotoxic.

In a 28-day immunotoxicity study in rats, which tested dietary flupyradifurone concentrations of 0, 125, 600 and 3000 ppm (equal to 0, 10, 50 and 230 mg/kg bw per day, respectively), no effects on the immune system were noted up to the highest dose tested. Reduced body weight gain and feed consumption were observed at 3000 ppm (equal to 230 mg/kg bw per day) (Repetto, 2011).

The Meeting concluded that flupyradifurone is not immunotoxic.

Toxicological data on metabolites and/or degradates

Toxicity tests were conducted on six flupyradifurone metabolites: (1) DFA, which is a major soil, water and plant metabolite and is also detected in rat urine at approximately 6% of the administered dose; (2) difluoroethyl-amino-furanone, which is a minor plant metabolite also present in rat urine at approximately 10% of the administered dose; (3) (6-chloro-3-pyridyl)methanol and (4) 6-chloronicotinic acid, which are plant metabolites not detected in rat metabolism studies and metabolites common to other pesticides; and (5) amino-furanone and (6) flupyradifurone-acetic acid, which are plant metabolites not detected in rat metabolism studies.

The LD_{50}s in rats were greater than 300 and less than 2000 mg/kg bw for DFA (Gerbeix, 2010), greater than 2000 mg/kg bw for difluoroethyl-amino-furanone (Rokh, 2011), 1483 mg/kg bw for (6-chloro-3-pyridyl)methanol (Mochizuki & Goto, 1997a) and greater than 5000 mg/kg bw for 6-chloronicotinic acid (Mochizuki & Goto, 1997b). Amino-furanone and flupyradifurone-acetic acid were not tested for acute toxicity.

In a 14-day range-finding study in rats, which tested dietary DFA concentrations of 0, 500, 2000 and 8000 ppm (equal to 0, 48, 187 and 745 mg/kg bw per day for males and 0, 51, 201 and 800 mg/kg bw per day for females, respectively), reduced blood glucose levels were observed at every dose (Kennel, 2011).

In a 90-day toxicity study in rats, which tested dietary DFA concentrations of 0, 200, 1000 and 6000 ppm (equal to 0, 12.7, 66.2 and 380 mg/kg bw per day for males and 0, 15.6, 78.7 and 472 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 12.7 mg/kg bw per day), based on reduced body weight, reduced blood glucose levels associated with increased urine volume and ketones, and the presence of black foci and glandular erosion in the stomach at 1000 ppm (equal to 66.2 mg/kg bw per day) (Kennel, 2012c).

In a 14-day range-finding study in rats, which tested dietary difluoroethyl-amino-furanone concentrations of 0, 1280, 3200, 8000 and 20 000 ppm (equal to 0, 135, 339, 736 and 1226 mg/kg bw per day for males and 0, 135, 335, 741 and 2254 mg/kg bw per day for females, respectively), reduced blood glucose levels occurred in females at every dose and in males at and above 3200 ppm (equal to 335 mg/kg bw per day). Reduced body weight gain and feed conversion efficiency occurred in both sexes at and above 3200 ppm (equal to 335 mg/kg bw per day) (Kubaszky, 2012a).

In a 28-day follow-up study in rats, which tested dietary difluoroethyl-amino-furanone concentrations of 0, 200, 800 and 3000 ppm (equal to 0, 17, 68 and 243 mg/kg bw per day for males and 0, 19, 76 and 273 mg/kg bw per day for females, respectively), the NOAEL was 3000 ppm (equal to 243 mg/kg bw per day), the highest dose tested (Kubaszky, 2012b).

In a 13-week toxicity study in rats, which tested dietary (6-chloro-3-pyridyl)methanol concentrations of 0, 160, 800, 4000 and 20 000 ppm (equal to 0, 9.9, 48.9, 250.1 and 1246.6 mg/kg bw per day for males and 0, 11.1, 55.9, 275.9 and 1173.7 mg/kg bw per day for females, respectively), the NOAEL was 800 ppm (equal to 48.9 mg/kg bw per day), based on the occurrence of eosinophilic intranuclear inclusions in the kidney of males at 4000 ppm (equal to 250.1 mg/kg bw per day) (Nukui & Ikeyama, 1997).

The six flupyradifurone metabolites were tested for genotoxicity in an adequate range of assays, both in vitro and in vivo (Mochizuki & Kanaguchi, 1997a,b; Hall, 2010a,b, 2011a,b,c; Sokolowski, 2011b, 2013a,b, 2014; Wollny, 2011; Bohnenberger, 2013, 2014). No evidence of genotoxicity was found, with the exception of difluoroethyl-amino-furanone, which was clastogenic in vitro in the absence of metabolic activation (Hall, 2011a). However, when further in vivo testing was undertaken, no evidence of genotoxicity was found (Hall, 2011b,c). On this basis, none of the six metabolites tested is likely to be genotoxic in vivo.

As limited toxicological data were provided for two of the metabolites, amino-furanone and flupyradifurone-acetic acid, supplementary analysis was undertaken using JMPR's Plant and Animal Metabolite Assessment Scheme (WHO, 2015). On the basis of this assessment and the fact that negligible levels of these metabolites are present in plant commodities, the Meeting concluded that neither of these metabolites poses a safety concern.

The Meeting concluded that difluoroethyl-amino-furanone, (6-chloro-3-pyridyl)methanol, 6-chloronicotinic acid, amino-furanone and flupyradifurone-acetic acid are of no greater toxicity than flupyradifurone, and therefore the ADI and ARfD established for flupyradifurone (see below) would adequately cover dietary exposure to these metabolites.

Based on a comparison of the NOAELs in rats over 90 days of dietary exposure (12.7 mg/kg bw per day for DFA versus 30.2 mg/kg bw per day for flupyradifurone), the Meeting concluded that DFA is approximately 2.5-fold more potent than flupyradifurone.

Human data

No information was provided on adverse health effects in workers involved in the manufacture or use of flupyradifurone. No information on accidental or intentional poisoning in humans is available.

The Meeting concluded that the existing database on flupyradifurone 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 0–0.08 mg/kg bw based on a NOAEL of 7.8 mg/kg bw per day for decreased maternal body weight, reduced female pup weight and pup weight gain at 39.2 mg/kg bw per day in a two-generation reproductive toxicity study in rats, with the application of a 100-fold safety factor. This NOAEL is supported by the NOAEL of 12 mg/kg bw per day for skeletal muscle degeneration in repeated-dose studies in the dog.

The Meeting established an acute reference dose (ARfD) of 0.2 mg/kg bw, based on the maternal toxicity NOAEL of 15 mg/kg bw for body weight loss and reduced feed consumption over the first few days of exposure in a rabbit developmental toxicity study, with the application of a 100-fold safety factor.

The Meeting concluded that the metabolite DFA is 3-fold (rounded) more toxic than flupyradifurone over 90 days of dietary exposure in rats. On this basis, it was concluded that a 3-fold potency factor should be applied to the residue levels for use in both the acute and chronic dietary exposure estimates for DFA and that these should be added to the dietary exposures for flupyradifurone and compared with the ARfD and ADI for flupyradifurone, respectively.

Both the ADI and ARfD are established for the sum of flupyradifurone and its metabolites (difluoroethyl-amino-furanone, (6-chloro-3-pyridyl)methanol, 6-chloronicotinic acid, amino-furanone, flupyradifurone-acetic acid and 3× DFA) and expressed as the parent flupyradifurone.

Levels relevant to risk assessment of flupyradifurone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	300 ppm, equal to 43 mg/kg bw per day	1 500 ppm, equal to 224 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 224 mg/kg bw per day[b]	–
Rat	Acute neurotoxicity studies[c,d]	Toxicity	35 mg/kg bw per day	50 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	400 ppm, equal to 15.8 mg/kg bw per day	2 000 ppm, equal to 80.8 mg/kg bw per day
		Carcinogenicity	2 000 ppm, equal to 80.8 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	500 ppm, equal to 39.2 mg/kg bw per day	1 800 ppm, equal to 140.2 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Parental toxicity	100 ppm, equal to 7.8 mg/kg bw per day	500 ppm, equal to 39.2 mg/kg bw per day
		Offspring toxicity	100 ppm, equal to 7.8 mg/kg bw per day	500 ppm, equal to 39.2 mg/kg bw per day
	Developmental toxicity studies[c,d]	Maternal toxicity	50 mg/kg bw per day	150 mg/kg bw per day
		Embryo and fetal toxicity	50 mg/kg bw per day	150 mg/kg bw per day
	Developmental neurotoxicity study[a]	Maternal toxicity	500 ppm, equal to 42.4 mg/kg bw per day	1 200 ppm, equal to 102 mg/kg bw per day
		Embryo and fetal toxicity	500 ppm, equal to 42.4 mg/kg bw per day	1 200 ppm, equal to 102 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	15 mg/kg bw per day	40 mg/kg bw per day
		Embryo and fetal toxicity	40 mg/kg bw per day[b]	–
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	400 ppm, equal to 12 mg/kg bw per day	1 000 ppm, equal to 28.1 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) (for sum of flupyradifurone and metabolites,[4] expressed as flupyradifurone)

0–0.08 mg/kg bw

Estimate of acute reference dose (ARfD)

0.2 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 flupyradifurone

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; > 80%
Dermal absorption	No data

[4] Difluoroethyl-amino-furanone, (6-chloro-3-pyridyl)methanol, 6-chloronicotinic acid, amino-furanone, flupyradifurone-acetic acid and 3× DFA.

Distribution	Rapid tissue distribution
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Rapid and complete
Metabolism in animals	Extensive; hydroxylation, conjugation and cleavage reactions
Toxicologically significant compounds in animals and plants	Flupyradifurone, DFA
Acute toxicity	
Rat, LD_{50}, oral	> 300 and < 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.67 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse, dermal sensitization	Not sensitizing (local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Muscle degeneration
Lowest relevant oral NOAEL	12 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	Reduced body weight and body weight gain, liver toxicity
Lowest relevant NOAEL	15.8 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	Reduced body weight, length of estrous cycle, implantation sites and litter size
Lowest relevant parental NOAEL	7.8 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	7.8 mg/kg bw per day (rat)
Lowest relevant reproduction NOAEL	39.2 mg/kg bw per day (rat)
Developmental toxicity	
Developmental target/critical effect	Slightly delayed ossification at maternally toxic doses
Lowest maternal NOAEL	15 mg/kg bw per day (rabbit)
Lowest embryo/fetal NOAEL	40 mg/kg bw per day (highest dose tested; rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	800 mg/kg bw (highest dose tested; rat)
Subchronic neurotoxicity NOAEL	143 mg/kg bw per day (highest dose tested; rat)
Developmental neurotoxicity NOAEL	102 mg/kg bw per day (highest dose tested; rat)

Other toxicological studies	
Immunotoxicity NOAEL	230 mg/kg bw per day (highest dose tested; rat)
Toxicological studies on DFA	
Oral LD_{50} (rat)	> 300 – < 2 000 mg/kg bw
Lowest relevant short-term NOAEL (rat)	12.7 mg/kg bw per day (90 d)
Genotoxicity	No evidence of genotoxicity
Toxicological studies on difluoroethyl-amino-furanone	
Oral LD_{50} (rat)	> 2 000 mg/kg bw
Lowest relevant short-term NOAEL (rat)	243 mg/kg bw per day (28 d)
Genotoxicity	Clastogenic in vitro; not genotoxic in vivo
Toxicological studies on (6-chloro-3-pyridyl)methanol	
Oral LD_{50} (rat)	1 483 mg/kg bw (females), 1 842 mg/kg bw (males)
Lowest relevant short-term NOAEL (rat)	48.9 mg/kg bw per day (90 d)
Genotoxicity	No evidence of genotoxicity
Toxicological studies on 6-chloronicotinic acid	
Oral LD_{50} (rat)	> 5 000 mg/kg bw
Genotoxicity	No evidence of genotoxicity
Toxicological studies on amino-furanone	
Genotoxicity	No evidence of genotoxicity
Toxicological studies on flupyradifurone-acetic acid	
Genotoxicity	No evidence of genotoxicity
Medical data	
	No data

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

Summary

	Value	Studies	Safety factor
ADI	0–0.08 mg/kg bw	Two-generation reproductive toxicity study (rat)	100
ARfD	0.2 mg/kg bw	Developmental toxicity study (rabbit)	100

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LUFENURON

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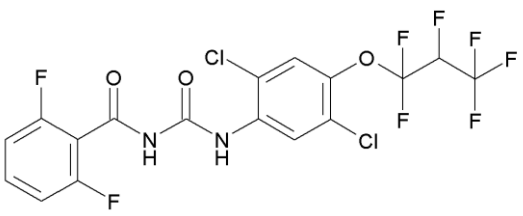
Explanation

Lufenuron (Fig. 1) is the International Organization for Standardization (ISO)–approved common name for (*RS*)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl)urea (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 103055-07-8.

Fig. 1. Structure of lufenuron



Lufenuron is an insecticide initially registered for use on a wide range of crops for the control of the larvae of many insect pests. Lufenuron inhibits chitin synthesis, probably through enzymatic interference, and prevents the larvae from moulting.

Lufenuron 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.

All critical studies contained statements of compliance with good laboratory practice (GLP).

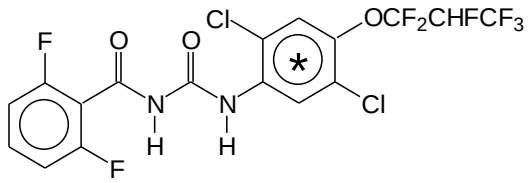
Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

To investigate the absorption, distribution and excretion of lufenuron, a single oral dose of [^{14}C]lufenuron (batch no. ILA-178 7A; > 98% radiochemical purity; see Fig. 2 for position of the radiolabel) was administered by gavage to four groups of four male rats (Alpk:AP$_f$SD, Wistar-derived) at a dose of 0.1, 1.0, 10 or 100 mg/kg body weight (bw), or a single intravenous dose of [^{14}C]lufenuron was administered to two groups of four male rats at a dose of 0.1 or 10 mg/kg bw. The dose vehicle was 7:3 (volume per volume [v/v]) polyethylene glycol (PEG) 400/ethanol. Urine and faeces were collected daily for 7 days following dosing. Subsequently, faeces were collected daily on days 8–21 following dosing, and urine was collected on days 10–11, 14–15 and 19–21. Serial blood samples were collected at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 288 and 384 hours after dosing and by cardiac puncture at termination. Twenty-one days after dosing, each rat was terminated.

Fig. 2. Position of radiolabel



* = ^{14}C position

The study was not performed according to GLP, but it met all criteria specified in the study guidelines (Organisation for Economic Co-operation and Development [OECD] Test Guideline 417).

Over the 21-day duration of the study, the mean total proportion of administered radioactivity excreted in urine following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw was 0.6%, 0.5%, 0.6% and 0.2%, respectively, whereas faecal excretion accounted for 80.6%, 72.9%, 80.4% and 91.9%, respectively. The mean total percentage of dose excreted in urine following an intravenous dose of 0.1 or 10 mg/kg bw was 0.6% and 0.8%, respectively, whereas faecal excretion accounted for 66.5% and 62.2%, respectively. A characteristic feature of faecal excretion was the slow, sustained period of elimination. Faecal excretion was still measurable 21 days following administration of a single oral or intravenous dose, irrespective of dose level (Table 1).

The area under the blood concentration–time curves over 120 hours ($AUC_{0–120\ h}$) after dosing, for both dose routes, clearly show that for dose levels between 0.1 and 10 mg/kg bw, systemic exposure is directly proportional to the administered dose. The systemic bioavailability of lufenuron can be directly determined by the classical method of calculating the ratio of AUC between an oral and an intravenous dose. This gives a value of approximately 70% for the 0.1 and 10 mg/kg bw dose levels. The amount of radioactivity in urine following oral and intravenous dosing can also be treated

Table 1. Comparison of kinetics following single oral or intravenous administration of lufenuron

Route	Dose (mg/kg bw)	Radioactivity (% of dose at 21 days after dosing)				$AUC_{0–120\ h}$ (h·µg eq/g)
		Urine	Faeces	Tissue and residual carcass	Total recovery	
Oral	0.1	0.64	80.59	21.00	102.93	0.40
	1.0	0.51	72.93	23.55	97.23	4.37
	10	0.57	80.37	14.83	96.05	41.25
	100	0.17	91.87	5.17	97.43	83.88
Intravenous	0.1	0.64	66.50	32.03	99.77	0.56
	10	0.82	62.23	34.73	98.11	60.69

$AUC_{0–120\ h}$: area under the blood concentration–time curve over 120 hours; bw: body weight; eq: equivalents
Source: Booth (2004)

similarly to obtain an estimate of absorption. For the 10 mg/kg bw dose level, the urinary data indicate an absorption value of 70%; for the 0.1 mg/kg bw dose level, the absorption is estimated at 100%.

The blood $AUC_{0–120\ h}$ increased with dose over the range 0.1–100 mg/kg bw. At the highest oral dose level, 100 mg/kg bw, evidence of saturation was observed, and the AUC was no longer proportional to the administered dose. Saturation was more evident in the relationship between blood $AUC_{0–\infty}$ and dose. The time to reach the maximum concentration (T_{max}) was 8 hours after oral administration and 2 hours after intravenous administration (Table 2).

The mean terminal half-life of faecal excretion following oral dosing was 256 hours, and following intravenous dosing, 232 hours. At termination of the study, the mean total proportion of administered radioactivity present in tissues and carcass following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw was 21.0%, 23.6%, 14.8% and 5.2%, respectively. The mean total proportion of dose present in tissues following an intravenous dose of 0.1 or 10 mg/kg bw was 32.0% and 34.7%, respectively. Radioactivity was located mainly in the fat and, to a much lesser extent, in residual carcass, followed by liver and kidneys. The total mean percentage recoveries of administered radioactivity, including excreta, tissues and residual carcasses, following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw were 102.9%, 97.2%, 96.0% and 97.4%, respectively. The total mean percentage recoveries of administered radioactivity, including excreta, tissues and residual carcasses, following an intravenous dose of 0.1 or 10 mg/kg bw were 99.8% and 98.1%, respectively.

Table 2. Blood kinetics data following a single oral or intravenous dose of [^{14}C]lufenuron

Nominal dose, route	Mean achieved dose (mg/kg bw)	$AUC_{0–120\ h}$ (h·µg/g)	$AUC_{0–\infty}$ (h·µg/g)	T_{max} (h)	C_{max} (µg/g)
0.1 mg/kg bw, oral gavage	0.10	0.40	0.49	8	0.008
1.0 mg/kg bw, oral gavage	1.08	4.37	10.73	8	0.097
10 mg/kg bw, oral gavage	10.52	41.25	90.44	8	0.89
100 mg/kg bw, oral gavage	101.85	83.88	216.36	8	1.341
0.1 mg/kg bw, intravenous	0.11	0.56	0.94	2	0.017
10 mg/kg bw, intravenous	10.35	60.69	153.76	2	1.907

$AUC_{0–120\ h}$: area under the blood concentration–time curve over 120 hours; $AUC_{0–\infty}$: area under the blood concentration–time curve from time 0 to infinity; bw: body weight; C_{max}; maximum concentration; T_{max}: time to reach maximum concentration
Source: Booth (2004)

From the AUC values determined for blood, the dose–response curve does not appear to be linear at the high dose level following oral gavage dosing (Booth, 2004).

To investigate the absorption, distribution and excretion of lufenuron, [^{14}C]lufenuron (batch no. Le-78.3A; approximately 99% radiochemical purity) was administered by gavage to groups of male and female rats (Tif: RAIf (SPF)) at a single dose of either 0.5 or 100 mg/kg bw following the study design indicated in Table 3.

Table 3. Terminal toxicokinetic studies in the rat – experimental scheme

Group	Number and sex	Route and dose level of [^{14}C]lufenuron	Sample collection times
B	5 males, 5 females	Single oral dose of 0.5 mg/kg bw 199 kBq/animal	Urine: 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h Faeces: 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h Volatiles: 0–8 and 8–24 h Expired air: 0–8 and 8–24 h Tissues: 7 days after administration of the ^{14}C-labelled material, tissues and organs were taken for analysis
C	5 males, 5 females	Single oral dose of 0.5 mg/kg bw [^{14}C]lufenuron preceded by 14 consecutive daily oral doses of 0.5 mg unlabelled lufenuron/kg bw 239 kBq/animal	Collection of urine, faeces, volatiles, expired air and tissues as in Group B
D	5 males, 5 females	Single oral dose of 100 mg/kg bw 7 785 kBq/animal (diluted with batch no. P1 to a specific activity of 385 kBq/mg)	Collection of urine, faeces, volatiles, expired air and tissues as in Group B
E1	3 males	Single oral dose of 0.5 mg/kg bw 196 kBq/animal	Blood: 1, 2, 4, 8, 12, 24, 32, 48 and 56 h after administration (determination of T_{max} and $T_{max/2}$ of this dose)
E2	3 males	Single oral dose of 100 mg/kg bw 1 423 kBq/animal (diluted with batch no. AMS 266/101 to a specific activity of 72 kBq/mg)	Blood: 1, 2, 4, 8, 12, 24, 32, 48, 56 and 72 h after administration (determination of T_{max} and $T_{max/2}$ of this dose)
F1 and F2	10 males	Single oral dose of 0.5 mg/kg bw 191 kBq/animal	Tissues: 8 h (T_{max}), 45 h ($T_{max/2}$), 5 days and 12 days after administration, selected tissues and organs were taken for analysis
F3 and F4	10 males	Single oral dose of 100 mg/kg bw 1 378 kBq/animal (diluted with batch no. AMS 266/101 to a specific activity of 70 kBq/mg)	Tissues: 8 h (T_{max}), 60 h ($T_{max/2}$), 5 days and 12 days after administration, selected tissues and organs were taken for analysis
G	5 males, bile duct cannulated under anaesthesia	Single oral dose of 0.5 mg/kg bw 237 kBq/animal	Urine: 0–24 and 24–48 h Faeces: 0–24 and 24–48 h Bile: 0–0.5, 0.5–1, 1–2, 2–4, 4–8, 8–18, 18–24, 24–42 and 42–48 h

bw: body weight; T_{max}: time to reach maximum concentration; $T_{max/2}$: time to decline to half the maximum concentration
Source: Bissig (1990)

Irrespective of dose level or sex, less than 1% of an oral dose of [^{14}C]lufenuron was excreted in urine, and less than 0.01% of the administered radioactivity was detected in exhaled volatile and carbon dioxide traps. The extent of absorption of a 0.5 mg/kg bw dose was 44%, based upon radioactivity present in urine and residual carcass and tissues at 7 days after administration. However, the extent of absorption and faecal excretion were dose dependent. Despite the similarities in faecal excretion of a 0.5 mg/kg bw dose by bile duct–cannulated and non-cannulated male rats and the low biliary elimination over 48 hours after dosing, the slow rate of faecal excretion by non-cannulated rats suggests that some faecal radioactivity was of biliary origin. The extent of absorption of a 100 mg/kg bw dose was much lower, accounting for at least 9% of the dose, based mainly on radioactivity present in tissues at the termination of the experiment. Even though some of the systemic dose was likely to have been eliminated in bile, the much lower proportion of dose retained in tissue and the very low urinary excretion indicate that at the high dose level, the capacity to absorb the test material was reduced (Table 4).

Table 4. Excretion of lufenuron by rats following an oral [^{14}C]lufenuron dose of 0.5 or 100 mg/kg bw

		Excretion (% of administered radioactivity)						
		B (single dose 0.5 mg/kg bw)		**C (repeated dose 0.5 mg/kg bw per day)**		**D (single dose 100 mg/kg bw)**		**G (bile duct cannulated, single dose 0.5 mg/kg bw)**
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
Bile	0–8 h	–	–	–	–	–	–	0.33
	8–24 h	–	–	–	–	–	–	0.54
	24–48 h	–	–	–	–	–	–	0.82
	Subtotal							1.70
Urine	0–24 h	0.27	0.29	0.19	0.31	0.10	0.14	0.09
	24–48 h	–	–	–	–	–	–	0.08
	24–96 h	0.35	0.27	0.25	0.28	0.10	0.10	–
	96–168 h	0.20	0.16	0.16	0.17	0.05	0.04	–
	Subtotal	0.82	0.72	0.60	0.75	0.26	0.28	0.17
Faeces	0–24 h	25.97	23.71	38.83	23.27	66.88	73.20	8.57
	24–48 h	–	–	–	–	–	–	43.03
	24–96 h	17.71	15.12	11.17	12.93	13.44	8.75	–
	96–168 h	8.26	8.92	5.31	7.84	2.10	1.32	–
	Subtotal	51.95	47.74	55.31	44.04	82.42	83.26	51.60
Expired air 0–24 h		Not measured	Not measured	Not measured	Not measured	< 0.01	< 0.01	Not measured
Total excretion		52.77	48.46	55.91	44.79	82.68	83.54	53.47
Cage wash		0.07	0.07	0.05	0.06	0.08	0.14	–
Tissue residues		5.41	4.95	5.87	8.36	1.96	1.50	–
Carcass		38.09	43.80	37.09	44.42	9.65	7.42	–
Total recovery 0–168 h		96.34	97.27	98.92	97.64	94.37	92.59	53.47

bw: body weight
Source: Bissig (1990)

The urinary excretion and tissue residue data showed that at least 44% and 9% of the orally administered lufenuron were absorbed from the intestinal tract into the systemic circulation by the low-dose rats (groups B and C) and the high-dose rats (group D), respectively.

Seven days after a single oral dose of 0.5 mg/kg bw, significant residues were present in most tissues in both sexes, predominantly in fat (1.91 parts per million [ppm] in males and 2.40 ppm in females). Residues above 0.1 ppm were also measured in ovaries (0.44 ppm), uterus (0.23 ppm), thyroid (0.22 ppm in males and 0.16 ppm in females), liver (0.13 ppm in males and 0.15 ppm in females), lungs (0.11 ppm in females) and kidneys (0.11 ppm in females). As a consequence of the slightly faster excretion by males, the residues in virtually all tissues in males in groups B and C were slightly lower than those in females, with the single exception of the thyroid in group B. As approximately 43–49% of a single 0.5 mg/kg bw dose of [^{14}C]lufenuron was present in tissues, predominantly fat, after 7 days and as similar proportions of a 0.5 mg/kg bw dose of radiolabelled lufeuron were present in tissues of rats pretreated with 14 daily doses of unlabelled lufenuron, substantial proportions of each unlabelled dose are also likely to be present in tissues. In contrast, 9–12% of a 100 mg/kg bw dose remained in tissues 7 days after dosing, reflecting the lower extent of absorption. Accordingly, at this dose level, tissue residues were only 40–60 times and 30–40 times higher in males and females, respectively. The only exception to this observation was thyroid in females, which contained approximately 100 times higher residue levels. As for the low dose level, the highest residue levels were present in the fat.

The blood kinetics of rats in groups E1 and E2 (single oral doses of 0.5 and 100 mg/kg bw, respectively) showed fairly rapid absorption of lufenuron from the intestinal tract into the systemic circulation. At both dose levels, the maximum blood levels (C_{max}) were reached about 8 hours after dosing. The blood concentrations declined to half of the maximum value ($C_{max/2}$) about 45 and 60 hours ($T_{max/2}$) after dosing at the low and high dose levels, respectively.

At the low dose level, the rate of tissue depletion followed first-order kinetics (45 hours – 12 days; with a coefficient of correlation $r^2 > 0.97$ for most tissues). Tissue concentrations for both low and high doses demonstrated similar depletion kinetics for all tissues. Half-lives of elimination, expressed over two time intervals, showed an initial faster rate followed by a slower terminal rate. Irrespective of dose level or tissue concentration, the initial rates of tissue elimination were similar across the range of tissues analysed, ranging from 2 to 6 days. At the low dose level, the terminal half-lives ranged between 5 and 13 days; the corresponding values at the high dose level ranged from 10 to 37 days (Bissig, 1990).

To investigate the absorption and depletion kinetics of lufenuron, a single oral dose of [^{14}C]lufenuron (batch no. Le-78.3A-1; 97% radiochemical purity) was administered by gavage to two groups of three male and three female rats (Tif: RAIf (SPF)) at a dose of 0.5 or 100 mg/kg bw.

In both sexes, the peak blood concentration of radioactivity was reached 8 and 12 hours after administration of the low and high dose levels, respectively. The ^{14}C concentration in blood declined to half of these values after 56 and 42 hours ($T_{max/2}$) at the low dose level and after 86 and 68 hours at the high dose level in males and females, respectively. Assuming first-order kinetics, a monophasic decline of radioactivity in blood was observed at both the low and high dose levels. When applying linear regression, the calculated half-lives of depuration were 84 and 97 hours at the low dose level and 119 and 111 hours at the high dose level for male and female rats, respectively (Table 5).

The shapes of the areas under the concentration–time curves ($AUC_{0–168\,h}$) were essentially identical for both sexes at both dose levels, accounting at the low dose level for 2.5 h·µg/g. At the high dose level, the respective values were 116 and 106 h·µg/g for male and female rats, respectively (Table 5). When comparing the AUCs at the low and high dose levels, a 46-fold increase in males and a 42-fold increase in females were observed, indicating that a smaller proportion of the dose was bioavailable at the higher dose level. The AUC values were virtually the same for both sexes, demonstrating similar absorption of lufenuron by both males and females (Müller, 1995).

Table 5. Blood kinetic data following a single oral [^{14}C]lufenuron dose of 0.5 or 100 mg/kg bw

	0.5 mg/kg bw		100 mg/kg bw	
	Males	**Females**	**Males**	**Females**
C_{max} (ppm)	0.033	0.035	1.331	1.176
T_{max} (h)	8	8	12	12
Calculated $t_{½}$ (h)	84	97	119	111
$AUC_{0–168\ h}$ (h·µg/g)	2.5	2.5	116.3	105.8

$AUC_{0–168\ h}$: area under the concentration–time curve over 168 hours; bw: body weight; C_{max}; maximum concentration; ppm: parts per million; $t_{½}$: half-life; T_{max}: time to reach maximum concentration
Source: Müller (1995)

To investigate the absorption, distribution and excretion of lufenuron after multiple oral administrations, [^{14}C]lufenuron (batch no. ILA-178.3; 97.0% radiochemical purity) was administered by gavage to four groups of four male rats (HanBrl: WIST (SPF)). The dosing was performed by 14 consecutive daily doses at a dose of 0.5 mg/kg bw. One group of four males was sacrificed after 1 day of dosing, another group of four males after 7 days of dosing, and the remaining two groups after 14 days of dosing.

Approximately 1% of the dose was excreted in urine, and 58% in faeces. Approximately 38% of the cumulative dose remained in tissues and the residual carcass at the conclusion of this study. The total recovery of administered radioactivity was greater than 97%. The unexcreted dose was accumulated in tissues, and the rate of faecal excretion suggested that such tissue residues would be eliminated slowly and predominantly in faeces. About 80% of the administered dose was found in tissues and residual carcass 1 day after a single oral dose, which indicates that absorption of a 0.5 mg/kg bw single oral dose was approximately 80%.

With the single exception of testes, all tissue concentrations of radioactivity increased to a maximum 1 day after the final dose. The results suggest that most tissue concentrations would plateau within 2–3 weeks of similar repeated dosing. The highest residues were present in fat (29 ppm lufenuron equivalents). Much lower residue levels were present in adrenals (4.2 ppm), pancreas (3.2 ppm), thyroid (3.0 ppm), liver (2.1 ppm), kidneys (1.3 ppm), heart (1.2 ppm), lungs (1.1 ppm) and thymus (1.1 ppm). All other tissues attained maximum levels below 1 ppm lufenuron equivalent. The calculated half-life ($t_{½}$) for the depuration of tissue ^{14}C residues, assuming a monophasic first-order kinetics, typically ranged from 7 to 12 days. A faster depletion was observed for thyroid ($t_{½}$ = 4 days). Elimination half-lives of residues from testes, lungs and fat ranged from 14 to 16 days, although testes showed lower residues after 14 days than after 7 days during the accumulation phase (Table 6).

Seven days after the last of 14 consecutive daily doses, tissue residues represented approximately 38% of the total administered radioactivity. There was a similar distribution pattern between these profiles and those seen 7 days after a similar single oral dose, and there was an approximate 10-fold difference in the comparative tissue concentrations (Table 6).

To conclude, the rates and routes of excretion and the tissue distribution did not change upon multiple dosing compared with single dosing. The tissue residues increased with ongoing dosing, not reaching a plateau within the dosing period. Indication is given that residue levels in most of the tissues will reach a plateau within a dosing period of 3 weeks (Hassler, 2003).

To investigate the absorption, excretion, tissue distribution and pharmacokinetics of lufenuron after multiple oral administrations, [^{14}C]lufenuron (batch no. ISL-158.1; 97.8% radiochemical purity) was administered by gavage to groups of male and female rats (Sprague Dawley Crj:CD(SD)) for 1, 7 or 14 days, respectively. The dosing was performed by 14 consecutive daily doses at a dose of 0.5 mg/kg bw. This study was not compliant with GLP, but it was conducted in Japan to standards apparently equivalent to GLP.

Table 6. Tissue residues of radioactivity during and following 14 daily oral [^{14}C]lufenuron doses of 0.5 mg/kg bw

	Tissue residues (µg eq/g tissue)					
	Single dose[a]	Multiple doses				$t_{½}$ (days)
	Day 7[b]	Day 1[b]	Day 7[b]	Day 14[b]	Day 20[b,c]	
Adrenals	n.a.	0.741 8	2.379 3	4.187 9	2.391 5	7
Blood	0.008 4	0.031 2	0.110 5	0.165 6	0.099 1	8
Bone	0.038 9	0.095 7	0.302 1	0.329 7	0.235 1	12
Brain	0.013 1	0.031 3	0.111 3	0.139 6	0.081 6	8
Fat	1.914 2	3.480 9	21.150 2	29.245 2	22.660 2	16
Heart	0.080 2	0.261 4	0.926 1	1.247 2	0.774 6	9
Kidneys	0.087 9	0.292 3	1.067 7	1.348 1	0.884 5	10
Liver	0.129 2	0.462 0	1.600 5	2.119 2	1.346 0	9
Lungs	0.094 2	0.299 4	0.826 6	1.096 3	0.833 8	15
Muscle	0.040 4	0.156 4	0.576 4	0.636 7	0.395 6	9
Pancreas	n.a.	0.595 7	2.164 5	3.169 6	2.165 1	11
Plasma	0.010 4	0.039 5	0.138 9	0.231 7	0.130 7	7
Spleen	0.046 5	0.160 0	0.547 7	0.724 2	0.512 6	12
Testes	0.026 0	0.082 2	0.367 3	0.279 0	0.207 6	14
Thymus	0.056 0	0.173 4	0.728 4	1.087 0	0.618 5	7
Thyroids	0.220 0	0.413 1	2.441 2	3.023 6	1.097 5	4

eq: equivalents; n.a.: not available; ppm: parts per million
[a] From Bissig (1990).
[b] Days after start of dosing.
[c] Seven days after last dose.
Source: Hassler (2003)

Concentrations of radioactivity in blood measured 8 hours after each dose showed a progressive increase during and following the 14-day repeated-dose experiment, corresponding to the peak blood concentration following a single similar dose. After an initial rapid increase, blood concentrations rose gradually to maximum values of 0.184 and 0.178 µg equivalents of lufenuron per millilitre after the final dose. Concentrations then declined gradually, with estimated elimination half-lives of 208 and 323 hours in males and females, respectively. There was considered to be no marked sex difference in accumulation or depletion profiles.

Excretion by 168 hours after the final dose in male and female rats administered 14 daily oral [^{14}C]lufenuron doses of 0.5 mg/kg bw was 58% of the cumulative dose in males and 64% in females. There was no pronounced sex difference in excretion. Urinary excretion accounted for only about 1% of the cumulative dose in both males and females, whereas faecal excretion accounted for the remainder. The unexcreted dose was accumulated in tissues, and the rate of faecal excretion suggested that such tissue residues would be eliminated slowly and predominantly in faeces.

Concentrations of radioactivity during and following 14 daily oral [^{14}C]lufenuron doses of 0.5 mg/kg bw showed an increase in all tissues until 8 hours after the 14th dose and then a decline. All tissue concentrations exceeded the plasma concentration at all time points, although concentrations in the cerebrum and cerebellum were only slightly higher than those in plasma. The greatest accumulation was observed in fat (28.1 µg eq/g in males and 34.7 µg eq/g in females), with markedly lower peak concentrations in other tissues. Progressively lower peak levels were present in adrenal

glands, liver, skin, thyroid, lungs, heart and pituitary. All other tissue concentrations attained peak values of less than 1 µg eq/g. Over the 7 days after the cessation of dosing, all tissue concentrations declined steadily, including fat residues. By this time, the residual carcasses contained 34.6% and 29.5% of the total administered radioactivity in males and females, respectively. There was no marked sex difference in either accumulation or tissue depletion.

Radioluminograms showed very little radioactivity in the brain 8 hours after the first dose. For both sexes, the concentration in the pituitary increased only by a factor of 2 between the first and 14th daily doses, followed by a decline, as observed for other tissues. Although the pineal body was not detected in sections for females, the concentrations in this tissue matched those in the pituitary in both sexes. The peak concentrations in these tissues were much lower than those observed in major organs, such as the liver, kidney, heart and lung (Okada, 1997).

In conclusion, lufenuron accumulates in fat, from which it is slowly released into faeces, with terminal half-lives in fat of 5–13 days at 0.5 mg/kg bw and 10–37 days at 100 mg/kg bw. This accumulation has to be taken into account when determining the oral absorption, as part of the faeces measured at 7–21 days after dosing will consist of the initially absorbed amount from tissues/carcass.

The oral absorption after a single low dose (0.1–0.5 mg/kg bw) is at least 70%, based on the ratio of AUC between oral and intravenous doses (Booth, 2004). This is confirmed by Hassler (2003), who found an oral absorption of around 80%, based on the concentration in tissues/carcass 1 day after a single dose of 0.5 mg/kg bw.

There are no studies with tissue/carcass measurements at 1 day to determine the oral absorption after a single high dose. Taking an average half-life of 14 days in carcass and tissues, the tissue/carcass concentrations at 21 days (Booth, 2004) or 7 days (Bissig, 1990) after a single high dose of 100 mg/kg bw would indicate an initial tissue/carcass concentration of around 15–20% of the administered dose. The faecal excretion in these studies at 1 day after dosing was around 70–80%. This figure also indicates that the oral absorption after a single high dose of 100 mg/kg bw would be around 20%.

1.2 Biotransformation

In a study described in section 1.1, a single major component was identified in faecal extracts after single oral doses of 0.1, 1.0, 10 or 100 mg/kg bw, and this was identified as parent lufenuron. The levels of administered radioactivity in urine were very low (< 1% of the dose), and metabolite identification was not performed on these samples (Booth, 2004).

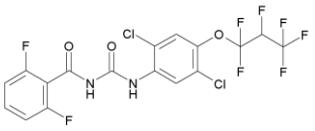
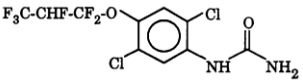
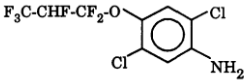
In Hassler (2003), described in section 1.1, chromatographic analysis of urine revealed a metabolite profile comprising at least 10 metabolite fractions. Although the total amount of radiolabelled metabolites was very low, based on very low urinary excretion, the patterns were qualitatively and quantitatively similar after single and repeated dosing.

Thin-layer chromatographic (TLC) analysis of the faecal extracts revealed at least four metabolite fractions. The extractability and the metabolite pattern of the faeces were essentially identical for all three selected sampling intervals, with slight quantitative variations mainly between the first time interval (0–1 day) and the later selected time intervals. The major metabolite fraction (3) corresponded to unchanged lufenuron, accounting for approximately 30% of the daily dose. Apart from unchanged parent, only minor metabolites were present in the faecal extracts (Hassler, 2003).

In Okada (1997), described in section 1.1, chromatographic analysis of metabolite profiles in plasma and cerebrum revealed the following. In plasma, 96% of the radioactivity was extracted into methanol and was resolved into four metabolites. The major component was unchanged lufenuron, and the other major metabolite was CGA 238277; one of the minor metabolites corresponded to

CGA 224443, and the other was unidentified (Table 7). The proportion of lufenuron in all samples was approximately 70–80% and appeared to be unaffected by the number of doses given or the sampling time. More than 94% of radioactivity in the cerebrum was extracted and comprised only unchanged lufenuron, representing more than 92%, and CGA 238277, accounting for 1.1% or less of the radioactivity (Okada, 1997).

Table 7. Summary of lufenuron-derived metabolites in the rat

Metabolite code	Chemical name	Structure
Lufenuron, (CGA 184699)	(*RS*)-1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl)urea	
CGA 238277	1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-urea	
CGA 224443	2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline	

Source: Thanei (1990)

In another study described in section 1.1 (Bissig, 1990), the urinary metabolite pattern after a single dose of either 0.5 or 100 mg/kg bw was not determined because of the low recovery of radioactivity in urine (< 1% of the dose). Analysis of faeces by one-dimensional TLC revealed no significant differences between the sexes and showed no significant influence of pretreatment with unlabelled lufenuron, but showed slightly dose-dependent differences. The metabolite pattern consisted of one dominant fraction (F3), which was characterized as unchanged lufenuron. As a consequence of the higher amount excreted in faeces at the high dose level, metabolite fraction F3 represented a higher percentage of the dose (77–79%) than at the low dose level (37–48%). In contrast, the most polar fractions (F1 and F2) were more pronounced at the low dose than at the high dose.

The faecal metabolite pattern of the bile duct–cannulated rats (group G) was similar to that of the animals of group B, but with a higher percentage of unchanged lufenuron. Radioactivity accounted for only 1.5% of the administered dose. Metabolite fraction G5, which represented 0.1% of the administered dose, co-chromatographed with unchanged lufenuron. The metabolite fraction G2 (corresponding to 0.1% of the dose) behaved as the authentic reference compound CGA 238277 (i.e. the free urea conjugate of the dichlorophenyl moiety). The least polar metabolite fraction G6 co-chromatographed with CGA 224443, the free amine derivative of the dichlorophenyl moiety (< 0.1% of the dose).

Samples of fat, liver, kidneys, lungs and carcass were pooled by sex and group for the analysis of ^{14}C residues. TLC analysis of organic solvent and aqueous extracts of tissues revealed a single metabolite fraction in all extracts, co-chromatographing with unchanged lufenuron (Bissig, 1990).

To further investigate the metabolism of lufenuron after a single oral administration, samples used in this study originated from a study described above (Bissig, 1990). Faeces and fat collected from male and female rats administered a single oral dose of [^{14}C]lufenuron at 100 mg/kg bw were used in the present study.

The 8- to 48-hour faecal pools (representing 76% of the dose) for male and female rats were extracted with methanol. Almost 100% of the radioactivity in faeces was extractable. The TLC analysis revealed one dominant metabolite fraction, accounting for 75% of the dose. This major metabolite fraction was identified by spectroscopic means to be unchanged lufenuron. The minor metabolite fractions accounted for less than 1% of the dose each. Lufenuron was metabolized to a very small extent. In addition, two metabolites were characterized by chromatographic comparison with authentic reference compounds (Table 7).

Organic solvent extraction of the single pool of fat from male and female rats extracted approximately 93% of the radioactivity present. Purification by preparative TLC yielded two fractions. The fraction containing almost all the radioactivity was further purified by high-performance liquid chromatography. It was shown by mass spectroscopy and by co-chromatography to be identical to the parent compound, lufenuron. By chromatographic comparison, the second fraction corresponded to metabolite F2.

Mostly unchanged lufenuron was excreted in faeces and was also retained in tissues, predominantly in fat, demonstrating that lufenuron was poorly metabolized. It is assumed that a minor route of degradation is cleavage of the benzamide moiety, yielding the urea CGA 238277 and 2,6-difluorobenzoic acid. Further cleavage of the ureido moiety of CGA 238277 leads to the aniline CGA 224443. It is established that rats excrete 2,6-difluorobenzoic acid largely unchanged. Based on the faecal and fat metabolites identified or characterized, which represented approximately 85% of the dose, the proposed metabolic pathway in the rat is summarized in Fig. 3 (Thanei, 1990).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with lufenuron administered orally, dermally or by inhalation are summarized in Table 8. All the studies were certified to comply with GLP and performed according to United States Environmental Protection Agency (USEPA) and OECD guidelines.

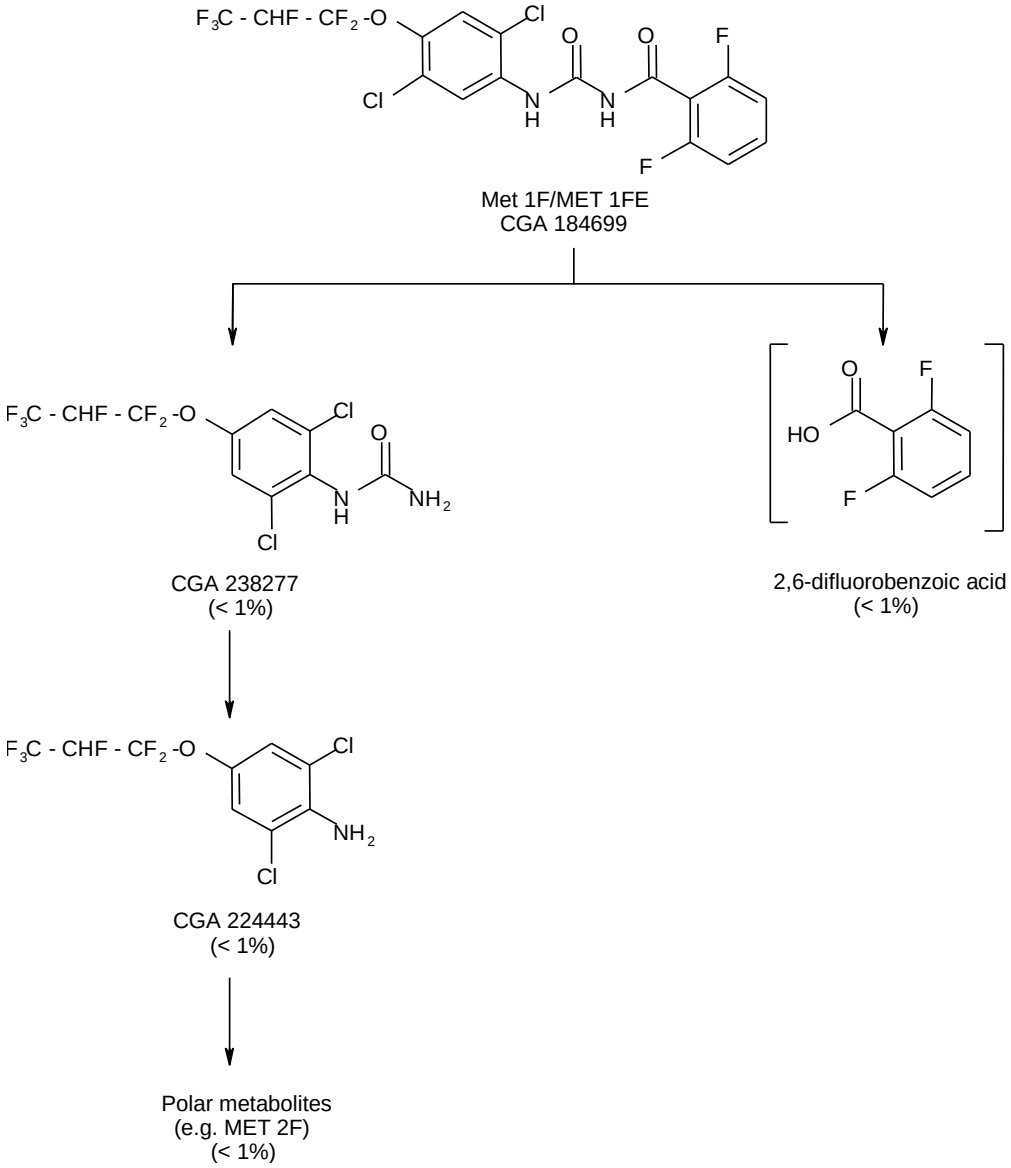
Lufenuron has low acute toxicity when administered orally, dermally and via inhalation to rats.

In an acute oral toxicity study, groups of five Tif:MAG f (SPF) mice of each sex received an oral dose of lufenuron (purity 95.2%) at 2000 mg/kg bw. The test material was suspended in Oleum arachidis Ph.H.VI. There were no deaths, and clinical signs were limited to piloerection, hunched posture and dyspnoea. Clinical signs were slight, and animals recovered within 6 days. Body weight gains were normal. There were no gross necropsy findings. The median lethal dose (LD_{50}) following oral exposure was greater than 2000 mg/kg bw (Hartmann, 1989).

In an acute oral toxicity study, groups of five Tif: RAIf (SPF) rats of each sex received an oral lufenuron (purity 95.2%) dose of 2000 mg/kg bw. The test was suspended in Oleum arachidis Ph.H.VI. There were no deaths, and clinical signs were limited to ruffled fur, dyspnoea, hunched posture and exophthalmos, ranging from moderate directly after treatment to slight up to 10 days after treatment. Body weight gains were normal. There were no gross necropsy findings. The LD_{50} following oral exposure was greater than 2000 mg/kg bw (Hartmann, 1988a).

In an acute dermal toxicity study, groups of five Tif: RAIf (SPF) rats of each sex were treated with lufenuron (purity 95.2%) via dermal exposure at a limit dose of 2000 mg/kg bw. The test material was suspended in Oleum arachidis Ph.H.VI and administered in 0.4 mL/kg bw to approximately 10% of the body surface area under an occlusive dressing for 24 hours. There were no deaths, and the animals showed ruffled fur, dyspnoea, abnormal body positions and reduced spontaneous activity. Clinical signs were slight, and animals recovered within 5 days. No skin

Fig. 3. Proposed metabolic pathway for lufenuron in the rat



Source: Thanei (1990)

Table 8. Acute toxicity of lufenuron

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Mouse	Tif:MAG f (SPF)	Male and female	Oral	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1989)
Rat	Tif: RAIf (SPF)	Male and female	Oral	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1988a)
Rat	Tif: RAIf (SPF)	Male and female	Dermal	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1988b)
Rat	Tif: RAIf (SPF)	Male and female	Inhalation	95.2	LC_{50} > 2.35 mg/L (maximal attainable concentration)	Hartmann (1988c)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

irritation was noted. Body weight gains were normal. There were no gross necropsy findings. The LD_{50} following dermal exposure was greater than 2000 mg/kg bw (Hartmann, 1988b).

In an acute inhalation toxicity study, groups of five Tif: RAIf (SPF) rats of each sex were exposed to lufenuron (purity 95.2%) for 4 hours via nose-only inhalation exposure at a test atmosphere concentration of 2.35 mg/L (nominal 3.0 mg/L, mass median aerodynamic diameter 1.9–2.8 μm, geometric standard deviation 2.2–2.5 μm). There were no deaths, and clinical signs recorded during exposure were moderate piloerection, slight hunched posture and moderate dyspnoea. All animals recovered within 2 days. The males exposed to the test article exhibited a significantly higher body weight increase during the second week after exposure. There were no notable necropsy findings at termination. The acute inhalation median lethal concentration (LC_{50}) was greater than 2.35 mg/L (Hartmann, 1988c).

(b) Dermal irritation

In a skin irritation study, three female New Zealand White (KFM-NZW) rabbits were treated with lufenuron (purity 95.2%) via dermal exposure at a dose of 0.5 g applied to the flank under an occlusive dressing for 4 hours. The (solid) test material was applied to the skin using a gauze patch that was moistened before application with distilled water containing 0.5% carboxymethyl cellulose and 0.1% polysorbate 80. Very slight erythema was observed in two animals at 1 hour after removal of the test substance. No other skin reactions were observed in any animal during the study. Body weight gains were normal (Schneider, 1988a).

(c) Ocular irritation

In an eye irritation study, three male New Zealand White (KFM-NZW) rabbits were treated with lufenuron (purity 95.2%) via ocular exposure at a dose of 0.1 mL (70 mg). Slight irritation of the cornea, iris and conjunctiva was observed at 1 hour after instillation in all three animals. Slight conjunctival redness was also found in one male 24 hours after instillation. No signs of eye irritation were present at the 48- and 72-hour examinations (Schneider, 1988b).

(d) Dermal sensitization

In a Magnusson and Kligman maximization test, lufenuron (purity 95.2%) was tested using 10 male and 10 female Pirbright White Strain (Tif: DHP) guinea-pigs in the test substance group and five male and five female guinea-pigs in the control group. No results of the intradermal injection/ topical induction are presented. Following challenge with lufenuron in petrolatum at 10% weight per weight (w/w), dermal responses were observed in 4/20 test animals at 24 hours after challenge and in 9/20 test animals at 48 hours after challenge. No skin reactions were observed in control animals. Up to 45% of the animals were sensitized by lufenuron under the experimental conditions employed. Therefore, lufenuron is considered to be a skin sensitizer in the guinea-pig. Sensitization of this strain of animals was positively tested with dinitrochlorobenzene (positive control), which gave very severe allergic reactions in all areas treated (Schneider, 1988c).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a dose range–finding study, lufenuron (purity 95.2%) was fed to groups of 10 male and 10 female mice (Tif: MAGf (SPF), hybrids of NIH × MAG) at a concentration of 0, 1000, 3000 or 9000 ppm (mean substance intakes: 0, 151, 449 and 1470 mg/kg bw per day for males and 0, 189, 517 and 1440 mg/kg bw per day for females, respectively) for up to 65 days. No haematological examinations were carried out, and no organ weights were measured.

As a result of high mortality and neurotoxic effects of the test substance (tonic-clonic seizures), the surviving animals at 3000 and 9000 ppm were sacrificed on day 49. Eleven animals (four males and seven females) treated at 3000 ppm and 19 animals (nine males and 10 females) treated at 9000 ppm were found dead between days 17 and 48. At 1000 ppm, there was a slight increase in mortality (one male and three females died between days 54 and 65). Tonic-clonic seizures were also observed in two animals in the 1000 ppm dose group. A small reduction in body weight gain and decreased feed consumption were observed in all treated males. It was concluded that the maximum tolerated dose was exceeded even at 1000 ppm (equal to 151 and 189 mg/kg bw per day for males and females, respectively) (Fankhauser, 1989a).

In a second dose range–finding study intended for test substance residue and blood level determination, lufenuron (purity 95.2%) was fed to groups of 30 female mice (Tif: MAGf (SPF), hybrids of NIH × MAG) at a concentration of 0, 4/8, 20, 100 or 1000 ppm (mean substance intakes: 0, 0.47/1.1, 2.94, 14.5 and 143 mg/kg bw per day, respectively) for up to 91 days (only 71 days for the high dose). From day 57 onwards, the diet of the low-dose group inadvertently contained 8 ppm instead of 4 ppm. Subgroups of nine mice were killed at weeks 9, 11 and 14; the remaining three animals per dose group were intended for eventual replacement or pharmacological investigations. Fat and blood samples were collected at 9 and 11 weeks, and brain samples were collected at terminal sacrifice at week 14. No haematological or clinical chemistry examinations were carried out, and no organ weights were measured.

There was a significant increase in mortality (eight animals) and signs of neurotoxicity (tonic-clonic seizures in four animals) at 1000 ppm. Therefore, the remaining animals at 1000 ppm (six from the last sacrifice group and the replacement group) were sacrificed on day 71. No effects on body weight or feed consumption were observed, and there were no macroscopic findings. Concentrations of lufenuron in blood, fat and brain were dose dependent, with equilibrium being reached by week 9 of the study. Lufenuron concentrations in fat were approximately 100-fold those in blood and brain (Table 9).

The no-observed-adverse-effect level (NOAEL) after 90 days in this dose range–finding study in female mice was 100 ppm (equal to 14.5 mg/kg bw per day), based on mortality and neurotoxicity at 1000 ppm (equal to 143 mg/kg bw per day) (Fankhauser, 1990).

Table 9. Blood, fat and brain concentrations of lufenuron observed in a 3-month range-finding study in female mice given lufenuron in the diet

Dietary concentration (ppm)	Blood (mg/L)			Fat (mg/kg)			Brain (mg/kg)
	Week 9	Week 11	Week 14	Week 9	Week 11	Week 14	Week 14
0	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.08
4/8	0.13	0.36	0.52	10.8	29.9	49.9	0.41
20	0.87	0.9	1.45	81.7	76.5	81.6	0.65
100	5.02	5.24	5.05	488.7	499.5	487.8	5.19
1 000	48.5	43.4	n.a.	4 537.7	5 400 (all survivors)	n.a.	187 (week 11)

n.a.: not applicable, as this dose group was terminated in week 11; ppm: parts per million
Source: Fankhauser (1990)

Rats

In a 28-day range-finding study, groups of five male and five female Tif: RAIf (SPF) rats were administered lufenuron (purity 95.2%) in the diet at a concentration of 0, 50, 400, 3000 or 20 000 ppm (mean substance intakes: 0, 4.10, 30.8, 254 and 1692 mg/kg bw per day for males and 0, 4.07, 32.6, 254 and 1741 mg/kg bw per day for females, respectively). Investigations included clinical signs, body weights, feed consumption, haematology and clinical chemistry at termination, urine analysis, organ weights, gross necropsy and histopathological examination.

No mortality and no treatment-related clinical findings were observed. At weeks 3 and 4, the mean body weight gain and feed consumption of males fed 20 000 ppm lufenuron were reduced, not statistically significantly, by about 13% and 12–16%, respectively. Feed utilization values were not affected in any group. There were no statistically significant differences in water consumption between the groups.

There were no treatment-related differences between the groups in haematological or blood clinical chemistry parameters; some minor differences did achieve statistical significance, but these were representative of the normal variation of the respective parameters. No treatment-related differences were observed in urinary clinical chemistry parameters.

There were no treatment-related differences in macroscopic findings postmortem. After exsanguination, the mean body weight of males fed 20 000 ppm lufenuron was about 15% lower than that of the control group. The increased mean testis to body weight ratio and decreased liver weights seen in these males are therefore considered unrelated to treatment. Absolute and relative thymus weights of animals fed 3000 or 20 000 ppm lufenuron were reduced by about 13–30% compared with controls. The thymus weight of 400 ppm males was only slightly reduced (9%), and the statistical significance was due to a rather low standard deviation at 400 ppm compared with the one for controls. Moreover, the thymus weight at 400 ppm in males was within the range of controls. The reduced thymus weight at 400 ppm was therefore not considered to be adverse. In females fed 20 000 ppm lufenuron, slightly increased ovary weights were noted (Table 10). There were no treatment-related changes seen during histopathological examination.

The NOAEL for lufenuron when fed to rats continuously for a 28-day period was 400 ppm (equal to 30.8 and 32.6 mg/kg bw per day for males and females, respectively), based on decreased thymus weight at 3000 ppm (equal to 254 mg/kg bw per day for both males and females) (Fankhauser, 1988).

In a 90-day toxicity study, groups of 20 male and 20 female Tif: RAIf (SPF) rats were fed diets containing 0 or 15 000 ppm lufenuron (purity 95.2%), and 10 male and 10 female rats were fed diets containing 25, 150 or 1500 ppm lufenuron (purity 95.2%), for 92 or 93 consecutive days. Mean substance intakes were 0, 1.6, 9.68, 101 and 998 mg/kg bw per day for males and 0, 1.7, 10.2, 103 and 1050 mg/kg bw per day for females at 0, 25, 150, 1500 and 15 000 ppm, respectively. In each dose group, 10 animals of each sex per group were killed at the end of the treatment period. In the control and highest-dose groups, 10 animals of each sex were kept for a 4-week recovery period before sacrifice. Clinical observations, body weights, and feed and water consumption were measured throughout the study. Ophthalmological examinations were made on control and highest-dose animals pre-study and towards the end of the treatment period and recovery period. Haematology and blood chemistry analyses were carried out on all animals towards the end of the treatment period. At the end of the scheduled period, all animals were killed and subjected to a postmortem examination, selected organs were weighed and specified tissues were taken for subsequent histopathology.

One female fed 15 000 ppm lufenuron was found dead on day 98, after showing signs of emaciated condition and motor disorder. There were no other treatment-related mortalities. Tonic-clonic seizures were observed in 9/20 males and 8/20 females fed 15 000 ppm lufenuron and in 1/10 females fed 1500 ppm lufenuron. Two males still exhibited tonic-clonic seizures in the last week of the recovery period. There was no evidence of any effect of treatment on the eyes. The mean body weight gain of males from groups fed 1500 or 15 000 ppm lufenuron was decreased from week 2 until

Table 10. Intergroup comparison of selected organ weights

	Absolute/relative (to body weight) organ weights									
	Males					Females				
Organ	**0 ppm**	**50 ppm**	**400 ppm**	**3 000 ppm**	**20 000 ppm**	**0 ppm**	**50 ppm**	**400 ppm**	**3 000 ppm**	**20 000 ppm**
Thymus										
Absolute (mg)										
Mean	792.9	749.5	725.0*	597.5	556.1	524.2	434.8	455.1	405.0	393.9
SD	239.9	103.3	89.22	116.7	110.6	214.1	106.7	67.25	69.45	59.65
Min	530.5	639.8	637.9	471.2	447.5	338.7	313.8	354.9	316.4	332.4
Max	1 086	872.4	836.2	752.7	733.6	799.4	601.6	536.5	492.7	492.1
Relative (%)										
Mean	2.530	2.451	2.337*	2.008	2.091	2.665	2.311	2.309	2.332*	2.109
SD	0.595	0.409	0.257	0.343	0.287	0.884	0.544	0.311	0.266	0.326
Min	1.882	1.919	1.993	1.660	1.792	1.933	1.616	1.767	2.105	1.781
Max	3.104	2.848	2.607	2.514	2.568	3.863	3.096	2.535	2.678	2.622
Ovary/testis										
Absolute (mg)	3.305	3.573	3.430	3.382	3.517	171.3	161.0	166.7	158.5	192.8
Relative (%)	10.68	11.63	11.08*	11.41	13.29*	0.886	0.856	0.844	0.912	1.031
Liver										
Absolute (g)	14.95	15.00	15.69	15.44	13.44	8.309	7.941	9.454	8.182	8.408
Relative (%)	48.30	48.61	50.32	51.96	50.74	43.13	42.16	48.02	47.25	44.92

Max: maximum; Min: minimum; ppm: parts per million; SD: standard deviation; *: $P < 0.05$
Source: Fankhauser (1988)

the end of treatment. At 15 000 ppm lufenuron, mean body weights comparable with control values were reached at the end of the recovery period. A similar tendency was seen in females at these dose levels. During the treatment period, slightly, but not statistically significantly, reduced feed consumption was seen in animals fed 1500 or 15 000 ppm lufenuron. A compensatory increase was seen in the animals retained for the recovery period. Water consumption was not affected in any group.

Haematocrit values and prothrombin time were increased slightly in females fed 1500 and 15 000 ppm lufenuron at the end of treatment, but were reversible within the recovery period. White blood cell counts in females fed 15 000 ppm lufenuron were at the upper end of the normal range at week 14 and at the end of the recovery period. Some changes in plasma phosphate and sodium concentrations and in alkaline phosphatase activity were seen in male and/or female animals fed 1500 or 15 000 ppm lufenuron; these changes were not reversible over the recovery period. Other minor changes in blood chemistry, which were reversible, were seen in cholesterol, albumin, protein and chloride levels and decreased albumin to globulin ratios. Alanine aminotransferase (ALAT) and alkaline phosphatase activities were slightly higher in females fed 15 000 ppm lufenuron. Other haematological and clinical chemistry parameters that achieved a statistically significant difference from control values were not biologically significant.

There were no treatment-related changes in macroscopic findings postmortem. Absolute and relative liver weights of females fed 15 000 ppm lufenuron were increased by 13–16% and 20–32% at the end of the treatment period and recovery period, respectively. In males at this dose, the ratio was increased by 13% at the end of the treatment period, with values comparable with control values after the recovery period. Adrenal weights in both sexes at this dose level were increased by 20–36%; in females at 1500 ppm lufenuron, they were increased by 11–16%. Adrenal weights were comparable with control values at all doses at the end of the recovery period. Other findings in organ weights that achieved a statistically significant difference from control values were not biologically significant. There were no treatment-related changes in microscopic findings postmortem.

In order to support the selection of dose levels for a 2-year toxicity and oncogenicity study and to study the accumulation of lufenuron in fat tissues, fat samples were collected at terminal sacrifice at week 14. The content of lufenuron in fat increased dose-dependently in the 25, 150 and 1500 ppm dose groups. The concentrations were similar at 1500 and 15 000 ppm, indicating that a solubility limit in fat had been reached (Table 11). Fat tissue concentrations were similar in males and females.

Table 11. Fat concentrations of lufenuron in rats after a 3-month treatment

	Concentrations of lufenuron (mg/kg fat)				
	0 ppm	**25 ppm**	**150 ppm**	**1 500 ppm**	**15 000 ppm**
Males	3.2[a]	47.2	378	3 025	2 710
Females	4.4[a]	44	447	3 875	3 176

ppm: parts per million
[a] Most probably due to contamination of samples.
Source: Fankhauser (1989b)

Some of the control samples contained traces of the test article. Reconstruction of the sampling procedure and a confirmational analysis of leftover samples indicated that this was most probably due to contamination of the samples rather than a feeding error. The interpretation of the analytical results was not considered to be compromised by this finding.

The NOAEL for lufenuron in rats when fed continuously in the diet for a period of up to 3 months was 150 ppm (equal to 9.68 and 10.2 mg/kg bw per day for male and female animals, respectively), based on clinical signs (tonic-clonic seizures), decreased body weight gain and feed consumption, slight changes in haematology and clinical chemistry parameters, and increased adrenal weights at 1500 ppm (equal to 101 and 103 mg/kg bw per day for male and female animals, respectively) (Fankhauser, 1989b).

Dogs

In a 4-week range-finding study, groups of two male and two female Beagle dogs received lufenuron (purity 95.2%) in their diet at a concentration of 200 or 50 000 ppm (equal to 8.43 and 2200 mg/kg bw per day for males and 10.1 and 2648 mg/kg bw per day for females, respectively). Blood samples were taken pretest and at intervals during the study and were analysed for proof of absorption. All the dogs were examined daily for clinical condition and mortality. Detailed clinical examination, body weights and feed consumption were measured or recorded at intervals during the study. Haematological and clinical chemistry examinations of blood were performed pretest and after 2 and 4 weeks. At the end of the study, all the animals were killed humanely and given a macroscopic examination postmortem. Final body weights and selected organ weights were recorded. A comprehensive range of tissues was examined histopathologically.

There were no mortalities, and no clinical signs of toxicity were seen during the study. No abnormalities were detected during the detailed clinical and physical examinations. There was no

effect on body weight or feed consumption. There were no toxicologically significant changes in any of the haematological or clinical chemistry parameters examined. No macroscopic or microscopic abnormalities were detected, and there was no effect on organ weights. The mean levels of lufenuron found in the blood were 4 and 16 ppm, and those in the fat were 615 and 2750 ppm, at the low and high doses, respectively.

The NOAEL was 50 000 ppm (equal to 2200 and 2648 mg/kg bw per day for males and females, respectively), the highest dose tested (Briffaux, 1989a).

In another 4-week range-finding study, groups of two male and two female Beagle dogs received an oral dose of 50 mg/kg bw per day of a water-based veterinary preparation containing 15% lufenuron. According to the report, this was 5 times the anticipated therapeutic dose. No further information on the test formulation was provided in the report; hence, the exact composition remains unclear. The following information is provided in the European Union Draft Assessment Report (Portugal, 2006):

> The Notifier informed that the test formulation used was a pilot formulation for a commercial veterinary product whose recipe is proprietary information now belonging to another company. The Notifier assumed that the main component is water, with the addition of glycerine and avicel (a mixture of microcrystalline cellulose and carboxymethylcellulose sodium, which acts as a water insoluble dispersible colloidal excipient) and on this basis found it reasonable to believe that the toxicity seen in this study is due to the 15% lufenuron in the formulation, and not to any of the formulation additives.

All the dogs were examined daily for clinical condition and mortality. Detailed clinical examination, ophthalmology, body weights and feed consumption were measured or recorded at intervals during the study. Haematological and clinical chemistry examinations of blood were performed pretest and after 2 and 4 weeks. Blood samples were taken at intervals and analysed for lufenuron concentration. At the end of the study, all the animals were killed humanely and given a macroscopic examination postmortem. Final body weights and selected organ weights were recorded. Tissues were not examined histopathologically.

The levels of lufenuron in blood showed a steady increase during the 4-week study. After 1 week, they ranged from 0.57 to 7.51 µg/mL, after 2 weeks, from 1.63 to 9.80 µg/mL, and after 4 weeks, from 2.46 to 16.0 µg/mL. There were no mortalities, and no clinical signs of toxicity were seen during the study. No abnormalities were detected during the detailed clinical and physical examinations, including ophthalmology. There was no effect on body weight or feed consumption. Haematological examination showed that platelet counts were slightly elevated in the males at 2 and 4 weeks compared with the pretest values. No other haematological or blood chemistry changes were detected. No significant macroscopic abnormalities were seen, and there was no effect on organ weights.

In this 4-week range-finding study in dogs, the NOAEL of a veterinary lufenuron formulation with 15% active substance was 50 mg/kg bw per day (corresponding to a lufenuron dose of 7.5 mg/kg bw per day), the only dose tested (Pavkov & Macaskill, 1989).

In a 90-day toxicity study, groups of four male and four female Beagle dogs received lufenuron (purity 95.2%) in their diet at a concentration of 0, 200, 3000 or 50 000 ppm (equal to 0, 7.8, 121.6 and 2023 mg/kg bw per day for males and 0, 7.9, 122.5 and 1933 mg/kg bw per day for females, respectively). An additional two animals of each sex in the control and high-dose groups were given a 4-week recovery period after the 90-day dosing period. Clinical observations (including ophthalmoscopy), body weights, feed consumption, haematology and clinical biochemistry were measured throughout the study. At the end of the scheduled periods, the animals were killed and subjected to a postmortem examination. Bone marrow smears were taken, selected organs were weighed and specified tissues were taken for subsequent histopathological examination.

There were no mortalities. No treatment-related clinical or ophthalmological findings were recorded. Feed consumption and body weight were not affected by treatment. No changes in haematology parameters attributable to a toxic effect of the compound were observed. A slight decrease in blood phosphorus level was observed in the 3000 and 50 000 ppm groups in both sexes at week 6 and in females at week 13. At week 17 (end of the recovery period), mean blood phosphorus levels were similar for control and 50 000 ppm animals. A slight decrease in blood potassium level was observed at week 13 in the 3000 and 50 000 ppm females, without a dose–response relationship. At week 17, mean blood potassium levels were similar for control and 50 000 ppm animals. A moderate to marked increase in total blood cholesterol level was observed at weeks 6 and 13 for both sexes at 3000 and 50 000 ppm. A moderate increase was still present at week 17 in the 50 000 ppm males, although this was not statistically significant. A moderate increase in alkaline phosphatase activity was seen at weeks 6 and 13 for females in the 200 or 3000 ppm groups and at week 13 for males in the 3000 ppm group, with no evidence of a clear dose–response relationship. At week 17, all individual alkaline phosphatase values were considered normal (Table 12). Statistically significant differences from controls were also seen in some glucose, albumin and transaminase values recorded, but these differences were considered not to be of toxicological significance and were not treatment related.

An apparent increase in the volume of urine associated with a decrease in specific gravity was seen at weeks 6, 13 and 17 in the 50 000 ppm males. When the values at pretest and in weeks 6, 13 and 17 were compared for individual dogs, no trends were evident. The semiquantitative estimations and the microscopic examination of the spun deposits in the urine did not reveal any treatment-related changes.

Absolute liver weights were increased at terminal sacrifice at week 13 in both sexes in the 3000 and 50 000 ppm groups (132% and 131% for males and 125% and 132% for females, respectively), reaching statistical significance only in 50 000 ppm males. Liver to terminal body weight ratios were increased statistically significantly at terminal sacrifice at week 13 in both sexes in the 3000 and 50 000 ppm groups (142% and 142% for males and 131% and 131% for females, respectively). Liver to brain weight ratios were statistically significantly increased in males at 50 000 ppm (135%). At the end of the recovery period, there was some evidence of reversibility, with none of the increases being statistically significant (Table 12).

No gross or microscopic changes were observed that correlated with the increased liver weights observed in the 3000 or 50 000 ppm dogs. There were no macroscopic or microscopic findings postmortem that could be attributed to treatment with lufenuron.

The NOAEL was 200 ppm (equal to 7.8 and 7.9 mg/kg bw per day for males and females, respectively), based on increased blood cholesterol levels and absolute and relative liver weights, reductions in blood potassium and phosphorus levels, and an increase in serum alkaline phosphatase activity for some animals at 3000 ppm (equal to 121.6 and 122.5 mg/kg bw per day for males and females, respectively) (Briffaux, 1989b).

In a 1-year toxicity study, groups of four male and four female Beagle dogs received lufenuron (purity 95.3%) in their diet at a concentration of 0, 100, 2000 or 50 000 ppm (equal to 0, 3.97, 65.4 and 1879 mg/kg bw per day for males and 0, 3.64, 78.3 and 1977 mg/kg bw per day for females, respectively). Clinical observations and feed consumption were recorded daily. Body weight was recorded weekly. A detailed veterinary examination and ophthalmoscopy were carried out at intervals during the study, and a range of haematological and clinical biochemistry (blood and urine) parameters was measured throughout the study. Lufenuron levels in the blood were analysed from all surviving dogs in weeks 34, 37 and 52. At the end of the scheduled period, the animals were killed and subjected to a postmortem examination. Terminal blood and bone marrow samples were taken, selected organs were weighed and specified tissues were taken for subsequent histopathology examination. In order to support the interpretation of the results obtained in this study and to demonstrate saturation, the concentrations of the test article were determined in blood, fat and brain

Table 12. Intergroup comparison of blood biochemistry (selected parameters) and liver weights in a 90-day dog study[a]

		Males				Females			
Parameter	**Week**	**0 ppm**	**200 ppm**	**3 000 ppm**	**50 000 ppm**	**0 ppm**	**200 ppm**	**3 000 ppm**	**50 000 ppm**
Phosphorus (mg/L)	0	75	77	72	71	70	71	72	70
	6	67	67	58**	56***	59	60	52***	49***
	13	59	56	48	50	54	52	41*	44**
	17	50	–	–	53	47	–	–	46
Potassium (meq/L)	0	4.7	4.9	5.0	4.9	4.8	5.1	4.7	4.8
	6	4.6	4.9	4.6	4.7	4.8	5.1	4.7	4.8
	13	5.0	5.2	4.8	4.7	4.9	4.9	4.2*	4.3*
	17	4.3	–	–	4.7	4.7	–	–	4.5
Cholesterol (g/L)	0	1.37	1.28	1.36	1.39	1.23	1.29	1.43	1.30
	6	1.20	1.45	1.95*	2.22***	1.35	1.48	2.17*	2.13*
	13	1.20	1.42	1.86*	1.98**	1.40	1.43	2.42*	2.09*
	17	1.12	–	–	1.93	1.43	–	–	1.64
Alkaline phosphatase (mU/mL)	0	284	244	253	248	244	263	294	241
	6	218	216	262	241	202	308*	395**	269
	13	168	168	260*	247	174	279*	674*	291
	17	99	–	–	187	140	–	–	200
Liver weight									
Absolute (g)	13	257	299	338	337*	258	247	323	340
Relative (%)									
- To body	13	2.6	3.0	3.7**	3.7**	2.9	2.9	3.8*	3.8**
- To brain	13	3.4	4.1	4.2	4.6**	3.4	3.4	4.3	4.4
Absolute (g)	17	241	–	–	341	254	–	–	319
Relative (%)									
- To body	17	2.6	–	–	3.2	2.8	–	–	3.3
- To brain	17	3.4	–	–	4.4	3.5	–	–	4.5

eq: equivalents; ppm: parts per million; U: units; week 0: pretest value; –: no measurements; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Because of the number of animals killed at week 17, no statistical analysis was performed for the recovery period

Source: Briffaux (1989b)

samples. Blood samples were taken at 34, 37 and 52 weeks of feeding. At final sacrifice after 24 months, fat and brain tissues were sampled from all surviving animals.

Mortality was observed in the mid-dose group of 2000 ppm: one male died during week 33, and one male and one female were killed moribund during week 37. Neuromuscular signs were noted for these three animals. This generally consisted of tremors, salivation, dyspnoea, unsteady gait and convulsions. There were no other mortalities. Neuromuscular clinical signs were noted in 3/4 animals of each group fed with 2000 and 50 000 ppm after at least 20 weeks of treatment. These signs generally included stiffness, convulsions (tonic or clonic) and salivation, followed by tremors, decreased activity and unsteady gait. The incidence of these signs was biologically similar in the 2000

and 50 000 ppm dose groups. At 2000 ppm, three animals died, but in dogs fed 50 000 ppm, neuromuscular signs were transient and reversible. A very high incidence of vomiting was noted throughout the treatment period for one female dosed at 50 000 ppm. The ophthalmological examination did not reveal any treatment-related changes. A reduction in mean body weight gain was seen in the 50 000 ppm males and females. At 2000 ppm, a slight decrease in body weight and a reduced body weight gain were seen, particularly in the males. Feed consumption was not affected.

An increase in platelet count was noted in groups fed 2000 and 50 000 ppm. In comparison with pretest and control values, blood chemistry analyses showed that there was a dose-related increase in cholesterol at several time points in the 2000 and 50 000 ppm groups. Phospholipids were elevated in males dosed at 2000 and 50 000 ppm and in females dosed at 50 000 ppm. A dose–response relationship was evident within the males. Alkaline phosphatase activity was higher in the 2000 ppm dose group (males and females) and in the 50 000 ppm males. No statistically significant changes in ALAT activity were noted. However, in comparison with control animals, elevated ALAT values were noted in males and females of the 2000 ppm dose group and in males dosed at 50 000 ppm. For one female at 2000 ppm, the elevated value noted at week 13 was associated with an increase in gamma-glutamyltranspeptidase (GGT) activity. These elevated ALAT and GGT values were considered to be treatment related (Table 13).

Increases in mean adrenal weights (absolute and relative to body and brain weights) were noted in males and females at 2000 and 50 000 ppm. Increases in mean thyroid weights (absolute and relative to body and brain weights) were noted in males of all dose groups and in females at 2000 and 50 000 ppm. Increases in liver weights (absolute and relative to body and brain weights) were noted in males from all treated groups (not dose related) and in females at 2000 and 50 000 ppm (not dose related). This increase was statistically significant in males and females at 2000 and 50 000 ppm for the relative values and in females at 2000 ppm for the absolute values (Table 13).

At the end of the treatment period, an enlargement of the adrenals was noted in one female at 2000 ppm, and pale area(s) in the lungs were observed for two animals at 2000 ppm and for 50 000 ppm animals. Histopathological lesions that could be attributed to treatment were seen in the liver, thyroid, adrenals and lungs. Hepatocytic hypertrophy was present in dogs fed 2000 and 50 000 ppm, associated with an increased intracellular dark pigmentation of the hepatocytes. Cell necrosis was noted in one female fed 2000 ppm. In the thyroid, follicular dilatation with increased eosinophilic staining of the colloid was seen in one dog at 100 ppm (grading: minimal), three dogs at 2000 ppm (grading: minimal [two animals] to slight [one animal]) and five dogs at 50 000 ppm (grading: minimal [one animal] to slight [four animals]). Cortical hyperplasia of the adrenal was present in dogs fed 2000 and 50 000 ppm, the highest severity being noted at 2000 ppm. In the lungs, intra-alveolar histiocytosis was seen generally associated with cholesterol clefts at 2000 and 50 000 ppm.

The blood, fat and brain tissue concentrations of lufenuron were similar at 2000 and 50 000 ppm. Thus, the blood and tissues seemed to be saturated to a similar extent at both of these feeding levels. At the low feeding level of 100 ppm, the blood and tissue concentrations were lower by a factor of approximately 10. The similar blood and tissue levels in the 2000 and 50 000 ppm groups were in good agreement with the absence of a dose–response relationship for the majority of treatment-related findings. Major clinical signs (neuromuscular signs, convulsions) were seen after at least 20 weeks of treatment in three animals at 2000 ppm and in three animals at 50 000 ppm. Although the incidence was similar in both groups, the animals at 2000 ppm were more severely affected. At 50 000 ppm, neuromuscular signs were transient and reversible. In view of the small number of animals and the similar blood and tissue levels, mortality could have occurred in the 2000 ppm group and not in the 50 000 ppm group only by chance (Table 14).

At 100 ppm, a slight increase in liver weight without any associated histopathological changes was observed, and one dog showed a slight increase in thyroid weight and histopathological evidence of minimal dilatation of the follicles. Considering the nature and low severity of these changes and that the histopathological changes in the thyroid were seen in only one animal, the NOAEL was 100 ppm (equal to 3.97 mg/kg bw per day for males and 3.64 mg/kg bw per day for females), based on mortality, neuromuscular signs, including convulsions, reduced body weight gains

Table 13. Haematology, clinical biochemistry and organ weights in a 52-week oral study in dogs

	Males				Females			
	0 ppm	**100 ppm**	**2 000 ppm**	**50 000 ppm**	**0 ppm**	**100 ppm**	**2 000 ppm**	**50 000 ppm**
Platelet count								
13	353	360	397	598**	325	356	383	421
26	317	343	448	629***	371	351	420	524
39	279	352	451*	622***	317	354	417	532**
52	289	363	532**	643***	346	345	469	572**
Cholesterol (g/L)								
13	1.27	1.53	1.71	2.21*	1.22	1.16	1.72	1.83
26	1.24	1.55	1.60	2.09*	1.32	1.49	1.59	1.92
39	1.34	1.61	1.85	1.91	1.27	1.21	1.45	1.96
52	1.23	1.49	1.73	2.24*	1.53	1.34	1.68	2.17*
Phospholipids (g/L)								
13	2.59	2.85	2.91	3.68*	2.42	2.36	3.00	3.26
26	2.66	2.99	3.04	3.45	2.71	3.08	2.90	3.48
39	2.74	2.78	3.16	3.36	2.33	2.48	2.64	3.45*
52	2.97	3.15	3.53	3.94	3.34	3.11	3.50	4.13
Alkaline phosphatase (IU/L)								
13	197	242	178	247	240	170	272	251
26	131	206	150	227	154	130	478	208
39	116	170	207	235	153	124	347	222
52	110	169	173	281*	154	151	323	254
ALAT (IU/L)								
13	40	42	36	48	48	38	67	34
26	44	40	35	38	43	33	54	35
39	40	38	35	35	41	35	44	33
52	43	42	40	33	37	37	63	33
Adrenal weight								
Absolute (g)	1.349	1.270	1.606	1.844*	1.462	1.412	2.686**	1.562
Relative (%)								
- To body	0.017 6	0.016 8	0.022 1	0.023 7*	0.019 7	0.020 4	0.036 7***	0.022 2
- To brain	0.012 7	0.013 0	0.016 9	0.019 6*	0.016 6	0.015 8	0.031 9***	0.020 1
Thyroid weight								
Absolute (g)	0.652	0.970	0.834	0.944	0.549	0.618	0.761	0.791
Relative (%)								
- To body	0.008 4	0.012 7	0.011 3	0.012 2	0.007 4	0.008 9	0.010 5	0.011 2

	Males				Females			
	0 ppm	100 ppm	2 000 ppm	50 000 ppm	0 ppm	100 ppm	2 000 ppm	50 000 ppm
- To brain	0.006 0	0.010 0	0.008 7	0.009 7	0.006 3	0.007 0	0.009 1	0.010 1
Liver weight								
Absolute (g)	261.7	331.8	432.7	387.2	262.5	281.0	419.3**	339.6
Relative (%)								
- To body	3.41	4.34	5.94**	4.96*	3.57	4.05	5.75***	4.81**
- To brain	2.42	3.42	4.54**	4.03**	3.01	3.13	4.96***	4.33***

ALAT: alanine aminotransferase; IU: International Units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Briffaux (1992)

Table 14. Blood, fat and brain concentrations in a 52-week oral toxicity study in dogs

Tissue	Week	Sex	0 ppm	100 ppm	2 000 ppm	50 000 ppm
Blood (µg/mL blood)	34	Males	< 0.1	5.8	31.7	29.2
		Females	< 0.1	4.3	29.7	26.7
	37	Males	< 0.1	5.6	33.3	33.7
		Females	< 0.1	4.7	29.2	30.7
	52	Males	< 0.1	5.1	42.5	55.5
		Females	< 0.1	6.3	34.7	41.7
Fat (mg/kg tissue)	52	Males	10.5[a]	670	6 000	5 025
		Females	9.2[a]	797	4 433	4 925
Brain (mg/kg tissue)	52	Males	< 2	15.5	250	242.5
		Females	< 2	9	282.5	197.5

ppm: parts per million
[a] Most probably due to contamination of samples.
Source: Briffaux (1992)

and changes in clinical pathology parameters, organ weights and histopathological lesions in adrenals, liver, thyroid and lungs observed at 2000 ppm (equal to 65.4 mg/kg bw per day for males and 78.3 mg/kg bw per day for females) (Briffaux, 1992).

In another 1-year toxicity study, groups of four male and four female Beagle dogs received lufenuron (purity 97.1%) in their diet at a concentration of 0, 10, 50, 250 or 1000 ppm (equal to 0, 0.31, 1.42, 7.02 and 29.8 mg/kg bw per day for males and 0, 0.33, 1.55, 7.72 and 31.8 mg/kg bw per day for females, respectively). Clinical observations and feed consumption were recorded daily. Body weight was recorded weekly. A detailed neurological examination and ophthalmoscopy were carried out at intervals during the study, and a range of haematological and clinical biochemistry (blood and urine) parameters was measured throughout the study. At the end of the scheduled period, the animals were killed and subjected to a postmortem examination. Terminal blood and bone marrow samples were taken, selected organs were weighed and specified tissues were taken for subsequent histopathological examination. In order to support the interpretation of the results obtained in this study and to demonstrate saturation, the concentrations of the test article were determined in blood, fat and brain samples. Blood samples were taken at 26 and 52 weeks of feeding. At final sacrifice after 24 months, fat and brain tissues were sampled from all surviving animals.

One female in the 1000 ppm group was found dead in week 31. Prior to death, this animal had shown marked clinical signs and deterioration of general state of health. A further one female and two males from this dose group were killed in weeks 28, 43 and 49 following clinical signs of convulsions. Treatment-related clinical signs were restricted to 1000 ppm animals that died or were killed prior to scheduled termination. These signs included convulsions, tremor, atactic gait, reduced locomotor activity, aggressiveness, nervousness, impeded respiration, vomiting and salivation. During the first third of the treatment period, body weight gain of treated animals was comparable with that of controls. After this time, loss in body weight was recorded for all 1000 ppm animals, resulting in an overall weight loss or depressed body weight gain. At the end of the treatment period, one male in the 250 ppm group lost weight from week 47 onwards (~5%: 10.4 kg in week 52 compared with ~11.0 kg during weeks 14–46), whereas the mean body weight of the 250 ppm group males in week 52 (12.00 kg) was comparable with the control value (12.08 kg). The results of the ophthalmoscopic examinations and measurement of neurological parameters showed no treatment-related effects.

Mean feed consumption for the treated groups was comparable with that of controls except for single periods of depressed feed intake in the 1000 ppm group caused by animals with clinical signs prior to early sacrifice. There were no statistically significant differences. Males and females in the 1000 ppm group had minimally but consistently higher platelet counts throughout the treatment period. Alterations of blood chemistry parameters were confined to the 1000 ppm animals. Higher activities of alkaline phosphatase were recorded in both sexes at weeks 26 and 52, and males also had higher GGT activities when measured at week 52. Plasma globulin levels were slightly increased in both sexes at the end of treatment and in males at week 26. Higher plasma cholesterol levels were observed in males at weeks 13, 26 and 52 and in females at week 52. Also, males had minimally lower plasma phosphate and thyroxine levels, whereas females showed higher plasma glucose levels at each assessment period during treatment (Table 15).

Table 15. Haematological and blood chemistry findings in a 52-week oral toxicity study in dogs

	Males					**Females**				
Parameter / week	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**
Platelets (g/L)										
−1	264.8	287.8	295.0	268.5	249.8	254.3	267.8	284.0	247.0	298.8
13	248.5	294.0	299.0	259.0	314.0	272.0	292.8	283.0	301.3	364.8*
26	243.0	354.8*	327.0*	337.8*	350.6*	294.8	322.3	308.0	299.8	454.5
52	274.5	381.3	311.5	386.3	405.0	305.3	307.8	356.0	383.0	654.0+
Alkaline phosphatase (U/L)										
−1	122.3	85.45	88.28	112.5	105.6	84.05	72.80	93.53	96.63	74.68
13	97.48	68.40	73.08	83.70	92.65	68.60	57.53	81.15	76.16	74.48
26	67.55	47.88	54.45	58.70	135.6*	48.15	46.50	64.33	70.18	113.9*+
52	53.30	40.53	46.50	59.23	144.1	48.03	47.90	65.78	67.68	173.9
GGT (U/L)										
−1	1.375	2.000	1.375	3.375*	0.725	1.375	2.200	0.550	2.950	2.925
13	4.450	4.325	4.625	3.500	4.800	4.175	3.800	4.300	4.175	4.625
26	4.700	4.150	4.050	3.625	4.550	4.150	3.225	3.625	4.313	5.338

	Males					Females				
Parameter / week	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**
52	2.850	2.700	3.325	3.025	5.100	1.750	3.350	3.350	3.888	3.650
Globulin (g/L)										
−1	21.01	22.72	21.34	21.28	22.71	21.18	20.76	21.60	19.45	20.50
13	24.02	25.10	23.05	23.86	24.47	22.70	23.34	22.21	21.87	22.17
26	24.44	25.23	23.99	24.22	27.02	22.38	23.57	22.62	21.83	22.36
52	25.89	28.09	25.14	26.95	30.61	23.88	25.95	24.99	24.25	27.40
Cholesterol (mmol/L)										
−1	3.475	3.408	3.630	3.593	4.061*	3.305	3.170	3.135	3.203	3.245
13	3.868	3.720	3.873	3.885	4.985*	3.513	3.708	3.588	3.515	3.805
26	3.840	3.113*	3.855	3.998	5.063*+	3.320	3.735	3.558	3.413	3.863
52	3.610	3.193	3.688	3.875	4.735	3.458	3.450	3.465	3.460	4.465
Phosphate (mmol/L)										
−1	2.138	2.010	2.225	2.035	2.015	1.813	1.945	1.890	1.973	1.920
13	1.628	1.533	1.675	1.588	1.350*	1.385	1.340	1.345	1.398	1.350
26	1.468	1.378	1.458	1.230*	1.114*−	1.306	1.298	1.298	1.124	1.045
52	1.185	1.275	1.175	1.083	0.810	1.063	1.115	0.858	0.985	0.828
Thyroxine (nmol/L)										
−1	37.84	36.73	31.25	33.32	35.00	33.48	35.06	36.10	32.35	41.65
13	35.20	32.26	28.40	32.40	27.03	33.50	35.15	35.27	32.54	31.02
26	33.75	34.37	27.00	25.17*−	25.42−	25.02	28.73	33.50	27.38	30.21
52	43.71	40.39	37.19	34.99	29.43	43.02	40.88	45.34	35.93	33.57
Glucose (mmol/L)										
−1	5.715	6.110	5.840	5.818	6.108	5.900	6.023	5.723	5.770	6.270
13	5.143	5.420	5.430	5.385	6.008*−	5.065	5.455	5.213	5.365	6.050*
26	5.420	5.323	5.495	5.350	6.185	5.275	5.953	5.560	5.665	8.820*
52	5.120	5.035	5.145	5.203	6.200	5.235	5.558	5.490	5.278	6.480

GGT: gamma-glutamyltranspeptidase; ppm: parts per million; U: units; *: $P < 0.05$ (Wilcoxon); +/−: $P < 0.01$ (Jonckheere trend test)
Source: Altmann (1995)

There was a dose-related increase in both absolute and relative (to body weight) liver weights for both sexes in the 250 ppm group (absolute: 126% of control values for both sexes; relative: 129% and 135% of control values for males and females, respectively) and in the surviving animals in the 1000 ppm group (absolute: 147% and 141% of control values for males and females, respectively; relative: 164% and 172% of control values for males and females, respectively). Mean absolute and relative adrenal weights were increased in the surviving animals in the 1000 ppm group. In addition, a

tendency to an increase in adrenal weights was recorded in one male and one female treated with 250 ppm, but without pathological correlate. Mean thymus weight was slightly decreased in the surviving animals in both 1000 ppm groups.

At necropsy, the liver was described as enlarged in one dog of each sex in the 1000 ppm group, and enlarged adrenal glands were found in most animals in this group. Microscopically, a minimal to moderate hypertrophy of hepatocytes was found in males and females in the 250 and 1000 ppm groups. Pigmentation of liver Kupffer cells was increased in incidence and severity in the 1000 ppm males. A minimal to marked diffuse adrenal cortical hyperplasia was seen in all animals in the 1000 ppm group. In a few females in this group, cortical haemorrhage was also present. A minimal to moderate depletion of lymphocytes of the Peyer's patches showed an increased incidence in the small intestine of the 1000 ppm animals. Depletion of lymphocytes in the mesenteric lymph node showed an increased incidence and severity in both 1000 ppm groups. Moderate to marked thymic atrophy showed an increased incidence and severity in 1000 ppm females. Aggregation of alveolar foam cells was found in the lungs of both sexes at 1000 ppm. Other findings noted at microscopic examination were considered to be unrelated to treatment. No effects were observed on thyroid weight or thyroid histopathology (Table 16).

The blood, fat and brain concentrations were similar in males and females. The blood levels at week 52 were similar to or only slightly higher than those at week 26, indicating that a plateau was reached. The blood to fat ratio was approximately 1:100 or slightly higher. In the brains, the concentrations were similar to those in the blood at the two lowest feeding levels and up to about 5 times higher than those in the blood at the two highest dose levels (Table 17).

The effects observed at a dose level of 250 ppm were confined to body weight loss in one male, a tendency to increased adrenal weight in one male and one female, without pathological correlates, and increased absolute and relative liver weights in males and females with hypertrophy of hepatocytes. The liver effect should be seen as an adaptive response, in the absence of further histopathological damage and relevant clinical chemistry changes. The effects on body weight and adrenals at this dose had no effect on the group means.

The NOAEL was 250 ppm (equal to 7.02 mg/kg bw per day for males and 7.72 mg/kg bw per day for females), based on treatment-related mortality and clinical findings, effects on body weight and effects on the liver and adrenals, with associated histopathology and/or clinical chemistry changes, at 1000 ppm (equal to 29.8 mg/kg bw per day for males and 31.8 mg/kg bw per day for females) (Altmann, 1995).

(b) Dermal application

Rats

In a 28-day dermal toxicity study, groups of five male and five female albino rats (Tif: RAIf (SPF), hybrids of RII/1 × RII/2) were treated dermally with lufenuron (purity 95.2%) moistened with 0.5% carboxymethyl cellulose in 0.1% aqueous polysorbate 80 (wetting agent) at a dose level of 0, 100, 300 or 1000 mg/kg bw per day, 5 days/week, for 4 weeks. The exposure period was 6 hours/day under an occlusive dressing. Mortality and signs of systemic toxicity were determined daily, and signs of local skin irritation were determined approximately 17 hours after removing the gauze patches. Body weights and feed consumption were recorded weekly. Laboratory investigations (haematology and blood chemistry) were carried out on all surviving animals of each dose group at the end of the treatment period. At the end of the test period, all control and treated rats were subjected to detailed necropsy. Besides the weight of the exsanguinated body, the following organs were weighed: brain, heart, liver, kidneys, adrenals, thymus, ovaries/testes and spleen. Selected organs and tissues were preserved; the following samples were subjected to microscopic examination: skin application site, skin remote site, brain, liver, kidneys, adrenal glands, thymus and spleen (histopathological evaluation of paired organs was performed on both of them).

There were no systemic or local effects at any dose level. The NOAEL of the study was 1000 mg/kg bw per day, the highest dose tested (Schneider, 1990).

Table 16. Pathology findings in a 52-week oral toxicity study in dogs

	Males (*n* = 4)					Females (*n* = 4)				
Parameter	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**
Mortality (weeks)	0	0	0	0	2 (43/49)	0	0	0	0	2 (28/31)
Body weight (kg)	11.56	10.88	11.10	11.44	10.53	10.64	10.14	10.42	9.905	8.740
Liver										
Liver weight										
- Absolute (g)	356.3	359.6	354.6	451.8	523.0*	326.1	299.4	334.3	410.6*	462.1
- Relative (%)	30.72	33.08	32.01	39.84	50.45	30.77	29.67	32.07	41.43*	52.95+
Enlarged liver	0	0	0	0	1	0	0	0	0	1
HH	0	0	0	4	3	0	0	0	3	1
KCP	0	1	1	1	2	0	0	0	0	0
Adrenals (both)										
Adrenal weight										
- Absolute (g)	1.470	1.461	1.583	1.732	2.485+	1.606	1.728	1.613	1.819	3.078
- Relative (%)	0.127	0.134	0.143	0.154	0.236+	0.151	0.173	0.155	0.184	0.356
Enlarged adrenals	0	0	0	0	3	0	0	0	0	4
ACH	0	0	0	0	4	0	0	0	0	4
Thymus										
Thymus weight										
- Absolute (g)	6.714	4.899	7.549	7.410	4.673	8.429	7.611	6.101	6.051	3.055
Atrophy	0	0	0	0	0	2	2	3	3	4
Thyroid										
Thyroid weight										
- Absolute (g)	1.162	1.088	1.034	1.077	1.079	0.820	1.040	0.960	0.981	1.020
- Relative (%)	0.010 1	0.010 0	0.009 3	0.009 4	0.010 7	0.007 7	0.010 7	0.009 2	0.009 9	0.011 8
Small intestine										
HPP	0	0	0	0	4	0	0	0	0	3
Mesenteric lymph node										
PC	4	3	4	4	4	0	0	0	0	0
HLT	0	1	0	0	3	1	1	0	1	3
Lungs										
AFC	0	0	0	0	3	0	0	0	0	1

ACH: adrenocortical hyperplasia; AFC: alveolar foam cells; HH: hypertrophy of hepatocytes; HLT: hypocellularity of lymphatic tissue; HPP: hypocellularity of Peyer's patch; KCP: Kupffer cell pigmentation; PC: phagocytic cells; ppm: parts per million; *: $P < 0.05$; +: $P < 0.01$
Source: Altmann (1995)

Table 17. Blood, fat and brain concentrations in a 52-week oral toxicity study in dogs

Tissue	Week	0 ppm	10 ppm	50 ppm	250 ppm	1 000 ppm
Blood (μg/mL blood)	26	< 0.1	0.19	1.7	10	41
	52	< 0.1	0.18	1.9	24	66
Fat (mg/kg tissue)	52	< 0.5	28	260	2 500	8 700
Brain (mg/kg tissue)	52	< 0.1	0.2	1.9	62	340

ppm: parts per million
Source: Altmann (1995)

(c) Exposure by inhalation

No study was submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a chronic toxicity and carcinogenicity study, groups of 60 male and 60 female mice (Tif:MAGf (SPF), hybrids of NIH × MAG) received lufenuron (purity 96.2%) in their diets at a concentration of 0, 2, 20, 200 or 400 ppm (equal to 0, 0.222, 2.25, 22.6 and 62.9 mg/kg bw per day for males and 0, 0.217, 2.12, 22.0 and 61.2 mg/kg bw per day for females, respectively) for 18 consecutive months. As a result of high mortality in the high-dose group, surviving animals in this dose group were terminated in weeks 9 and 10. Samples were taken from selected animals at weeks 53 and 78 for haematology. Clinical chemistry parameters were not assessed. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed. Gross necropsies were performed for all animals that died prior to scheduled termination (found dead or euthanized moribund). Samples of selected tissues were processed for histopathological evaluation from all surviving test animals and from any unscheduled deaths that occurred during the study.

During the first 9 weeks of the study, five males and 29 females in the 400 ppm group were found dead. All survivors in this group were sacrificed early in weeks 9 and 10. The mortality recorded for the 2 and 20 ppm groups was similar to control values, but there was a higher mortality rate than for controls in the 200 ppm group. The percentage of animals surviving to termination at 18 months was 76%, 74%, 84% and 64% for the males and 82%, 82%, 88% and 48% for the females in the 0 (controls), 2, 20 and 200 ppm groups, respectively. Tonic-clonic convulsions, occurring spontaneously or in response to external stimuli such as handling, were exhibited by a number of animals of both sexes in the 200 and 400 ppm groups. The convulsions were of short duration (up to 30 seconds), during which time forelimb paddling, uncoordinated movement, jumping and straub tail occurred. Convulsions recorded in the other dose groups, including controls, were of a shorter duration and less intense (tonic phase only) and were regarded as spontaneous events, unrelated to treatment. No other clinical signs of reaction to treatment were observed.

No effects on body weight were recorded for either sex in the 2 and 20 ppm groups or for males in the 200 ppm group. Females in the 200 ppm group showed a small reduction in mean body weights (which did not attain statistical significance) compared with controls during the last 6 months of the study. Feed consumption for both sexes in the 2, 20 and 200 ppm groups was not adversely affected by treatment. No treatment-related haematological changes were noted in either sex at either 12 months or study termination (18 months). Slight increases in absolute and relative (to body weight) adrenal weights were observed in the 200 ppm females compared with controls (absolute weight: 113%; relative weight: 122%). However, all values were within the range recorded among controls, and microscopy revealed no morphological changes in this organ. Macroscopic postmortem examination revealed a higher incidence of single nodules of the lung in male mice of the 2 ppm group and of single and multiple nodules of the lung in male mice of the 200 ppm group compared

with controls. The incidence at 20 ppm was similar to that of controls, indicating no dose–response relationship.

Microscopic examination revealed higher incidences of fatty liver compared with controls, accompanied by necrotic changes in females of the 200 ppm group. Similar findings were seen in the 400 ppm group. Males in the 200 ppm group had a higher incidence of inflammatory changes in the prostate. Males in this group also showed an increased incidence, compared with controls, of both single and multiple lung adenomas, relating to the increased numbers of lung nodules seen macroscopically. The incidences observed across the treated groups in this study were not dose related, suggesting that the variation in incidence was spontaneous rather than as a result of treatment. In addition, the incidence of lung adenomas is known to be very variable in aged mice, and thus these isolated higher incidences are not considered to indicate an effect of treatment (Table 18).

Table 18. Pathology findings in an 18-month oral toxicity and carcinogenicity study in mice

	Incidence of finding									
	Males					**Females**				
Observation	**0 ppm**	**2 ppm**	**20 ppm**	**200 ppm**	**400 ppm**	**0 ppm**	**2 ppm**	**20 ppm**	**200 ppm**	**400 ppm**
Number examined	60	60	60	60	60	60	60	60	60	60
Lung										
Adenoma	12	19	4	19	0	8	5	8	7	0
Liver										
Fatty change	42	41	38	53	37	46	39	46	56	52
Recent necrosis	4	3	1	0	0	8	6	8	13	20
Hepatocellular necrosis	4	10	7	7	1	5	2	5	12	6
Prostate										
Total inflammatory lesions	3	6	4	10	1	–	–	–	–	–

ppm: parts per million
Source: Bachmann (1993a)

The NOAEL for systemic toxicity was 20 ppm (equal to 2.25 mg/kg bw per day for males and 2.12 mg/kg bw per day for females), based on increased mortality, clinical signs (tonic-clonic convulsive episodes), increased incidences of fatty liver (in females accompanied by necrotic changes) and a higher incidence of inflammatory changes in the prostate at 200 ppm (equal to 22.6 mg/kg bw per day for males and 22.0 mg/kg bw per day for females).

No treatment-related tumours were observed in male or female mice (Bachmann, 1993a).

Rats

In a combined chronic toxicity and carcinogenicity study in rats, groups of 80 male and 80 female Tif: RAIf (SPF) rats (70 of each sex in main groups, 10 of each sex in satellite groups) received lufenuron (purity 96.2%) in their diets at a concentration of 0, 5, 50, 500 or 1500 ppm (equal to 0, 0.19, 1.93, 20.4 and 108 mg/kg bw per day for males and 0, 0.23, 2.34, 24.8 and 114 mg/kg bw per day for females, respectively) for 104 weeks. As a result of overt toxicity at 1500 ppm, all animals in this group were terminated in week 14. Clinical observations (including ophthalmology), body weights, and feed and water consumption were measured throughout the study. Haematology, blood chemistry and urine analysis were carried out on selected animals periodically throughout the study

and at the end of the treatment period. At the end of the scheduled period, all animals were killed and subjected to a postmortem examination, selected organs were weighed and specified tissues were taken for subsequent histopathology. Samples of fat and blood were taken from all 1-year sacrifice animals and from selected surviving animals at study termination for analytical investigation, the results of which were reported as a separate study that was not submitted.

As a result of overt toxicity recorded in the 1500 ppm group, all animals in this group were terminated in week 14. The mortality rate in the remaining treated groups was similar to that of the controls. The percentage of animals surviving to termination at 18 months was 50%, 46%, 57% and 59% for the males and 51%, 57%, 59% and 61% for the females in the 0 (controls), 5, 50 and 500 ppm groups, respectively. A total of 46 males and 57 females in the 1500 ppm group and 47 males and 58 females in the 500 ppm group exhibited whole-body tonic-clonic convulsions from weeks 6 to 7. The convulsive episodes, lasting approximately 30–90 seconds, were observed mainly during and after handling and occurred repeatedly over several weeks in the majority of affected animals. As the study progressed, the incidence of observed convulsions in the 500 ppm animals diminished. A low frequency of spontaneous convulsive episodes was seen in the control and low-dose groups. Several females in the 500 ppm group had vaginal discharge during the latter part of the treatment period.

Males and females in the 1500 ppm group had body weights approximately 10% lower than those of controls in week 12 prior to early sacrifice. Slightly lower mean body weights were recorded for both sexes in the 500 ppm group up to week 27. Thereafter, an increased body weight gain in the females resulted in mean body weights approximately 20% higher than control values by the end of the study. The males in the 500 ppm group continued to have body weights slightly lower than control values to the end of the study (92% of controls). Higher feed intake, compared with controls, was recorded in week 1 for both sexes in the 500 and 1500 ppm groups (111–114% of controls). Thereafter, the feed intake of the 1500 ppm group tended to be lower than that of controls up to the early termination of this group. Lower feed intake, compared with controls, was recorded in the 500 ppm males for the next 26 weeks (95% of controls), after which time the feed intakes were similar to control values. Females in the 500 ppm group showed an 18% increase in feed intake from week 23 to termination.

The week 13 examination of the blood, 1 week prior to the termination of the 1500 ppm animals, showed 8/20 males in this group with platelet counts above the concurrent control range and 3/20 females with white blood cell counts above the control range. Other parameters in this group were not affected by treatment, and subsequent examinations revealed no evidence of a treatment-related influence on the haematological profile of treated rats. Slightly lower levels of plasma protein and albumin and higher levels of plasma potassium and inorganic phosphorus were recorded for females in the 1500 ppm group prior to termination of this group in week 14. No clear effect of treatment on the blood chemistry profile of males or females was seen in the 5, 50 or 500 ppm groups throughout the study. A number of differences between the mean values attained a level of statistical significance, but the differences were small and considered to be of no biological relevance. No effects on the urinary parameters were observed.

No treatment-related effects on organ weights were recorded in this study. Compared with the controls, levels of statistical significance were achieved for absolute and relative adrenal gland weights for females in the 50 and 500 ppm groups sacrificed at week 53. However, no changes were detected in the adrenals by microscopy. Therefore, the difference at week 53 was considered to have occurred by chance.

At macroscopy, higher incidences of mottled lungs in males and females of the 500 ppm group were considered to be treatment related. Microscopic examination revealed an increased incidence of pulmonary alveolar foam cells in both sexes of the 500 and 1500 ppm groups, with a higher severity level in the 500 ppm animals. This lesion was considered to be associated with a dilatation of the right heart ventricle developing due to increased pulmonary pressure caused by massive aggregations of foam cells in some females in the 500 ppm group.

Large adrenal glands were reported in 2/80, 5/80, 5/80 and 8/80 males and 3/80, 4/80, 2/80 and 11/80 females at 0, 5, 50 and 500 ppm, respectively. On microscopic examination, various

lesions, such as cysts, sinusoidal cystic dilatations, and hyperplasias or tumours of cortex or medulla, were found in these adrenals. The incidences of these lesions in the different groups did not indicate any treatment-related effect. The microscopic verification of some other macroscopic findings reported in higher numbers of treated animals revealed no treatment-related gross pathological effects.

An increased incidence of ulcerative and inflammatory lesions was seen in the non-glandular stomachs of males and females in the 500 ppm group. Focal haemorrhagic, necrotic, ulcerative and inflammatory lesions were found in the caecum and/or colon of both males and females in the 500 and 1500 ppm groups. An increased incidence of fatty change was seen in the perilobular region of the liver in females in the 500 ppm group. In addition, inflammation of the female urinary tract was markedly increased in 500 ppm animals (Table 19).

Table 19. Selected microscopic findings in a 2-year oral toxicity and carcinogenicity study in rats

	Incidence of finding									
	Males (*n* = 80)					**Females (*n* = 80)**				
Observation	**0 ppm**	**5 ppm**	**50 ppm**	**500 ppm**	**1 500 ppm**	**0 ppm**	**5 ppm**	**50 ppm**	**500 ppm**	**1 500 ppm**
Non-neoplastic findings										
Lung: foam cells										
Minimal	20	24	19	24	61	21	25	21	22	69
Moderate	12	12	10	19	6	14	15	16	24	5
Marked	5	4	5	11	0	8	3	6	19	0
Heart: dilatation of right ventricle	6	5	3	4	0	4	1	4	12	0
Non-glandular stomach: ulceration	0	0	1	4	0	1	0	1	2	0
Stomach: chronic inflammation	2	0	1	4	0	0	1	4	1	11
Caecum/colon: lesions total count	0	0	0	5	10	1	1	1	5	6
Liver (centrilobular): fatty change	0	1	0	0	0	2	2	1	36	0
Urinary bladder: inflammation	10	5	3	1	0	1	2	2	22	0
Kidney: inflammation of pelvis	11	5	2	2	0	2	4	2	20	0
Neoplastic findings										
Testis: benign interstitial cell tumour	2	2	1	5	–	–	–	–	–	–
Cerebral meninges: benign granular cell tumour	1	0	1	3	–	0	0	0	1	–

ppm: parts per million
Source: Bachmann (1993b)

No treatment-related increase in the incidence of hyperplastic or neoplastic lesions was found. The incidence of animals (*n* = 80) bearing primary tumours was 49, 52, 55, 42 and 0 for males and 57,

67, 54, 41 and 0 for females in the 0, 5, 50, 500 and 1500 ppm groups, respectively. Evidence of an apparently statistically significant dose-related effect at 500 ppm was shown for the benign interstitial cell tumour of testis found at incidences of 2/80 at 0 ppm, 2/80 at 5 ppm, 1/80 at 50 ppm and 5/80 (6.25%) at 500 ppm, as well as the benign granular cell tumour of the cerebral meninges found in male animals at incidences of 1/80 at 0 ppm, 0/80 at 5 ppm, 1/80 at 50 ppm and 3/80 (3.75%) at 500 ppm. Both tumour incidences were in line with the historical control data presented in the study report, even though the historical control data were not completely within JMPR standards (i.e. rat strain not specified and presented studies were from 1978 to 1989, which is not within 2 years of the in-life phase of the lufenuron study). The historical control data from the laboratory and from the Registry of Industrial Toxicology Animal-data (RITA) database show a variable incidence, with no obvious change in incidence over the period 1978–1989. On this basis, it can be considered that all these data are applicable historical control data for the lufenuron 2-year rat study, which started in January 1990 (see Appendix 1).

Treatment with lufenuron for 24 months resulted in whole-body tonic-clonic convulsions at 500 and 1500 ppm. The extent of the reaction showed that the maximum tolerated dose (MTD) was exceeded at the high dose level of 1500 ppm, and this group was terminated in week 14. At 500 ppm, an initially reduced body weight gain was observed, which in females reversed to a marked increase in body weights from week 27 onwards, associated with a marked increase in feed intake. Histopathological changes at 500 and/or 1500 ppm included an increase in the incidence of pulmonary alveolar foam cells, ulcerative and inflammatory lesions in the non-glandular stomach and focal lesions in the caecum and/or colon. Additionally, increased incidences of fatty change in the liver and inflammation of the urinary tract were detected in females. There was no evidence of a tumorigenic response.

The NOAEL for systemic toxicity was 50 ppm (equal to 1.93 mg/kg bw per day for males and 2.34 mg/kg bw per day for females), based on tonic-clonic convulsions, decreased body weight and (histo)pathological effects on lungs, liver, non-glandular stomach, intestines and urinary tract at 500 ppm (equal to 20.4 mg/kg bw per day for males and 24.8 mg/kg bw per day for females). There was no evidence of a tumorigenic response (Bachmann, 1993b).

2.4 Genotoxicity

The results of studies of genotoxicity with lufenuron are summarized in Table 20. All the studies were certified to comply with GLP and performed according to internationally accepted guidelines. No evidence of genotoxicity was observed.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation study on reproductive toxicity, groups of 30 male and 30 female Sprague-Dawley-derived Tif: RAIf (SPF) rats received lufenuron (purity 96.2%) in their diets at a concentration of 0, 5, 25, 100 or 250 ppm over the course of two generations (P and F_1). Mean lufenuron intakes for combined P and F_1 generations during the premating period were 0, 0.41, 2.1, 8.3 and 20.9 mg/kg bw per day for males and 0, 0.44, 2.2, 8.9 and 22.2 mg/kg bw per day for females, respectively.

After 10 weeks, the animals were mated (1:1) within each dose group and allowed to rear the ensuing F_1 litters to weaning. Litters were culled to four male and four female pups, where possible, on day 4 postpartum. The breeding programme was repeated with the F_1 parents selected from the F_1 offspring. Test diets were fed continuously throughout the study. Parental feed consumption and body weights were measured throughout the study. Reproductive performance, pup survival and developmental parameters were measured. Gross necropsy findings and histopathological observations in target organs of parental animals and pups not selected for mating were recorded.

Table 20. Results of studies of genotoxicity with lufenuron

End-point	Test object	Concentration	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, T1537	20–5 000 µg/0.1 mL (±S9)	95.2	Negative	Deparade (1988)
Mammalian cell gene mutation	V79 Chinese hamster cells, HPRT test	37.5–900 µg/mL (+S9) and up to 25–500 µg/mL (−S9)	95.2	Negative	Dollenmeier (1988)
Chromosomal aberration	CCL 61 CHO cells	200–1 600 µg/mL (+S9) and up to 25–200 µg/mL (−S9)	95.2	Negative	Strasser (1989)
Unscheduled DNA synthesis	Male Tif: RAIf (SPF) rat hepatocytes	2–2 000 µg/mL	95.2	Negative	Hertner (1988)
Unscheduled DNA synthesis	Human fibroblasts	28.40–6 900 µg/mL	95.2	Negative	Meyer (1988)
Unscheduled DNA synthesis	Cultured human MRC-9 lung cells	0.15–5 µg/mL (±S9)	97.1	Negative	Tanaka (1997)
In vivo					
Mouse micronucleus	Tif: MAGF, SPF mice	0, 1 250, 2 500 or 5 000 mg/kg bw (gavage)	95.2	Negative	Meyer (1989a)
Unscheduled DNA synthesis	Male Tif: RAIf (SPF) rats	0, 1 250, 2 500 or 5 000 mg/kg bw (gavage)	97.1	Negative	Hertner (1994)
Unscheduled DNA synthesis	Male HanIbM: WIST (SPF) rats	0, 1 000 or 2 000 mg/kg bw (gavage)	97.1	Negative	Ogorek (2000)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* fraction from Aroclor 1254 or phenobarbital/5,6-benzoflavone–induced pretreated rat liver homogenate

There were no mortalities or treatment-related clinical observations in either generation of parents. There was an increased incidence of skin wounds/crust on the head/trunk in the F_1 male 250 ppm group. Body weights and feed consumption were unaffected by treatment for the P parents. Body weights of the F_1 250 ppm animals of both sexes were significantly higher than control values during the premating period. Feed consumption was also slightly higher than control values during this period in the same groups, indicating that the increased body weight was a direct result of increased feed consumption.

For both generations, male and female mating and fertility indices, maternal gestation and parturition indices, and duration of gestation were unaffected by treatment. There were no treatment-related observations at gross necropsy in either generation of adults. No effects on organ weights were seen in the P parents. Organ weights were higher in the 250 ppm male and female F_1 parents, consistent with the higher body weights in this group, but there were no statistically significant differences from control values in the organ to body weight ratios in this group. There were no treatment-related histopathological changes in the reproductive system or in any organs examined in both the P and F_1 parental generations.

In both the F_1 and F_2 offspring, live births, postnatal survival indices and litter weights were unaffected by treatment. The mean time of appearance of the surface righting reflex was slightly

delayed by about 0.2–0.4 day in the 250 ppm group compared with controls for both generations. There were no treatment-related observations at postmortem examination in the F_1 or F_2 offspring.

In this study, the highest dose did not cause any toxicity, which is required according to OECD Test Guideline 416. However, from the 2-year rat study, it is clear that 1500 ppm was above the MTD (animals euthanized in week 14 because of the extent of tonic-clonic convulsions) and 500 ppm was the lowest-observed-adverse-effect level (LOAEL), based on, for example, tonic-clonic convulsions. A highest dose level of 250 ppm is therefore considered acceptable for this two-generation reproductive toxicity study.

The NOAEL for parental and reproductive effects was 250 ppm (equal to 20.9 mg/kg bw per day for males and 22.2 mg/kg bw per day for females), the highest dose tested.

The NOAEL for offspring toxicity was 100 ppm (equal to 8.3 mg/kg bw per day for males and 8.9 mg/kg bw per day for females), based on the slight delay of 0.2–0.4 day in righting reflex in pups at 250 ppm (equal to 20.9 mg/kg bw per day for males and 22.2 mg/kg bw per day for females) (Fitzgerald & Khalil, 1992).

(b) Developmental toxicity

Rats

In a study of developmental toxicity, groups of 25 mated female Sprague-Dawley (Crl:CD® (SD) BRVAF/Plus™) rats were administered lufenuron (purity 94.7%) at a dose of 0, 100, 500 or 1000 mg/kg bw per day in 3% cornstarch and 0.5% Tween 80 via oral gavage on gestation day (GD) 6 to GD 15 (inclusive). On day 21 of gestation, the rats were killed and examined for macroscopic changes in the oral cavity and all organs of the thoracic and abdominal cavities. The ovaries and uterus with cervix were removed, trimmed, weighed intact and examined. Corpora lutea were counted. The uterus was opened and examined for the number and distribution of live fetuses and intrauterine deaths. All fetuses were weighed, sexed and examined for external variations and malformations. Live fetuses were killed, and half of the fetuses from each litter were eviscerated and subsequently processed and examined for skeletal variations and malformations. The remaining fetuses from each litter had the heads removed and fixed in Bouin's fixative for examination of the structure by the serial cross-section technique. The trunks of these fetuses were given an internal examination of the soft tissue, then eviscerated and processed for skeletal examination.

Twenty-five sperm-positive female rats were assigned to the 0, 100, 500 or 1000 mg/kg bw per day dose levels, of which 22, 23, 19 and 25 animals, respectively, were pregnant. No animals died prior to scheduled sacrifice, and no significant treatment-related signs of toxicity were seen in the individual clinical observations recorded. Minimal maternal toxicity occurred at 1000 mg/kg bw per day, with slight, but statistically significant, reductions in body weight gain on GDs 7–9 and in feed consumption on GDs 6–9.

All pregnant females had live fetuses at termination, and no animals delivered prior to scheduled sacrifice. No embryo/fetal toxicity was apparent at any of the dose levels tested. Statistically significant reductions in the mean number of implantations per dam at 100 mg/kg bw per day and in the number of live fetuses per dam at 100 and 1000 mg/kg bw per day were considered not to be adverse effects of treatment. The reduction at 100 mg/kg bw per day reflected a slight reduction in the number of corpora lutea (developed before treatment), and the less than 4% reduction in mean number of live fetuses at 1000 mg/kg bw per day, compared with concurrent controls, exceeded the mean values for controls from the previous five studies.

The historical control data were presented in the study report as data from control CD rats. No further details were provided; the data were apparently from the performing laboratory, but the exact rat strain is not stated.

There were no significant increases in the test groups, compared with controls, in the occurrences of external, soft tissue or skeletal observations either singularly or collectively when classified as variations or malformations. The mean number of malformed live fetuses in treated

groups was similar to control values. Malformations were observed in six, six, three and four fetuses from five, four, two and four litters in the 0, 100, 500 and 1000 mg/kg bw per day groups, respectively. All of these malformations were considered to have occurred spontaneously.

The NOAEL for maternal toxicity was 500 mg/kg bw per day, based on a transient reduction in body weight gain on GDs 7–9 and in feed consumption on GDs 6–9 at 1000 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested. Lufenuron did not show teratogenic potential in rats (Gilles, 1989).

Rabbits

In a study of developmental toxicity, groups of 16 mated Hra: New Zealand White SPF female rabbits were dosed by gavage at 0, 100, 500 or 1000 mg/kg bw per day with lufenuron (purity 94.7%) in aqueous 3% cornstarch and 0.5% Tween 80 on GD 7 to GD 19 (inclusive). The females were monitored daily for clinical signs. Body weights and feed consumption were recorded at selected intervals during gestation. On day 29 of gestation, the rabbits were killed and examined for macroscopic changes in all organs of the thoracic and abdominal cavities. The ovaries and uterus with cervix were removed, trimmed, weighed intact and examined. Corpora lutea were counted. The uterus was opened and examined for the number and distribution of live fetuses and intrauterine deaths. All fetuses were weighed and examined for external variations and malformations. Each fetus was examined for both soft tissue and skeletal malformations and variations. A transverse section of the brain was made, and organ structure was examined. The trunks of the fetuses were given an internal examination of the soft tissue, sexed, and then eviscerated and processed for skeletal examination.

One female in the control group died on day 18, and one female in the 100 mg/kg bw per day group was found dead on day 11. Both of these deaths were a result of trauma induced by gavage dosing. Pregnancy was observed at scheduled sacrifice in 13, 15, 12 and 15 females in the 0 (controls), 100, 500 and 1000 mg/kg bw per day dose groups, respectively. There was no treatment-related effect on fertility. The fertility index was 88%, 100%, 75% and 94% in the 0 (controls), 100, 500 and 1000 mg/kg bw per day dose groups, respectively. No significant treatment-related signs of toxicity were seen in the individual clinical observations recorded, and there were no treatment-related effects on body weight, body weight gain or feed consumption. At necropsy of the dams, there were no findings that appeared to be treatment related, and no effects of treatment on mean absolute or relative organ weights were observed.

All surviving pregnant females had live fetuses on GD 29. There were no statistically significant changes in fetal weights or other intrauterine parameters. There was no significant increase, relative to controls, in the occurrence of any external, soft tissue or skeletal malformation or variation in fetuses from does treated with lufenuron.

The NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Meyer, 1989b).

2.6 Special studies

(a) Neurotoxicity

In a repeated-dose neurotoxicity study, groups of 10 male Tif: RAIf (SPF) rats received lufenuron (purity 96.2%) in their diet at 0, 5, 25, 100 or 500 ppm (equal to 0, 0.26, 1.22, 5.43 and 27.0 mg/kg bw per day, respectively) for 4 months. An additional 10 males in the control and high-dose groups served as recovery animals. The investigation was limited to males, as previous studies conducted in rats showed that males were as sensitive as or slightly more sensitive than females to the effects of lufenuron. Animals were observed daily except for weekends throughout the study. Detailed clinical observations were not made "blind" (trained observers unaware of the actual treatment group at reading). No data were presented to provide evidence of the ability to detect and quantify, as appropriate, changes in the different end-points recommended for observation, such as autonomic signs, sensory reactivity, limb grip strength and motor activity (use of positive controls).

Ophthalmological examination was not carried out in this study, but data are already available from other studies of similar duration and at similar dose levels. Any observable gross changes were apparently not recorded. The deviations are considered not to compromise the scientific validity of the study. In five animals per dose group, the lufenuron concentrations in fat and blood were determined.

There were no treatment-related mortalities during the study. In the 500 ppm group, single episodes of hyper-reactivity to handling, fasciculations and tonic-clonic convulsions were observed in one animal each between weeks 13 and 18. There were no other compound-related clinical signs in the treated animals. Mean body weight and feed consumption were comparable for all groups over the whole study period. No compound-related effects were noted in any group throughout the study period in any of the neurology parameters measured. Motor activity parameters were not affected in any group, and mean startle habituation reactions were comparable for all groups throughout the study. The maze learning error scores did not differ between controls and treated groups at the end of the treatment period or at the end of the recovery period.

Following intraperitoneal injection with 25 mg/kg bw of pentylenetetrazol at the end of the treatment period, generalized tonic-clonic convulsions involving the hindlimbs were induced in top-dose animals only. Comparable motor seizures limited to the head, neck and forelimb areas were induced in all other groups. After a generalized tonic-clonic convulsion, subsequent seizure activity was inhibited, whereas motor seizures were shown frequently throughout the 30-minute observation period. At the end of the recovery period, the incidence rate for generalized convulsions in top-dose animals was comparable with that seen at the end of the treatment period. Median convulsion scores in the top-dose animals were lower than at the end of the treatment period, but still higher than in control animals, thus indicating partial recovery from the proconvulsive effect induced by the test article.

At the end of the treatment period, the concentrations of lufenuron in fatty tissue were 16, 150, 660 and 2600 mg/kg tissue for the 5, 25, 100 and 500 ppm groups, respectively. Corresponding concentrations in whole blood were 0.1, 0.6, 2.6 and 17 mg/L, indicating a preferential accumulation in fat. In top-dose animals, concentrations of 1600 mg/kg in fat and 4.3 mg/L in blood were measured at the end of the 2-month recovery period.

No treatment-related macroscopic or microscopic changes were seen in the central or peripheral nervous system or in muscles.

The NOAEL for systemic toxicity was 500 ppm (equal to 27.0 mg/kg bw per day), the highest dose tested. The NOAEL for neurotoxicity was 100 ppm (equal to 5.43 mg/kg bw per day), based on spontaneous tonic-clonic convulsions or fasciculations observed in weeks 13–18 and facilitated pentylenetetrazol-induced generalized convulsions at 500 ppm (equal to 27.0 mg/kg bw per day), (Classen, 1992).

(b) Mechanistic studies

A study was performed to determine the effects of treatment of lufenuron in the diet for 3 weeks on the estrous cycle in female rats and various plasma hormone levels in male and female rats. Forty-five rats (Crj: Sprague-Dawley (SD)) of each sex were randomly allocated to three groups: 0, 500 or 1500 ppm (equal to 0, 30.5 and 92.5 mg/kg bw per day for males and 0, 39.4 and 120.1 mg/kg bw per day for females, respectively). All animals were checked twice a day for mortality and clinical signs. Body weights were recorded at the start of the study and weekly thereafter. Two-day feed consumption per cage was measured weekly, and mean daily feed consumption values per rat were calculated. Vaginal smears were taken each morning from 10 females per group. Proestrous, estrous, metestrous and diestrous stages were determined, and vaginal cytology was examined. Each smear was scored for the relative density of cornified and nucleated cells. At necropsy, 10 mL blood samples were collected under ether anaesthesia from the abdominal aorta of 10 animals per group for analysis of plasma hormone levels (estradiol, progesterone, corticosterone, aldosterone, prolactin, luteinizing hormone [LH], follicle stimulating hormone [FSH], adrenocorticotropic hormone [ACTH] and testosterone). The left ovaries, adrenals and testes from 10 animals per group were taken for the

analysis of hormone levels in tissue. Gross pathology of these animals consisted of macroscopic examination of the organs and tissues in the thoracic and abdominal cavities and removal of uterus, ovaries, vagina, testes, adrenals and pituitary for weighing and/or processing for microscopic examination. After 3 weeks of administration of test diet, cholinesterase activity in plasma erythrocyte and brain samples from five rats per group was determined.

No deaths or changes of general condition related to lufenuron administration were observed. With the exception of body weight gain retardation in the 1500 ppm females at week 3 (96% of controls), there were no differences in mean body weight of either sex between the groups given lufenuron and the control groups. There was no difference between the feed consumption of test and control animals of both sexes.

No statistically significant differences in mean estrous cycle length were found between the treated groups and controls, although there were two cases in the 500 ppm group of 7 and 10 days' length, respectively, and one case in the 1500 ppm group of 4.6 days' length, demonstrating prolongation. There was no statistically significant difference in the relative density of cornified and nucleated epithelial cells between the treated and control animals. Statistically significant elevation of mean prolactin, FSH and ACTH levels was noted for the 1500 ppm male group. For prolactin, 8/10 animals in the 1500 ppm group had plasma levels that were within the control range; therefore, the higher mean value for this group was not considered to be biologically significant. The slightly higher FSH levels in the 1500 ppm group were also not considered large enough to be biologically significant. The increase in ACTH levels is relatively small and may indicate a stress response in these animals that is unrelated to treatment. No other statistically significant differences in hormone levels were found between any of the groups given lufenuron and controls (Table 21).

Table 21. Hormone level data: selected parameters in a 3-week mechanistic study in rats

	Hormone levels					
	Males			**Females**		
Parameter	**0 ppm**	**500 ppm**	**1 500 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**
Prolactin (ng/mL)	20.200	22.635	33.420*	85.610	< 80.460	95.870
FSH (MIU/mL)	19.4	21.64	22.47**	18.26	16.72	21.50
ACTH (pg/mL)	41.35	50.95	67.88**	115.22	130.57	118.29
Corticosterone (ng/mL)	295.30	325.15	336.46	950.10	977.20	953.30

ACTH: adrenocorticotropic hormone; FSH: follicle stimulating hormone; ppm: parts per million; IU: international units; *: $P < 0.05$; **: $P < 0.01$
Source: Tamano (1997)

No gross pathological abnormalities were observed in any of the groups, other than inner dilatation of the uterus in one rat in the 1500 ppm group. Significantly lower relative uterus weights were noted in the 500 ppm group compared with controls. This was unlikely to be treatment related, as the effect was slight, and there was no dose dependence. There were no statistically significant differences between test and control animals in the weights of the ovaries, testes, adrenals or pituitary.

Angiectasis in the adrenals was observed in one to three female rats in each group and one male rat fed 1500 ppm lufenuron. Fatty metamorphosis in the adrenals was also noted in one or two male rats in each group. Inner dilatation of the uterus was observed in one rat in each of the control and 1500 ppm groups. No abnormal histopathological findings were noted for the pituitary, ovaries, vagina or testes in any of the groups.

No statistically significant differences in any of the cholinesterase activity samples were noted between the groups of rats given lufenuron and the controls.

The results of this investigation, which focused on the pituitary, adrenal and genital organs, suggest that there is no effect of lufenuron on the endocrine system in rats of either sex. This conclusion is supported by the reproductive toxicity study in rats, which showed no effect of lufenuron on any reproductive end-point (Tamano, 1997).

3. Observations in humans

The sponsor submitted the following information:

Manufacturing employees in Switzerland are medically examined by a company physician at the beginning of their employment and then routinely once a year according to the criteria of the Swiss Accident Insurance Institution (SUVA). Routine medical examinations include:

- Anamnesis
- Physical examination including blood pressure
- Blood analysis: hemoglobin, erythrocytes, leukocytes, thrombocytes, complete blood count, blood sedimentation rate, blood sugar, blood pressure, cholesterol, triglycerides, ALAT, ASAT, alkaline phosphatase, bilirubin, creatinine, uric acid
- Urine analysis

The Occupational Health group of Syngenta (formerly ICI/Zeneca Agrochemical) has maintained a database of incidents involving chemical exposure of workers since 1983. Since 1994, data has been collected on the occurrence of occupationally related illnesses from all our manufacturing, formulation and packaging sites around the world. Analysis of the Syngenta internal database confirmed that no cases of adverse reactions that related to the handling of lufenuron have been reported up to July 2015.

In conclusion, lufenuron has been handled in large quantities over the last 22 years at a number of sites. With the use of appropriate control strategies, no adverse health effects associated with the material have been reported in the workforce.

In companion animals lufenuron is used for flea control. From 1995-1997, 57 incidents of human exposure involving accidental ingestion and/or therapeutic mishaps ("<1/4 tablet" to 409mg (1.0–26.4mg/kg)) were reported to 2 poison centres in the US. At the time of the call a 15 month old male vomited within 2 hours, without further symptoms and a 13 year old female experienced diarrhoea that resolved within 8 hours. The other patients remained asymptomatic for up to 2 hours post ingestion. Lufenuron has shown no significant toxicity in humans from the limited amounts ingested to date. (Ref: J. of Toxicol Clin Toxicol, Vol.36, No.5, pp. 491)

A breast feeding mother accidentally ingested an anti-flea preparation of lufenuron. The infant was exposed to an average dose of 0.032 mg/kg bw per day, which is only 3% of the reported acute overdose. No adverse effects were reported during 7 months of follow-up. (Ref: Bar-Oz (2000), J. of Human Lactation, 16 (3), 229-30)

Comments

Biochemical aspects

Lufenuron is only partially absorbed following a single oral dose, with the extent of absorption being dose related; approximately 20% of a single 100 mg/kg bw dose appears to be absorbed, compared with about 70% of a single 0.1 or 0.5 mg/kg bw dose. A large proportion of the absorbed dose partitions into fat, with very much lower uptake by other tissues, including the brain. All tissue concentrations of radioactivity increased to a maximum 1 day after the last of 14 repeated low doses. The results suggest that most tissue concentrations would plateau within 2–3 weeks of similar repeated dosing. The fat depot is slowly released, with a terminal half-life of up to 5–13 days at 0.5 mg/kg bw and 10–37 days at 100 mg/kg bw, leading to an increase in concentrations of lufenuron in the brain over long periods (see below). Excretion of the absorbed dose is predominantly via faeces, with only about 1% of the dose being excreted in urine, independent of the dose.

Metabolism of lufenuron is minimal, with only about 1% of an oral dose being metabolized by deacylation followed by cleavage of the ureido group. There is no marked sex difference in absorption, tissue distribution, metabolism or excretion. The pattern of excretion and metabolism is not affected by repeated dosing (Bissig, 1990; Thanei, 1990; Müller, 1995; Okada, 1997; Hassler, 2003; Booth, 2004).

Toxicological data

Lufenuron has low acute toxicity when administered orally or dermally (LD_{50} > 2000 mg/kg bw) or via inhalation (LC_{50} > 2.35 mg/L, maximal attainable concentration) to rats (Hartmann, 1988a,b,c, 1989). Lufenuron produced very slight skin and eye irritation in rabbits and is considered to be a skin sensitizer in the guinea-pig (Schneider, 1988a,b,c).

The most significant toxicological end-point for lufenuron is convulsions, observed after prolonged treatment at high dose levels. Convulsions are observable in all species treated with lufenuron at daily doses of more than 20 mg/kg bw for extended periods (2–3 months in rodents, > 3 months in dogs). As lufenuron is a very lipophilic compound (log octanol–water partition coefficient [K_{ow}] = 5.12), it has the potential to accumulate in fatty tissues. It has been shown in toxicity studies with rats, mice and dogs that following prolonged exposure to high doses of 20 mg/kg bw per day or more, fat compartments may become saturated. If exposure is continued after saturation occurs, concentrations in the brain increase, leading to tonic-clonic convulsions.

In a dose range–finding study, lufenuron was fed to mice at a concentration of 0, 1000, 3000 or 9000 ppm (equal to 0, 151, 449 and 1470 mg/kg bw per day for males and 0, 189, 517 and 1440 mg/kg bw per day for females, respectively) for up to 65 days. As a result of mortality and neurotoxic effects of the test substance (tonic-clonic seizures) at all dose levels, it was concluded that the MTD was exceeded even at 1000 ppm (equal to 151 mg/kg bw per day) (Fankhauser, 1989a).

In a second dose range–finding study, intended for test substance residue and blood level determination, lufenuron was fed to female mice at a concentration of 0, 4/8, 20, 100 or 1000 ppm (equal to 0, 0.47/1.1, 2.94, 14.5 and 143 mg/kg bw per day, respectively) for up to 91 days (only 71 days for the high dose). From day 57 onwards, the diet of the low-dose group inadvertently contained 8 ppm instead of 4 ppm. The NOAEL was 100 ppm (equal to 14.5 mg/kg bw per day), based on mortality and neurotoxicity (tonic-clonic seizures) at 1000 ppm (equal to 143 mg/kg bw per day) (Fankhauser, 1990).

In a 28-day range-finding study, rats were administered lufenuron in the diet at a concentration of 0, 50, 400, 3000 or 20 000 ppm (equal to 0, 4.10, 30.8, 254 and 1692 mg/kg bw per day for males and 0, 4.07, 32.6, 254 and 1741 mg/kg bw per day for females, respectively). The NOAEL was 400 ppm (equal to 30.8 mg/kg bw per day), based on decreased thymus weight at 3000 ppm (equal to 254 mg/kg bw per day) (Fankhauser, 1988).

In a 90-day toxicity study, rats were fed diets containing 0, 25, 150, 1500 or 15 000 ppm lufenuron (equal to 0, 1.6, 9.68, 101 and 998 mg/kg bw per day for males and 0, 1.7, 10.2, 103 and 1050 mg/kg bw per day for females, respectively). The NOAEL was 150 ppm (equal to 9.68 mg/kg bw per day), based on clinical signs (tonic-clonic seizures), decreased body weight gain and feed consumption, slight changes in haematology and clinical chemistry parameters and increased adrenal weights at 1500 ppm (equal to 101 mg/kg bw per day) (Fankhauser, 1989b).

In a 4-week range-finding study, dogs received lufenuron in their diet at a concentration of 200 or 50 000 ppm (equal to 8.43 and 2200 mg/kg bw per day for males and 10.1 and 2648 mg/kg bw per day for females, respectively). The NOAEL was 50 000 ppm (equal to 2200 mg/kg bw per day), the highest dose tested (Briffaux, 1989a).

In a 90-day toxicity study, dogs received lufenuron in their diet at a concentration of 0, 200, 3000 or 50 000 ppm (equal to 0, 7.8, 121.6 and 2023 mg/kg bw per day for males and 0, 7.9, 122.5 and 1933 mg/kg bw per day for females, respectively). The NOAEL was 200 ppm (equal to 7.8 mg/kg bw per day), based on increases in blood cholesterol levels and absolute and relative liver weights,

reductions in blood potassium and phosphorus levels, and an increase in serum alkaline phosphatase activity for some animals at 3000 ppm (equal to 121.6 mg/kg bw per day) (Briffaux, 1989b).

In a 1-year toxicity study, dogs received lufenuron in their diet at a concentration of 0, 100, 2000 or 50 000 ppm (equal to 0, 3.97, 65.4 and 1879 mg/kg bw per day for males and 0, 3.64, 78.3 and 1977 mg/kg bw per day for females, respectively). The main target organs were the brain, adrenals, liver, thyroid and lungs. The NOAEL was 100 ppm (equal to 3.64 mg/kg bw per day), based on mortality, neuromuscular signs, including convulsions, reduced body weight gains, changes in clinical pathology parameters and histopathological lesions in adrenals, liver, thyroid and lungs observed at 2000 ppm (equal to 65.4 mg/kg bw per day) (Briffaux, 1992).

In another 1-year toxicity study, dogs received lufenuron in their diet at a concentration of 0, 10, 50, 250 or 1000 ppm (equal to 0, 0.31, 1.42, 7.02 and 29.8 mg/kg bw per day for males and 0, 0.33, 1.55, 7.72 and 31.8 mg/kg bw per day for females, respectively). The NOAEL was 250 ppm (equal to 7.02 mg/kg bw per day), based on treatment-related mortality and clinical findings, including convulsions, effects on body weight and effects on the liver and adrenals, with associated histopathology and/or clinical chemistry changes, at 1000 ppm (equal to 29.8 mg/kg bw per day) (Altmann, 1995).

An overall NOAEL of 250 ppm (equal to 7.02 mg/kg bw per day) can be identified on the basis of the two 1-year dog studies. The 90-day dog study should not be included in the overall NOAEL, as the observed effects (blood parameters and liver weights) are far less severe than the effects in the 1-year dog studies (e.g. mortality) at similar dose levels. This can be explained by the fat accumulation, which is not yet saturated in the 90-day study; this leads to higher concentrations of the parent compound in the brain in the longer-term studies.

In an 18-month dietary toxicity and carcinogenicity study, mice received lufenuron at a concentration of 0, 2, 20, 200 or 400 ppm (equal to 0, 0.222, 2.25, 22.6 and 62.9 mg/kg bw per day for males and 0, 0.217, 2.12, 22.0 and 61.2 mg/kg bw per day for females, respectively). As a result of high mortality in the high-dose group, surviving animals in this dose group were terminated in weeks 9 and 10. The NOAEL was 20 ppm (equal to 2.12 mg/kg bw per day), based on increased mortality, clinical signs (tonic-clonic convulsive episodes), increased incidences of fatty liver (in females accompanied by necrotic changes) and a higher incidence of inflammatory changes in the prostate at 200 ppm (equal to 22.0 mg/kg bw per day). No treatment-related tumours were observed (Bachmann, 1993a).

In a 2-year dietary toxicity and carcinogenicity study, rats received lufenuron at a concentration of 0, 5, 50, 500 or 1500 ppm (equal to 0, 0.19, 1.93, 20.4 and 108 mg/kg bw per day for males and 0, 0.23, 2.34, 24.8 and 114 mg/kg bw per day for females, respectively). As a result of overt toxicity at 1500 ppm, all animals in this group were terminated in week 14. The NOAEL was 50 ppm (equal to 1.93 mg/kg bw per day), based on clinical signs (tonic-clonic convulsions), decreased body weight and (histo)pathological effects on lungs, liver, non-glandular stomach, intestines and urinary tract at 500 ppm (equal to 20.4 mg/kg bw per day). No treatment-related tumours were observed (Bachmann, 1993b).

The Meeting concluded that lufenuron is not carcinogenic in mice or rats.

Lufenuron 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 lufenuron 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 lufenuron is unlikely to pose a carcinogenic risk to humans.

In a two-generation study on reproductive toxicity, rats received lufenuron in their diet at a concentration of 0, 5, 25, 100 or 250 ppm (equal to 0, 0.41, 2.1, 8.3 and 20.9 mg/kg bw per day for males and 0, 0.44, 2.2, 8.9 and 22.2 mg/kg bw per day for females, respectively, based on mean intakes for combined P and F_1 generations during the premating period). The NOAEL for parental and reproductive effects was 250 ppm (equal to 20.9 mg/kg bw per day), the highest dose tested. The

NOAEL for offspring toxicity was 100 ppm (equal to 8.3 mg/kg bw per day), based on the slight delay in righting reflex in pups at 250 ppm (equal to 20.9 mg/kg bw per day) (Fitzgerald & Khalil, 1992).

In a study of developmental toxicity, rats were administered lufenuron via gavage at a dose of 0, 100, 500 or 1000 mg/kg bw per day. The NOAEL for maternal toxicity was 500 mg/kg bw per day, based on a transient reduction in body weight gain on GDs 7–9 and feed consumption on GDs 6–9 at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Gilles, 1989).

In a study of developmental toxicity, rabbits were dosed at 0, 100, 500 or 1000 mg/kg bw per day with lufenuron via gavage. The NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Meyer, 1989b).

The Meeting concluded that lufenuron is not teratogenic.

In a repeated-dose neurotoxicity study, male rats received lufenuron in their diet at 0, 5, 25, 100 or 500 ppm (equal to 0, 0.26, 1.22, 5.43 and 27.0 mg/kg bw per day, respectively) for 4 months. No systemic toxicity was observed. The NOAEL for neurotoxicity was 100 ppm (equal to 5.43 mg/kg bw per day), based on spontaneous tonic-clonic convulsions or fasciculations observed in weeks 13–18 and facilitated pentylenetetrazol-induced generalized convulsions at 500 ppm (equal to 27.0 mg/kg bw per day) (Classen, 1992).

Convulsions are observed in all species after prolonged treatment with lufenuron, owing to saturation of the accumulation in fatty tissues, with subsequent increased lufenuron levels in the brain. The neurotoxic effects are not expected to occur after a single dose. The Meeting concluded that lufenuron is not acutely neurotoxic, but is neurotoxic after prolonged treatment.

A study was performed to determine the effects of treatment of lufenuron for 3 weeks on the estrous cycle in female rats and various plasma hormone levels (estradiol, progesterone, corticosterone, aldosterone, prolactin, LH, FSH, ACTH and testosterone) in male and female rats administered a dietary concentration of 0, 500 or 1500 ppm (equal to 0, 30.5 and 92.5 mg/kg bw per day for males and 0, 39.4 and 120.1 mg/kg bw per day for females, respectively). The results of this investigation, focused on the pituitary, adrenal and genital organs, suggest that there is no effect of lufenuron on the endocrine system in rats of either sex (Tamano, 1997). This conclusion is supported by the reproductive toxicity study in rats, which showed no effect of lufenuron on any reproductive end-point (Fitzgerald & Khalil, 1992).

No specific studies on immunotoxicity were submitted. The available repeated-dose studies do not indicate an immunotoxic potential for lufenuron following exposure by the oral route.

Toxicological data on metabolites and/or degradates

No metabolites of concern were identified.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. Several incident reports indicate no significant toxicity in humans.

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

Toxicological evaluation

An acceptable daily intake (ADI) of 0–0.02 mg/kg bw was established on the basis of the NOAEL of 1.93 mg/kg bw per day for tonic-clonic seizures and findings in lungs, gastrointestinal tract, liver and urinary tract in the 2-year dietary study in rats, using a safety factor of 100.

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

Levels relevant to risk assessment of lufenuron

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equal to 2.12 mg/kg bw per day	200 ppm, equal to 22.0 mg/kg bw per day
		Carcinogenicity	400 ppm, equal to 61.2 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 1.93 mg/kg bw per day	500 ppm, equal to 20.4 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 108 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	250 ppm, equal to 20.9 mg/kg bw per day[b]	–
		Parental toxicity	250 ppm, equal to 20.9 mg/kg bw per day[b]	–
		Offspring toxicity	100 ppm, equal to 8.3 mg/kg bw per day	250 ppm, equal to 20.9 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	500 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
	Four-month neurotoxicity study[a]	Neurotoxicity	100 ppm, equal to 5.43 mg/kg bw per day	500 ppm, equal to 27.0 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Dog	One-year studies of toxicity[a,d]	Toxicity	250 ppm, equal to 7.02 mg/kg bw per day	1 000 ppm, equal to 29.8 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)

0–0.02 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 lufenuron

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	~70% within 24 h at 0.1 or 0.5 mg/kg bw; ~20% within 24 h at 100 mg/kg bw
Dermal absorption	No data
Distribution	Widely distributed; highest concentrations in fat; a plateau within 2–3 weeks of dosing is suggested for all tissues. Brain levels are initially low, but rise after prolonged exposure due to saturation of fat storage.
Potential for accumulation	High fat accumulation, with slow release (terminal half-life 5–13 days at 0.5 mg/kg bw and 10–37 days at 100 mg/kg bw)
Rate and extent of excretion	Predominantly in faeces, with < 1% in urine, independent of dose
Metabolism in animals	Minimal
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 2.35 mg/L, maximal attainable concentration
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Sensitizing (Magnusson and Kligman maximization test)
Short-term studies of toxicity	
Target/critical effect	Mortality and neurotoxicity (tonic-clonic convulsions)
Lowest relevant oral NOAEL	7.02 mg/kg bw per day (1 year; dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (28 d; highest dose tested; rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Neurotoxicity (tonic-clonic convulsions), (histo)pathological findings in lungs, liver, non-glandular stomach, intestines and urinary tract
Lowest relevant NOAEL	1.93 mg/kg bw per day (2 years; rat)

Carcinogenicity	Not carcinogenic in mice or rats[a]
Genotoxicity	
	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Slight delay in righting reflex in pups
Lowest relevant parental NOAEL	20.9 mg/kg bw per day (highest dose tested; rat)
Lowest relevant offspring NOAEL	8.3 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	20.9 mg/kg bw per day (highest dose tested; rat)
Developmental toxicity	
Target/critical effect	Transient reduction in maternal body weight gain and feed consumption (rat)
Lowest relevant maternal NOAEL	500 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	1 000 mg/kg bw per day (rat, rabbit; highest dose tested)
Neurotoxicity	
Acute neurotoxicity NOAEL	No evidence of acute neurotoxicity
Subchronic neurotoxicity NOAEL	5.43 mg/kg bw per day (4 months; rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	No metabolites of concern were identified
Medical data	
	No evidence of adverse effects in personnel exposed to lufenuron; several incident reports indicate no significant toxicity in humans

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

Summary

	Value	**Study**	**Safety factor**
ADI	0–0.02 mg/kg bw	Two-year toxicity and carcinogenicity study (rat)	100
ARfD	Unnecessary	–	–

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Appendix 1. Historical control data for 2-year rat study

Table A-1. Historical control data for benign interstitial cell tumour of testis in the 2-year rat study

Laboratory database				RITA database for SD rats, 1985–1995, 2-year oral studies			
Study number	**Date of first dose**	**Incidence of benign interstitial cell tumours**	**Number of testes examined**	**Study number**	**Date of first dose**	**Incidence of benign interstitial cell tumours**	**Number of testes examined**
785271	Oct 78	2	90	1	Mar 85	4	68
801440	Mar 81	6	80	40	Sep 88	2	50
800218	Jun 82	1	58	45	Jul 89	2	55
820872	Aug 82	1	79	46	Jul 86	2	49
820650	Apr 83	2	80	47	Feb 89	4	71
82648	Oct 83	0	79	50	Sep 89	1	55
821482	Oct 83	2	79	52	Jul 86	1	50
820559	Feb 84	1	80	58	Nov 87	0	50
821638	Apr 84	3	70	62	Aug 87	6	60
830732	Nov 84	2	79	70	Jul 86	2	48
840171	Mar 85	4	78	96	Nov 93	4	60
850335	Feb 86	6	80	101	Oct 92	0	55
850853	May 86	1	80	102	Oct 92	1	55
874076	May 87	1	50	106	Nov 93	1	60
860087	Sep 87	3	80	111	Jul 95	5	59
850703	Jan 88	1	79	140	Oct 94	1	60
876047	May 88	2	70	141	Oct 94	2	60
861139	Feb 89	4	80				
861154	Mar 89	0	80				
886178	Aug 89	2	70				

Laboratory database				RITA database for SD rats, 1985–1995, 2-year oral studies			
Study number	**Date of first dose**	**Incidence of benign interstitial cell tumours**	**Number of testes examined**	**Study number**	**Date of first dose**	**Incidence of benign interstitial cell tumours**	**Number of testes examined**
901463	Dec 90	4	80				
891326	Feb 91	1	60				
901483	May 92	2	60				
911123	Aug 92	0	60				

RITA: Registry of Industrial Toxicology Animal-data; SD: Sprague-Dawley

Table A-2. Historical control data (laboratory database) for benign granular cell tumour of the cerebral meninges in the 2-year rat study

Study number	Date of first dose	Incidence of benign granular cell tumours in the meninges	Number of organs examined
785271	Oct 78	0	88
800218	Jun 82	0	60
801440	Mar 81	0	79
820559	Feb 84	0	80
820648	Oct 83	1	78
820650	Apr 83	0	80
820872	Aug 82	0	80
821482	Oct 83	1	79
821638	Apr 84	1	68
830732	Nov 84	1	77
840171	Mar 85	1	78
850703	Jan 88	3	80
860087	Sep 87	3	80
861139	Feb 89	4	80
861154	Mar 89	1	80
874076	May 87	2	50
876047	May 88	2	70
886178	Aug 89	0	70

PENCONAZOLE

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Explanation

Penconazole is the International Organization for Standardization (ISO)–approved common name for 1-(2,4-dichloro-β-propylphenethyl)-1*H*-1,2,4-triazole (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 66246-88-6. Penconazole is a systemic triazole fungicide with preventive and curative properties for the control of powdery mildew. It stops the development of fungi by interfering with the biosynthesis of sterols in cell membranes and is used on grapes, pome and stone fruit, cucurbits and strawberries.

Penconazole was previously evaluated for toxicology by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1992, when the Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) on the basis of a no-observed-adverse-effect level (NOAEL) of 3 mg/kg bw per day in a 1-year study in dogs.

In 2008, a group of manufacturers of triazole fungicides formed a task force known as the "Triazole Derivative Metabolite Group" and made a joint submission of toxicological data on the common metabolites 1,2,4-triazole, triazole acetic acid and triazole alanine to JMPR. Triazole alanine and triazole acetic acid residues are primarily associated with plant commodities, whereas 1,2,4-

triazole is mainly associated with animal commodities, lesser amounts of this compound being found in plant commodities.

In 2008, the Meeting established an ADI of 0–0.2 mg/kg bw for 1,2,4-triazole, based on a NOAEL of 16 mg/kg bw per day in a two-generation reproductive toxicity study in rats. The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw for 1,2,4-triazole, based on a NOAEL of 30 mg/kg bw per day in a developmental toxicity study in rabbits.

In 2008, the Meeting established a group ADI of 0–1.0 mg/kg bw for triazole alanine and triazole acetic acid (alone or in combination), based on a NOAEL of 100 mg/kg bw per day in a developmental toxicity study in rats administered triazole alanine. The 2008 Meeting concluded that it was unnecessary to establish an ARfD for triazole alanine and triazole acetic acid.

Penconazole was re-evaluated by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues. Both the new data and previously submitted studies with penconazole were considered by the present Meeting. New data on the common rat and plant metabolites 1,2,4-triazole, triazole acetic acid and triazole alanine were considered by the present Meeting to evaluate whether a revision of the ADIs or ARfDs for these compounds was necessary.

All critical studies contained statements of compliance with good laboratory practice (GLP). The Meeting considered that the database was adequate for the risk assessment.

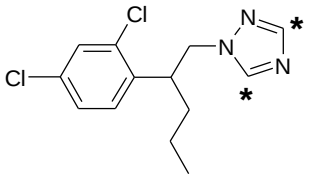
Evaluation for acceptable intake

1. Biochemical aspects

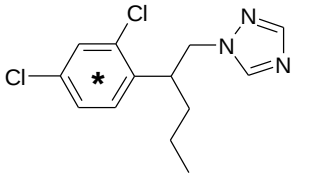
Two ^{14}C-radiolabelled penconazole molecules were used for the toxicokinetic studies (Fig. 1).

Fig. 1. Structure of penconazole with positions of radiolabel

[3,5-^{14}C-triazole]penconazole



[U-^{14}C-phenyl]penconazole



1.1 Absorption, distribution and excretion

Mice

Groups of five male and five female CD-1 (ICR) mice received technical-grade penconazole (purity 98.7%; batch no. FL-840833) in the diet at a dose level of 0, 10, 100, 300, 500, 1000 or 2400 parts per million (ppm) for at least 90 days before a single oral or intravenous dose of 25 µg [^{14}C]penconazole (radiochemical purity > 98%; batch no. GAN-IX-83) in polyethylene glycol (PEG) 200/deionized water (1:1) as dose vehicle. Urine was collected at daily intervals over 48 hours after the ^{14}C dose, and a single 0- to 48-hour collection of faeces was made. Radioactivity in urine and cage wash was determined by direct liquid scintillation counting. Aqueous homogenates of faeces were prepared for scintillation counting by sample oxidation.

The excretion of radioactivity in urine and faeces and recoveries in terminal cage washes following an intravenous or oral dose are presented in Table 1 (intravenous dose) and Table 2 (oral

Table 1. Excretion of radioactivity by mice administered a single intravenous dose of 25 µg [^{14}C]penconazole following pretreatment with dietary doses of unlabelled penconazole for at least 90 days

	% of administered radioactivity					
	10 ppm	**100 ppm**	**300 ppm**	**500 ppm**	**1 000 ppm**	**2 400 ppm**
Males						
Urine						
0–24 h	52.40	42.25	50.95	40.76	50.39	61.28
24–48 h	4.15	5.00	5.46	7.37	4.82	4.49
0–48 h	56.55	47.25	56.41	48.12	55.21	65.78
Faeces						
0–48 h	21.75	31.31	25.35	19.96	25.34	25.69
Total excreted						
0–48 h	78.30	78.56	81.76	68.08	80.55	91.47
Cage wash, 48 h	3.09	5.29	4.34	3.69	4.18	2.07
Total dose recovered, 0–48 h	81.39	83.85	86.10	71.77	84.73	93.54
Females						
Urine						
0–24 h	66.49	64.77	74.55	70.71	69.26	62.84
24–48 h	3.55	2.72	2.32	3.39	2.73	4.42
0–48 h	70.04	67.49	76.88	74.10	71.99	67.26
Faeces						
0–48 h	12.50	11.26	8.65	10.56	10.54	13.90
Total excreted						
0–48 h	82.54	78.75	85.53	84.66	82.53	81.16
Cage wash, 48 h	1.88	2.86	2.65	2.32	3.37	2.61
Total dose recovered, 0–48 h	84.42	81.61	88.18	86.98	85.90	83.77

ppm: parts per million
Source: Hiles (1987a)

dose). For both sexes, there was no marked difference in excretion profiles either across the range of dietary predosing levels or between the oral and intravenous routes of administration of the single radiolabelled dose. Both male and female mice readily absorbed an oral dose of 25 µg [^{14}C]penconazole. There was a difference between the sexes, with females excreting a higher proportion of the dose in urine and less via faeces, compared with males. No change in the distribution of radioactivity was observed between the urine and faecal compartments, from an oral or intravenous dose of [^{14}C]penconazole, following pretreatment with increasing dietary dose levels of technical-grade penconazole from 10 to 2400 ppm (Hiles, 1987a).

Table 2. Excretion of radioactivity by mice administered a single oral dose of 25 µg [^{14}C]penconazole following pretreatment with dietary doses of unlabelled penconazole for at least 90 days

	% of administered radioactivity					
	10 ppm	**100 ppm**	**300 ppm**	**500 ppm**	**1 000 ppm**	**2 400 ppm**
Males						
Urine						
0–24 h	53.07	56.59	54.02	42.74	47.26	54.96
24–48 h	5.75	4.27	7.98	4.05	7.10	6.23
0–48 h	58.82	60.86	62.01	46.79	54.35	61.18
Faeces						
0–48 h	27.73	21.67	18.60	25.29	27.19	23.48
Total excreted						
0–48 h	86.55	82.53	80.61	72.08	81.54	84.66
Cage wash, 48 h	1.83	1.52	1.67	5.58	2.56	2.49
Total dose recovered, 0–48 h	88.38	84.05	82.28	77.66	84.10	87.15
Females						
Urine						
0–24 h	69.73	75.15	71.77	73.24	58.63	53.44
24–48 h	3.24	2.75	3.43	3.23	4.15	12.35
0–48 h	72.97	77.90	75.21	76.48	62.78	65.79
Faeces						
0–48 h	13.07	12.34	13.82	11.46	15.04	16.52
Total excreted						
0–48 h	86.04	90.24	89.03	87.94	77.82	82.31
Cage wash, 48 h	2.33	3.05	4.62	2.17	6.94	4.46
Total dose recovered, 0–48 h	88.37	93.29	93.65	90.11	84.76	86.77

ppm: parts per million
Source: Hiles (1987a)

Rats

The absorption, distribution and excretion of [3,5-^{14}C-triazole]penconazole (radiochemical purity > 98%; batch number not reported), dissolved in ethanol/PEG 200/water (2:3:5 by volume), were studied in groups of two male and two female Tif: RAI f (SPF) rats (a Sprague-Dawley-derived strain) dosed orally by gavage at a single dose of 0.5 or 25 mg/kg bw. Urine, faeces and expired air were collected at 24-hour intervals. After 6 days, the rats were killed, and brain, fat, heart, kidneys, liver, lungs, muscle, ovaries, plasma, spleen, testes, whole blood and residual carcass were sampled and analysed for radioactivity. All excreta and tissue samples were analysed for radioactivity by liquid scintillation counting either directly or following sample oxidation. Urinary metabolite profiles were investigated by thin-layer chromatography (TLC). These data are presented in section 1.2 (Hamböck, 1980, 1985).

Table 3. Group mean excretion data following single oral administration of [3,5-^{14}C-triazole]penconazole to rats

	% of administered dose			
	0.5 mg/kg bw		25 mg/kg bw	
	Males	**Females**	**Males**	**Females**
Urine				
0–24 h	49.86	68.20	47.72	81.05
24–48 h	10.00	3.80	7.74	2.86
48–72 h	3.32	0.74	4.07	0.62
72–144 h	2.43	0.39	2.58	0.27
Subtotal	62.26	73.12	62.10	84.79
Faeces				
0–24 h	17.19	26.38	25.94	10.13
24–48 h	13.17	3.91	6.81	3.08
48–72 h	4.42	0.87	3.93	0.50
72–144 h	2.65	0.37	1.84	0.32
Subtotal	37.32	31.53	38.51	14.20
Expired air				
0–24 h	0.10	0.10	0.04	0.03
24–48 h	0.02	0.02	0.02	0.01
48–144 h	0.02	0.01	0.02	0.00
Subtotal	0.14	0.12	0.08	0.04
Total excretion	99.72	104.77	100.69	99.03
Excretion within 0–24 h	67.05	94.58	73.66	90.68
Tissues	0.09	0.05	0.08	0.04
Cage wash	0.53	0.61	0.63	0.35
Total recovery	100.34	105.41	101.39	99.41

bw: body weight
Source: Hamböck (1980, 1985)

The excretion of radioactivity, expressed as a percentage of the administered dose, is presented in Table 3.

Following a single oral dose of [3,5-^{14}C-triazole]penconazole, absorption was extensive, and the majority of the absorbed dose was excreted rapidly in urine. At both doses, excretion in urine was higher in females, in particular at the high dose. Also, excretion in urine and faeces occurred more rapidly in females than in males. Excretion in expired air was negligible, and excretion was virtually complete by 6 days after dosing. At 6 days after dosing with 0.5 or 25 mg/kg bw, total tissue levels accounted for less than 0.1% of the administered dose. Highest concentrations were found in liver, lungs and kidneys (Hamböck, 1980, 1985).

The blood kinetics and tissue distribution of radioactivity were investigated in Tif: RAI f (SPF) rats administered a single oral dose of [U-^{14}C-phenyl]penconazole, dissolved in a mixture of PEG 200/ethanol/water (5:3:2 by volume) at 50 mg/kg bw. In a group of three males and three

females, blood was collected at 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours after dosing. In a second group of rats, one male and one female each were subjected to whole-body radioluminography at 4, 6, 8, 12, 24 and 48 hours after dosing.

The peak blood concentration (8 mg/kg in males and 7 mg/kg in females) was attained 4 and 6 hours after dosing in males and females, respectively. The blood concentration declined to half the maximum value within 13 and 3 hours in males and females, respectively. Radioluminography indicated that most tissue concentrations reached a maximum after 6 hours in males and after 4 hours in females. The highest concentration was found in the penis (probably related to contamination with urinary radioactivity), followed by organs associated with metabolism and excretion (i.e. liver and kidney). Maximum tissue levels and depletion half-lives in tissues of males and females are presented in Table 4.

Table 4. Maximum tissue concentrations and depletion half-lives in male and female rats administered a single oral [^{14}C]penconazole dose of 50 mg/kg bw

	Males		Females	
Tissue	**Concentration (ppm equiv)**	**$t_{½}$ (h)**	**Concentration (ppm equiv)**	**$t_{½}$ (h)**
Adrenal gland	38.3	8	41.3	6
Blood	12.9	11	5.5	4
Bone	4.9	9	2.2	4
Bone marrow	10.0	9	6.1	3
Brain	9.7	8	7.2	3
Fat (abdominal)	27.1	7	24.8	5
Heart	14.7	9	10.5	4
Kidney cortex	53.1	12	28.6	7
Kidney medulla	23.6	8	30.9	3
Liver	73.9	10	36.4	7
Lungs	14.3	12	6.8	8
Muscle (skeletal)	9.2	7	6.0	3
Ovaries (females)	n.a.	n.a.	10.7	3
Penis (males)	115.2	22	n.a.	n.a.
Plasma	13.6	10	7.2	6
Salivary gland	15.8	9	11.5	3
Spinal cord	9.1	8	8.5	4
Spleen	13.1	9	17.7	3
Testes (males)	8.6	8	n.a.	n.a.
Thymus	8.5	8	6.1	4
Thyroid	13.4	8	8.6	4
Uterus (females)	n.a.	n.a.	12.0	4

bw: body weight; equiv: equivalents; n.a.: not applicable; ppm: parts per million; $t_{½}$: half-life
Source: Hassler (1999)

In males, the half-life of elimination from each tissue, assuming first-order kinetics, ranged from 7 to 12 hours, with the single exception of the penis, which had an elimination half-life of 22 hours. In females, half-lives ranged from 3 to 8 hours (Hassler, 1999).

The kinetics of the distribution and elimination of an oral or intravenous dose of [3,5-^{14}C-triazole]penconazole (radiochemical purity > 98%; batch no. GAN-IX-83) was investigated following dietary exposure to unlabelled penconazole (purity 98.7%; batch no. FL-840833) for at least 90 days. Groups of five male and five female Sprague-Dawley: Crl:CD (SD) BR rats received technical-grade penconazole in the diet at a dose level of 0, 10, 100, 300, 500, 1000 or 2400 ppm for at least 13 weeks before a single oral or intravenous dose of 0.1 mg [^{14}C]penconazole in PEG 200/distilled water (1:1) as dose vehicle. The radiolabelled dose was equivalent to a nominal 5 ppm dietary dose. Urine was collected at daily intervals over 48 hours after the ^{14}C dose, and a single 0- to 48-hour collection of faeces was made. Radioactivity in urine and cage wash was determined by direct liquid scintillation counting. Aqueous homogenates of faeces were prepared for scintillation counting by sample oxidation.

The excretion of radioactivity in urine and faeces and recoveries in terminal cage washes following an intravenous or oral dose are presented in Tables 5 and 6, respectively.

Table 5. Excretion of radioactivity by rats administered a single intravenous dose of 0.1 mg [^{14}C]penconazole following pretreatment with dietary doses of unlabelled penconazole for at least 90 days

	% of administered radioactivity					
	10 ppm	**100 ppm**	**300 ppm**	**500 ppm**	**1 000 ppm**	**2 400 ppm**
Males						
Urine						
0–24 h	42.54	44.04	42.28	42.11	39.38	42.43
24–48 h	10.25	8.47	7.84	8.55	9.91	9.91
0–48 h	52.79	52.51	50.12	50.66	49.29	52.34
Faeces						
0–48 h	21.87	25.04	25.31	28.61	27.17	28.81
Cage wash, 48 h	1.80	1.36	1.60	1.09	1.30	1.49
Total recovery, 0–48 h	76.46	78.91	77.03	80.36	77.76	82.64
Females						
Urine						
0–24 h	66.61	68.25	72.11	68.36	66.97	68.30
24–48 h	6.04	5.45	5.25	4.75	7.06	5.77
0–48 h	72.65	73.70	77.36	73.11	74.03	74.07
Faeces						
0–48 h	13.42	11.68	13.12	16.83	15.41	15.26
Cage wash, 48 h	3.14	2.32	2.04	1.29	3.54	3.34
Total recovery, 0–48 h	89.21	87.70	92.52	91.23	92.98	92.67

ppm: parts per million
Source: Hiles (1987b)

Table 6. Excretion of radioactivity by rats administered a single oral dose of 0.1 mg [^{14}C]penconazole following pretreatment with dietary doses of unlabelled penconazole for at least 90 days

	% of administered radioactivity					
	10 ppm	**100 ppm**	**300 ppm**	**500 ppm**	**1 000 ppm**	**2 400 ppm**
Males						
Urine						
0–24 h	45.47	50.74	40.39	45.08	39.55	43.31
24–48 h	7.11	7.83	8.12	9.36	8.20	9.86
0–48 h	52.58	58.57	48.51	54.44	47.75	53.17
Faeces						
0–48 h	28.26	26.45	31.36	25.88	30.59	27.79
Cage wash, 48 h	1.54	1.81	1.70	1.30	1.30	1.90
Total recovery, 0–48 h	82.38	86.83	81.57	81.62	79.64	82.86
Females						
Urine						
0–24 h	69.47	71.52	74.76	70.07	74.17	69.15
24–48 h	4.57	5.55	4.23	4.13	4.62	5.31
0–48 h	74.04	77.07	78.99	74.20	78.79	74.46
Faeces						
0–48 h	13.34	13.30	12.95	15.19	14.63	16.19
Cage wash, 48 h	2.44	3.18	1.31	2.74	1.60	2.98
Total recovery, 0–48 h	87.38	90.37	91.94	89.39	93.42	90.65

ppm: parts per million
Source: Hiles (1987b)

For both sexes, there was no marked difference in excretion profiles either across the range of dietary predosing levels or between the oral and intravenous routes of administration of the single radiolabelled dose. Females excreted a higher proportion of the dose in urine and less via faeces, compared with males. The mean recoveries of administered radioactivity were also higher in females (92%) than in males (81%) over the 48-hour time course of this study. These results show that the pretreatment of male and female rats with dietary doses of penconazole ranging from 10 to 2400 ppm had no apparent effect on the rate or extent of urinary or faecal excretion of a single intravenous or oral dose of 0.1 mg [^{14}C]penconazole (Hiles, 1987b).

The effect of dose level on the kinetics of the distribution and elimination of an oral dose of [^{14}C]penconazole (radiochemical purity 98%; batch no. GAN-IX-83) was investigated in Sprague-Dawley: Crl:CD(SD) BR rats. Groups of five male and five female rats received a single oral dose of technical-grade penconazole equivalent to a dietary dose of 0, 10, 100, 300, 500, 1000 or 2400 ppm. Each rat was then immediately administered a single oral dose of 0.1 mg [3,5-^{14}C-triazole]penconazole in PEG 200/distilled water (1:1 by volume) as dose vehicle. The radiolabelled dose was equivalent to a nominal 5 ppm dietary dose. Urine was collected at daily intervals over 48 hours after the ^{14}C dose, and a single 0- to 48-hour collection of faeces was made. Radioactivity in urine and cage wash was determined by direct liquid scintillation counting. Aqueous homogenates of faeces were prepared for scintillation counting by sample oxidation.

Table 7. Excretion of radioactivity by rats administered a single oral dose of 0.1 mg [3,5-^{14}C-triazole]penconazole, preceded by a single oral dose of unlabelled penconazole across a range of dose levels equivalent to dietary doses of 0–2400 ppm

	% of administered radioactivity						
	0 ppm	**10 ppm**	**100 ppm**	**300 ppm**	**500 ppm**	**1 000 ppm**	**2 400 ppm**
Males							
Urine							
0–24 h	45.28	38.20	43.91	44.76	45.81	39.69	33.85
24–48 h	8.02	7.80	7.80	7.82	9.56	11.69	13.97
0–48 h	53.31	46.00	51.71	52.58	55.37	51.38	47.82
Faeces							
0–48 h	27.28	26.89	26.31	26.51	27.17	24.09	18.82
Cage wash, 48 h	3.21	2.73	2.60	2.65	3.59	2.80	3.78
Total recovery, 0–48 h	83.80	75.62	80.62	81.74	86.13	78.27	70.42
Females							
Urine							
0–24 h	76.93	68.83	77.58	79.48	86.13	79.69	55.58
24–48 h	3.55	3.87	2.39	2.83	4.12	4.00	13.14
0–48 h	80.48	72.20	79.97	82.32	90.25	83.70	68.73
Faeces							
0–48 h	12.68	12.61	11.99	11.07	9.35	10.02	9.98
Cage wash, 48 h	2.25	1.89	2.56	2.14	2.48	2.72	3.35
Total recovery, 0–48 h	95.41	87.20	94.52	95.53	102.08	96.44	82.06

ppm: parts per million
Source: Levan (1987)

The excretion of radioactivity in urine and faeces and recoveries in terminal cage washes are presented in Table 7.

For both sexes, there was no marked difference in excretion profiles across the range of dose levels investigated. There was a difference between the sexes, with females excreting a higher proportion of the dose in urine and less via faeces, compared with males. The mean recoveries of administered radioactivity were also higher in females (93%) than in males (80%) over the 48-hour time course of this study. These results show no apparent relationship between the pretreatment gavage dose level over an equivalent dietary dose range of penconazole from 10 to 2400 ppm and the kinetics of the distribution and elimination of a single 0.1 mg gavage dose of [^{14}C]penconazole between the urine and faecal compartments (Levan, 1987).

The absorption, tissue distribution and excretion of penconazole were investigated in Wistar, KFM-WIST outbred, SPF rats administered a single oral dose of [U-^{14}C-phenyl]penconazole (radiochemical purity 98.2%; batch no. GB-XXIX-57 B1). The identification of penconazole and its metabolites in tissues and excreta is described in section 1.2 (Van Dijk, 1987). The experimental design is presented in Table 8. In the repeated-dose part of the study, rats were treated with unlabelled

Table 8. Experimental design of a study on the absorption, distribution and excretion of penconazole in rats

Group	Number and sex	Route and dose level of [U-^{14}C-phenyl]penconazole	Sample collection
1	5 males	Single oral dose (50 mg/kg bw)	Expired $^{14}CO_2$ collections at 8, 24 and 48 h after dosing Excreta collections at 8, 24, 48, 72 and 96 h after dosing Blood and selected tissues taken for analysis
2	5 males	Single oral dose (0.5 mg/kg bw)	Excreta collections at 8, 24, 48, 72 and 96 h after dosing Blood and selected tissues taken for analysis
3	5 females	Single oral dose (0.5 mg/kg bw)	
4	5 females	Single oral dose (50 mg/kg bw)	
5	3 males (bile duct cannulated)	Single oral dose (0.5 mg/kg bw)	Bile collected at 3 h intervals Urine and faeces collected at 24 and 48 h
6	3 females (bile duct cannulated)	Single oral dose (0.5 mg/kg bw)	Gastrointestinal tract removed, but no tissues taken for analysis
7	5 males	Single oral dose (0.5 mg/kg bw) following 14 consecutive daily 0.5 mg/kg bw oral doses of unlabelled penconazole	Excreta collections at 8, 24, 48, 72 and 96 h after radiolabel dosing Blood and selected tissues taken for analysis
8	5 females		

bw: body weight
Source: Van Dijk (1987)

penconazole (purity > 99%; batch no. P2). At the termination of rats in groups 1–4 and 7–8, the following tissues were taken for radioactivity analysis: bone (femur), brain, fat, heart, intestinal tract (including contents), kidneys, liver, lungs, muscle, ovaries, pancreas, plasma, skin, spleen, stomach, testes, thyroid, uterus, whole blood and residual carcass. All samples were counted for radioactivity by liquid scintillation counting either directly or following tissue digestion or sample oxidation.

The excretion of radioactivity by rats across the eight dose groups is presented in Table 9. Penconazole was very well absorbed by both male and female rats. In bile duct–cannulated rats that were administered a dose of 0.5 mg/kg bw, less than 5% of the dose was excreted in faeces. Males excreted similar proportions of a 0.5 mg/kg bw dose between urine (47%) and faeces (44%), whereas females eliminated a greater proportion of the dose in urine (69%) than in faeces (21%). This difference could be attributed in part to the higher biliary elimination by males (55% of the dose) compared with females (40%). The rate of biliary elimination was fast in both sexes, with 49% of the dose in 0- to 9-hour bile collections in male rats and 29% in female bile over the same interval. The comparative biliary and faecal excretion data of an equivalent dose (0.5 mg/kg bw) in non-cannulated rats indicate that some biliary metabolites were subject to reabsorption. No marked differences in excretion profiles between the low and high dose levels were observed. Furthermore, pretreatment with unlabelled penconazole for 14 days caused no marked difference in excretion profiles. No volatile radiolabelled metabolites were trapped from expired air, which is consistent with the location of the radiolabel in the phenyl ring.

Tissue residues were low by 4 days after dosing, irrespective of sex or dose level (Table 10). Following a 0.5 mg/kg bw dose, most residues were at or below the limit of quantification, with measurable residues in liver, kidneys, blood and residual carcass. Following a 50 mg/kg bw dose level, the highest tissue residues in males were present in liver, kidneys and adrenal glands, with all other tissue concentrations below that found in blood. In females at 50 mg/kg bw, the highest residue appeared in the thyroid; however, thyroid tissue concentrations were below the limit of quantification in all other male and female groups. Progressively lower residues were present in the adrenal glands, liver and kidneys, with concentrations generally lower in females than in males. In the repeated-dose

Table 9. Mean absorption and excretion data following single oral administration of [U-^{14}C-phenyl]penconazole to rats

	% of dose							
	Group 1 (M)	**Group 2 (M)**	**Group 3 (F)**	**Group 4 (F)**	**Group 5 (M)**	**Group 6 (F)**	**Group 7 (M)**	**Group 8 (F)**
	50 mg/kg bw	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**50 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**
Urine								
0–8 h	19.4	31.9	56.3	36.5	22.3	46.3	24.8	49.9
8–24 h	15.1	9.6	10.5	31.5	5.9	1.6	14.2	18.7
24–48 h	5.0	3.7	1.7	3.6	n.a.	n.a.	2.0	1.2
48–96 h	1.5	1.7	0.5	0.6	n.a.	n.a.	1.2	0.4
Subtotal	41.1	46.9	69.0	72.2	28.2	47.9	42.2	70.2
Bile								
0–24 h	n.a.	n.a.	n.a.	n.a.	54.6	38.6	n.a.	n.a.
24–48 h	n.a.	n.a.	n.a.	n.a.	0.97	1.6	n.a.	n.a.
Subtotal	n.a.	n.a.	n.a.	n.a.	54.6	40.2	n.a.	n.a.
Faeces								
0–8 h	0.4	2.8	0.9	0.1	3.2	0.9	1.6	2.0
8–24 h	30.7	26.6	16.2	13.5	1.5	1.1	33.5	18.3
24–48 h	9.7	8.7	3.0	3.6	n.a.	n.a.	11.8	2.6
48–96 h	6.1	1.5	1.0	0.9	n.a.	n.a.	4.9	0.8
Subtotal	47.0	43.5	21.1	18.1	4.7	2.0	51.8	23.7
Expired air								
0–48 h	< 0.01	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cage wash	4.0	8.5	7.7	7.9	0.8	4.2	2.1	3.6
Total excretion	92.1	98.9	97.8	98.2	88.3	94.3	96.1	97.5
GIT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Tissues	1.6	2.2	0.5	0.2	n.a.	n.a.	1.4	0.2
Digestive tract	n.a.	n.a.	n.a.	n.a.	1.7	0.5	n.a.	n.a.
Carcass	n.a.	n.a.	n.a.	n.a.	0.9	3.5	n.a.	n.a.
Total recovery	93.7	101.1	98.3	98.4	90.9	98.3	97.5	97.7

bw: body weight; F: females; GIT: gastrointestinal tract; M: males; n.a.: not applicable
Source: Van Dijk (1987)

study, most radiolabelled tissue concentrations were below the limit of quantification (Van Dijk, 1987).

1.2 Biotransformation

Rats

The metabolism of [3,5-^{14}C-triazole]penconazole (radiochemical purity > 98%; batch number not reported), dissolved in ethanol/PEG 200/water (2:3:5 by volume), was studied in groups of two male and two female Tif: RAI f (SPF) rats dosed orally by gavage at a single dose of 25 mg/kg bw. In

Table 10. Group mean tissue residues of radioactivity 4 days after a single oral dose of [U-^{14}C-phenyl]penconazole

	Concentration (ppm penconazole equivalents)					
	Group 1 (M)	**Group 2 (M)**	**Group 3 (F)**	**Group 4 (F)**	**Group 7 (M)**	**Group 8 (F)**
	50 mg/kg bw	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**50 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**
Heart	0.16	< LOQ	< LOQ	0.11	< LOQ	< LOQ
Lungs	0.19	< LOQ	< LOQ	0.17	< LOQ	< LOQ
Liver	1.46	0.019	0.006	0.34	0.015	< LOQ
Spleen	0.16	< LOQ	< LOQ	0.19	< LOQ	< LOQ
Kidneys	0.71	0.007	0.004	0.23	< LOQ	< LOQ
Muscle	0.08	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Bone (femur)	0.07	0.007	0.006	< LOQ	LOQ	< LOQ
Brain	0.06	< LOQ	< LOQ	LOQ	< LOQ	< LOQ
Fat	0.33	< LOQ	< LOQ	0.027	< LOQ	< LOQ
Pancreas	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Stomach	0.10	< LOQ	< LOQ	0.09	< LOQ	< LOQ
Intestinal tract	4.08	0.054	0.006	0.45	0.061	0.005
Adrenal glands	0.56	< LOQ	< LOQ	0.49	< LOQ	< LOQ
Uterus/ovaries	n.a.	n.a.	< LOQ	< LOQ	n.a.	< LOQ
Testes	0.07	< LOQ	n.a.	n.a.	< LOQ	n.a.
Thyroid gland	< LOQ	< LOQ	< LOQ	1.11	< LOQ	< LOQ
Skin chest	0.27	LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Skin back region	0.15	< LOQ	< LOQ	0.05	< LOQ	< LOQ
Carcass	0.19	0.003	LOQ	0.06	LOQ	< LOQ
Blood	0.45	0.006	< LOQ	0.08	0.006	< LOQ
Plasma	0.30	0.004	< LOQ	0.08	LOQ	< LOQ

bw: body weight; F: females; LOQ: limit of quantification; M: males; n.a.: not applicable; ppm: parts per million
Source: Van Dijk (1987)

urine collected over the first 24-hour interval, metabolite profiles were investigated by TLC. The study design and toxicokinetics are described in section 1.1 (Hamböck, 1980, 1985).

Chromatographic analysis of urine samples is presented in Table 11. The chromatographic analysis showed the presence of several polar metabolites and no unchanged parent penconazole. The general pattern of metabolites was qualitatively similar both between the sexes and between the dose levels. However, the relative proportions of radioactivity in metabolite fractions differed considerably between males and females. The polar metabolite fraction (U_{01} and U_{02}) accounted for 45% of the dose in females and just 3% in males. The "medium polar" metabolites (U_{03} and U_{06}) represented 12% of the dose in males, but only 2% in females. These "medium polar" metabolites included free triazole, which accounted for 7% of the dose in males and only 1% in females. No marked differences were apparent in the proportions of other fractionated metabolites (Hamböck, 1980, 1985).

Table 11. Quantitative distribution of urinary metabolites in urine collected over 24 hours after a single oral [3,5-^{14}C-triazole]penconazole dose of 25 mg/kg bw

	% of dose		
TLC fraction code	**Males**	**Females**	**Structural identification**
U_{01}	1	7	Conjugates with glucuronic acid
U_{02}	2	38	
U_{03}	7	1	Free 1,2,4-triazole
U_{06}	5	1	Several carboxylic acid metabolites
U_{07}	21	23	
Other minor fractions	11	11	
Start zone	1	0	
Total	48	81	

bw: body weight; TLC: thin-layer chromatography
Source: Hamböck (1985)

The metabolism of [3,5-^{14}C-triazole]penconazole (radiochemical purity > 98%; batch number not reported), dissolved in ethanol/PEG 200/water (2:3:5 by volume), was studied in 20 male Tif: RAI f (SPF) rats dosed orally by gavage at a single dose of 22.8 mg/kg bw. Urine and faeces were collected over 48 hours. Urinary metabolite profiles were investigated by two-dimensional TLC. The major metabolites were isolated by extraction, column chromatography, preparative TLC and high-performance liquid chromatography (HPLC). Acidic metabolites were acidified and methylated to facilitate purification, and the hydroxylated derivatives were acetylated, thereby preventing potential losses due to chemical instability. Isolated metabolites were analysed by mass spectroscopy and nuclear magnetic resonance (NMR) to elucidate their structures.

In urine and faeces, 62% and 33% of the administered dose were found, respectively. The relative proportions of identified radioactivity are presented in Table 12.

TLC analysis showed an array of metabolites, and the profile was not markedly changed following treatment of metabolites with β-glucuronidase or sulfatase. The amounts of these conjugates were therefore shown to be low.

At least four metabolic reactions were involved in the biotransformation of penconazole:

1. cleavage of the triazole ring (15% of the dose);
2. oxidation of the ω-position of the alkane chain to form the respective carboxylic acid (30% of the dose);
3. oxidation of the 3- or 4-position of the alkane chain to form monohydroxy and dihydroxy derivatives (2.5% of the dose); and
4. oxidation of the triazole ring in the 3- or 5-position (0.7% of the dose).

Secondary metabolic reactions included:

- α-oxidation of the carboxylic acids to form α-hydroxy carboxylic acids (4.4% of the dose);
- decarboxylation following oxidation to α-ketocarboxylic derivative (9% of the dose);
- oxidation of the 3,4-dihydroxy derivatives to produce the corresponding 3- or 4-keto derivatives (0.5% of the dose);
- conjugation of all alkanol derivatives with glucuronic acid (2.5% of the dose); and
- β-oxidation of ω-carboxylic acids, which may also reduce the chain length.

The stepwise reduction of the alkyl chain and the cleavage of the molecule to form free triazole are the two main metabolic reactions. Free triazole was the only metabolite present in both urine and faeces. Oxidation of the triazole ring was a minor metabolic reaction, and no

biotransformation occurred on the dichlorophenyl ring. Sequential methanol extraction of faeces yielded 82% extraction of faecal radioactivity. Free triazole was present and accounted for approximately 1% of the dose, and unchanged penconazole accounted for less than 1% of the dose, which was considered to represent unabsorbed penconazole. Other faecal metabolites (not identified) did not correspond with urinary metabolites.

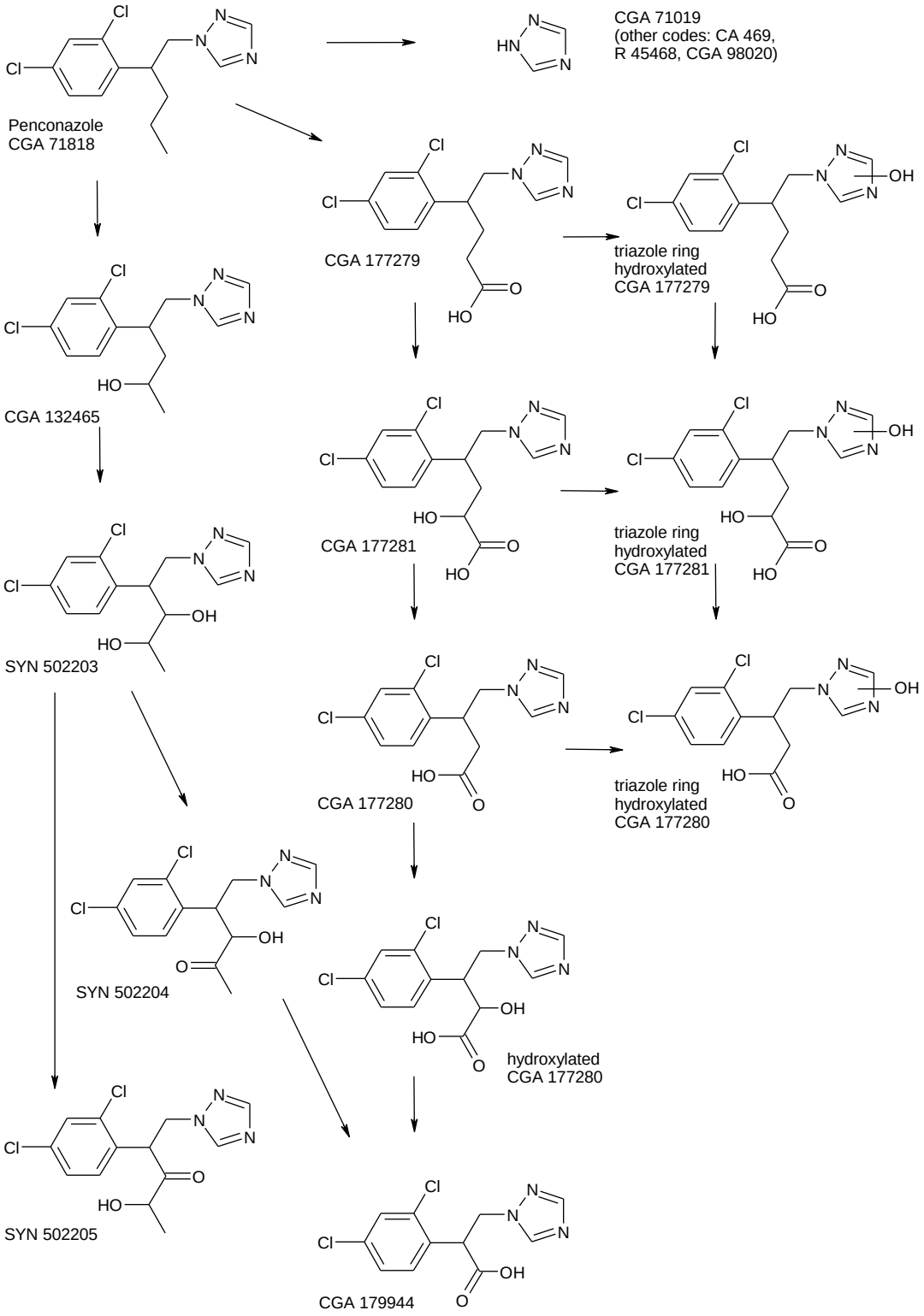
Table 12. Characterization and quantification of metabolites of penconazole in urine and faeces

		% of dose	
Reference code	**Nomenclature**	**Urine**	**Faeces**
CGA 71818	1-[2-(2,4-Dichloro-phenyl)-pentyl]-1*H*-[1,2,4]triazole	–	0.8
CGA 71019 (other codes: CA 469, R 45468, CGA 98020)	1*H*-[1,2,4]Triazole	14	1
CGA 177279	4-(2,4-Dichloro-phenyl)-5-[1,2,4]triazol-1-yl-pentanoic acid	16	–
Triazole ring hydroxylated CGA 177279		0.6	–
CGA 177281	4-(2,4-Dichloro-phenyl)-2-hydroxy-5-[1,2,4]triazol-1-yl-pentanoic acid	4.4	–
CGA 177280	3-(2,4-Dichloro-phenyl)-4-[1,2,4]triazol-1-yl-butyric acid	7.8	–
CGA 177281 (hydroxylated CGA 177280)		< 0.1	–
Triazole ring hydroxylated CGA 177280		Not quantified	–
Triazole ring hydroxylated CGA 177281		0.1	–
CGA 179944	2-(2,4-Dichloro-phenyl)-3-[1,2,4]triazol-1-yl-propionic acid	0.9	–
CGA 132465	4-(2,4-Dichloro-phenyl)-5-[1,2,4]triazol-1-yl-pentan-2-ol		
SYN 502203	4-(2,4-Dichloro-phenyl)-5-[1,2,4]triazol-1-yl-pentane-2,3-diol		
SYN 502204	4-(2,4-Dichloro-phenyl)-3-hydroxy-5-[1,2,4]triazol-1-yl-pentan-2-one	2.5	–
SYN 502205	2-(2,4-Dichloro-phenyl)-4-hydroxy-1-[1,2,4]triazol-1-yl-pentan-3-one		
Not identified	–	16	31

Source: Hamböck (1982, 1984)

The proposed metabolic pathway of penconazole in rats is shown in Fig. 2 (Hamböck, 1982, 1984).

Fig. 2. The proposed biotransformation of penconazole in the rat (urine and faeces)



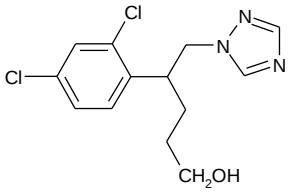
Source: Hamböck (1984)

The metabolism of [U-^{14}C-phenyl]penconazole (radiochemical purity 98.2%; batch no. GB-XXIX-57 B1) was studied in groups of Wistar, KFM-WIST outbred, SPF rats. The experimental designs and toxicokinetics are described in section 1.1 (Van Dijk, 1987). All samples were counted for radioactivity by liquid scintillation counting either directly or following tissue digestion or sample oxidation. Metabolite profiles were investigated by two-dimensional TLC in urine (0- to 24-hour pools), bile (0- to 6-hour, 6- to 12-hour and 12- to 18-hour pools), solvent extracts of faeces (0- to 48-hour pools, except group 8, 0- to 24-hour pool), liver and kidney. Metabolite fractions were treated with enzymes to cleave metabolite conjugates. The relative proportions of the administered dose in resolved metabolite fractions were determined.

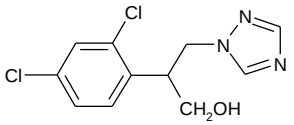
At least eight metabolites were resolved in urine, and 12 in faecal extracts. The solvent extraction of pooled faecal samples yielded about an 85% extraction of radioactivity. No unchanged parent compound was found in excreta. Two of the resolved metabolites in excreta and tissues were characterized as CGA 127841 and CGA 189659 (Fig. 3). Conjugates of these metabolites were also present. All other metabolites remained unidentified in this study. Conjugated CGA 127841 was found in urine, faeces and bile. Unconjugated CGA 127841 was found in faeces, bile, liver and kidney. CGA 189659 was identified in faeces, liver and kidney (Van Dijk, 1987).

Fig. 3. Structural characterization of metabolites of penconazole

CGA 127841



CGA 189659



Source: Van Dijk (1987)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with penconazole are summarized in Table 13.

(b) Dermal irritation

In an acute dermal irritation study, the intact or abraded skin of three male and three female New Zealand White rabbits was exposed for 24 hours under occlusion to 0.5 g penconazole (purity 88.4%; batch no. P.2+3) moistened with PEG/water (70:30 by volume). Dermal irritation was scored at 24 and 72 hours after patch removal.

Minimal erythema on the intact and abraded skin was observed at 24 hours. No signs of dermal irritation were observed at 72 hours (Ullmann, 1980).

(c) Ocular irritation

In an acute eye irritation study, 100 mg of penconazole (purity not specified; batch no. FL840833) was instilled into the conjunctival sac of one eye of each of three male and six female New Zealand White rabbits. The untreated eye served as a control. The treated eyes of three female rabbits were washed with deionized water 30 seconds after application. The eyes were examined

Table 13. Results of studies of acute toxicity with penconazole

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50}/LC_{50}	Reference
Mouse	Tif: MAG (SPF)	M/F	Oral	PEG 400	88.4	2 444 mg/kg bw (M/F)	Sarasin (1980)[a]
Rat	Tif: RAI f (SPF)	M/F	Oral	PEG 400	88.4	2 125 mg/kg bw (M/F)	Bathe (1980a)[b]
Chinese hamster	–	M/F	Oral	PEG 400	88.4	~5 000 mg/kg bw (M/F)	Bathe (1980b)[c]
Rabbit	New Zealand White	M/F	Oral	Carboxymethyl cellulose	88.4	971 mg/kg bw (M/F)	Kobel (1981)[d]
Rat	Tif: RAI f (SPF)	M/F	Dermal	PEG 400	88.4	> 3 000 mg/kg bw (M/F)	Bathe (1980c)[e]
Rat	Tif: RAI f (SPF)	M/F	Inhalation	–	96.1	> 4.0 mg/L (M/F)	Hartmann (1987)[f]

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male; PEG: polyethylene glycol

[a] Study design resembles Organisation for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were given a dose of 1500, 2000, 3000 or 5000 mg/kg bw. At 3000 and 5000 mg/kg bw, 4/5 and 5/5 males died, respectively. At 1500, 3000 and 5000 mg/kg bw, 1/5, 5/5 and 5/5 females died, respectively. At all doses, sedation, dyspnoea, ruffled fur and curved or ventral/lateral body position were observed. The first onset of symptoms occurred within 1 hour after dosing in all groups. The longest duration of symptoms in surviving animals was 7 days after 1500, 2000 and 3000 mg/kg bw. Recovery was complete in survivors of all groups by day 8 post-dosing. Necropsy revealed no treatment-related findings. Batch no. P.2+3.

[b] Study design resembles OECD Test Guideline 401. The rats were given a dose of 500, 1000, 2000 or 4000 mg/kg bw. At 2000 and 4000 mg/kg bw, 3/5 and 5/5 males died, respectively. At 1000 and 4000 mg/kg bw, 2/5 and 4/5 females died, respectively. At 500 mg/kg bw, sedation, dyspnoea, curved or lateral/ventral body position and ruffled fur were observed. At higher doses, diarrhoea was also observed. At the high dose, symptoms lasted up to 9 days. Gross pathology revealed no treatment-related findings. Batch no. P.2+3.

[c] Study design resembles OECD Test Guideline 401. The hamsters were given a dose of 2000, 4000 or 5000 mg/kg bw. At 4000 and 5000 mg/kg bw, 1/5 and 1/5 males died, respectively. At 4000 and 5000 mg/kg bw, 2/5 and 3/5 females died, respectively. At 2000 mg/kg bw, sedation, dyspnoea, curved body position, ruffled fur and exophthalmos were observed. At higher doses, salivation and lateral/ventral body position were also observed. At the high dose, symptoms lasted up to 8 days. Gross pathology revealed no treatment-related findings. Batch no. P.2+3.

[d] Study design resembles OECD Test Guideline 401. The rabbits were given a dose of 600, 1000 or 2000 mg/kg bw. At 1000 and 2000 mg/kg bw, 2/3 and 3/3 males died, respectively. At 1000 and 2000 mg/kg bw, 2/3 and 3/3 females died, respectively. At 600 mg/kg bw, ataxia and ruffled fur were observed. At higher doses, sedation, dyspnoea, curved or lateral body position, dacryorrhoea and tremor were also observed. At 1000 mg/kg bw, symptoms lasted up to 6 days. Gross pathology of dead rabbits revealed partly congested organs. Batch no. P.2+3.

[e] Study design resembles OECD Test Guideline 402. The rats were given a dose of 2000, 2500 or 3000 mg/kg bw. No deaths occurred. Slight symptoms of toxicity were observed in all treatments. The symptoms included dyspnoea, ruffled fur and curved body position. Gross pathology revealed no treatment-related findings. Batch no. P.2+3.

[f] Study design resembles OECD Test Guideline 403. Rats were exposed nose only to an actual penconazole concentration of 4.0 mg/L. Vehicle was "F1", a powdered mixture of aluminium oxide and Sipernat 50 S (3:1 weight per weight). No mortality was observed. All rats displayed sedation, dyspnoea, ruffled fur and curved body position. In the vehicle control group, the symptoms were graded as slight to moderate, whereas in the penconazole group, they were graded as slight to severe. Macroscopic examination showed mottled lungs and thymus haemorrhages in the vehicle control group. In the penconazole group, no effects were observed. Mass median aerodynamic diameter was 3.5–5.4 (± 2.0) µm. Batch no. EN 603012.

macroscopically for signs of irritation at 1, 24, 48 and 72 hours and at days 4, 7 and 10 post-instillation. The study design resembles Organisation for Economic Co-operation and Development (OECD) Test Guideline 405.

An overview of the mean irritation scores of the unwashed eyes is presented in Table 14.

Table 14. Eye irritation in rabbits – Group mean scores at the 24-, 48- and 72-hour readings (unwashed eye)

	Males (*n* = 3)			Females (*n* = 3)			Males and females (*n* = 6)		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Corneal opacity	0.33	0.67	0.67	1.0	1.0	1.0	0.67	0.83	0.83
Iris lesions	1.0	0.0	0.33	1.0	0	0	1.0	0.0	0.17
Conjunctival redness	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Chemosis	1.0	1.0	0.33	1.0	1.0	0.33	1.0	1.0	0.33

Source: Kuhn (1988)

In the unwashed eyes, partial corneal opacity (grade 1) was seen in the majority of the animals between 24 and 72 hours, lasting for up to 4 days in two rabbits. Grade 1 iris lesions were not noted after 72 hours. Slight swelling (grade 1) of the conjunctiva was observed in all animals up to 48 hours and in two rabbits at 72 hours, whereas redness lasted for up to 7 days in three rabbits. Recovery was complete after 10 days. In washed eyes, no effects on the cornea or iris were observed. Corneal chemosis lasted for 48 hours, and redness for up to 72 hours only. The eyes were free of irritation on day 4 (Kuhn, 1988).

(d) Dermal sensitization

In a dermal sensitization study using the Magnusson and Kligman maximization test, performed in accordance with OECD Test Guideline 406, penconazole (purity 96%; batch no. EN 603012) was tested in 20 female GOHI (Himalayan spotted) guinea-pigs. The vehicle control group consisted of 10 animals. In the induction phase, the animals received 5% penconazole followed by epidermal treatment with 50% penconazole on day 8. The challenge on day 21 was performed with epidermal application of 20% penconazole. Benzocaine was used as a positive control.

After challenge application, skin reactions were evident at the application site in some animals at 24 and 48 hours. The sensitization rate was 15%. Under the conditions of this study, penconazole was not a skin sensitizer (Cantoreggi, 1998).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day dietary study, penconazole (purity 98.7%; batch no. FL-840833) was administered to groups of 15 male and 15 female Crl:CD®-1(ICR)BR mice at 0, 10, 100, 300, 500, 1000 or 2400 ppm (equal to 0, 1.7, 17, 52, 85, 163 and 423 mg/kg bw per day for males and 0, 2.5, 24, 72, 116, 237 and 614 mg/kg bw per day for females, respectively). Mice were observed daily for mortality and clinical signs. A detailed physical examination was performed weekly. Body weight and feed consumption were measured weekly. Ophthalmoscopic examinations were done before study initiation on all animals and on control and high-dose animals near study termination. Prior to termination, blood and urine samples were taken from all animals for haematology, blood biochemistry and urine analysis. All mice underwent complete necropsy. Testes, ovaries, liver, heart, brain, kidneys and spleen were weighed. A wide range of organs of all mice was examined microscopically. The study design resembles OECD Test Guideline 407.

No treatment-related mortality or clinical signs were observed. Body weight, feed consumption, and ophthalmoscopic and haematological parameters were not affected. Males given 1000 or 2400 ppm had lower total protein (7–9%). Alanine aminotransferase (ALAT) activity was significantly increased (+170% versus controls) in the 2400 ppm males. Significant reductions in

gamma-glutamyltranspeptidase (GGT) activity at the 500, 1000 and 2400 ppm levels were considered not toxicologically relevant. Females at 2400 ppm had significantly lower total protein (10%) and albumin levels (14%) and albumin to globulin ratios (13%). Cholesterol level was decreased in both male and female mice at 1000 ppm (31–36%) and 2400 ppm (40–61%). Absolute and relative liver weights were significantly higher in the 2400 ppm male (41%) and female rats (32%) and in males at 500 ppm (10%) and 1000 ppm (16%). No treatment-related macroscopic changes were observed. Histopathological examination revealed centrilobular hypertrophy of hepatocytes in males at 500 and 1000 ppm and in both sexes at 2400 ppm. Degeneration of the hepatocytes around the central vein and hepatocellular vacuolation were observed in males at 2400 ppm. Focal coagulative necrosis was found in the liver of some of the 1000 and 2400 ppm males and in a few females. Frozen sections of liver stained with Oil Red O revealed no difference between control and treated rats in the amount of lipid material present. In the absence of other signs of liver toxicity, the increased hepatocyte hypertrophy in males at 500 ppm is not considered adverse. There were no other microscopic findings that could be related to the treatment.

The NOAEL was 500 ppm (equal to 85 mg/kg bw per day), based on lower total protein and cholesterol levels and focal coagulative necrosis in the liver of both sexes at 1000 ppm (equal to 163 mg/kg bw per day) (Hiles, 1987a).

In another 90-day dietary study, penconazole (purity 97.7%; batch no. WS007001) was administered to groups of 10 male and 10 female C57BL/10JfCD-1 mice at 0, 100, 500, 1500, 3000 or 5000 ppm (equivalent to 0, 14, 69, 229, 437 and 837 mg/kg bw per day for males and 0, 18, 87, 274, 545 and 983 mg/kg bw per day for females, respectively). Mice in the 5000 ppm group were killed for humane reasons during the second week of the study. Mice were observed daily for mortality and clinical signs. A detailed physical examination and body weight measurements were performed daily during the first 2 weeks and weekly thereafter. Feed consumption was measured weekly. At termination during week 2 for the 5000 ppm mice and on day 92 for all other surviving mice, blood was sampled for clinical chemistry. All mice underwent complete necropsy. Testes, epididymides, uterus, liver, heart, brain, kidneys, adrenals and spleen were weighed. Adrenals, brain, epididymides, ovary, kidney, liver and testis from the control and 3000 ppm groups and livers from the 100, 500 and 1500 ppm groups were examined microscopically.

At 5000 ppm, the mice lost weight (8–11%) during the first week of treatment. The mice in this group were killed for humane reasons in week 2. No treatment-related mortality was observed in the other dose groups. At 3000 ppm, the mice initially lost weight (up to 4–5%). At the end of the study, the body weights of males at 1500 and 3000 ppm were 6% and 15% lower than control male values, respectively. Females at 3000 ppm had a 11% lower body weight than control females at termination. Feed consumption was reduced in both sexes at 3000 ppm (25–26%) and 5000 ppm (26–44%) on day 1. Feed utilization was less efficient in both sexes at 3000 ppm and in males at 1500 ppm than in controls. Significant reductions in cholesterol levels were observed at 1500 ppm (42–43%), 3000 ppm (52–54%) and 5000 ppm (67–71%). Small reductions in cholesterol level were seen at 500 ppm (10–29%). In the 3000 ppm group, plasma alkaline phosphatase (ALP) activity was slightly increased (22–25%). At 5000 ppm, ALP activity was increased in both sexes by 51–95%. Plasma albumin and plasma total protein were slightly lower (6–8%) in females at 1500 and 3000 ppm. In females at 5000 ppm, albumin levels were decreased by 17%. There was a slight reduction (20%) in triglyceride levels in both sexes at 3000 ppm and a more marked reduction (42–52%) at 5000 ppm. Relative liver weights were increased by 12%, 33% and 48% for males at 500, 1500 and 3000 ppm, respectively, and by 10% and 27% for females at 1500 and 3000 ppm, respectively. Hepatocyte hypertrophy and increased nuclear pleomorphism were present in all males at 1500 and 3000 ppm. Hepatocyte hypertrophy was also observed in 4/10 females treated with 3000 ppm. A small increase in the incidence of mononuclear cell infiltration of the liver observed in males and females treated with 3000 ppm was considered not to be of toxicological significance in view of it being a common spontaneous change in mice. Adrenal weights adjusted for body weight were higher than control values in females receiving 3000 ppm owing to high values for three females. When adrenal weights

for these animals were excluded, there were no differences between adrenal weights in treated and control animals.

The NOAEL was 500 ppm (equal to 69 mg/kg bw per day), based on reductions in cholesterol levels in both sexes, a reduction in total protein and albumin levels in females, and a reduction in body weight gain and increased nuclear pleomorphism in hepatocytes in males at 1500 ppm (equal to 229 mg/kg bw per day) (Milburn, 2002).

Rats

In a 28-day toxicity study, penconazole (purity 91.7%; batch no. P.11-14) was administered by gavage to groups of 10 male and 10 female Tif: RAI f (SPF) rats at 0, 20, 100 or 500 mg/kg bw per day. The vehicle was aqueous methyl cellulose. From day 8 of the study, doses were increased to 0, 100, 500 and 1000 mg/kg bw per day, respectively. Animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed and water consumption were measured weekly. Ophthalmoscopy was performed before treatment started and towards termination. Blood and urine were sampled at termination for haematology, clinical biochemistry and urine analysis. All rats were necropsied, and weights of liver, spleen, kidneys, heart, brain, thyroid, thymus, gonads and adrenals were recorded. These organs were also examined microscopically.

No deaths were observed. At 1000 mg/kg bw per day, marked apathy and lateral body position after administration of the test substance on day 10 of dosing were observed in three rats. Body weight gain was reduced in both sexes from week 2 onward at 500 mg/kg bw per day (up to 15–17%) and 1000 mg/kg bw per day (up to 21–35%). Feed consumption during weeks 2–4 was decreased at 500 mg/kg bw per day (8–9%) and 1000 mg/kg bw per day (12–19%). Water consumption was increased (31%) in high-dose females. Slight decreases in haemoglobin and haematocrit were observed in females at 500 and 1000 mg/kg bw per day. In males and females at 500 and 1000 mg/kg bw per day, glucose, cholesterol, protein, sodium and calcium levels and the activities of ALP and ALAT were slightly increased, whereas potassium and chloride levels were decreased. In addition, in females at these doses, glucose and inorganic phosphate levels were increased, and creatinine levels were slightly decreased. Urine volume was increased in both sexes at the middle and high doses. Absolute and relative weights of the liver, kidneys and adrenals were dose-dependently increased at 500 and 1000 mg/kg bw per day in both sexes. In high-dose females, absolute and relative thyroid weights were also slightly increased. Enlarged livers and hepatocyte hypertrophy were observed in some mid-dose rats and all high-dose rats.

The data indicate that various adverse effects, in particular liver toxicity, occur at doses above 100 mg/kg bw per day. However, owing to the change in dose levels after week 1, no definite NOAEL could be identified from this study (Basler, 1984).

In a 28-day toxicity study, the toxicities of two batches of penconazole were compared. Penconazole "batch A" (purity 96.2%; batch no. Op.3-23.01.90) or penconazole "batch B" (purity 96.1%; batch no. EN 603012) was administered by gavage to groups of 10 male and 10 female Tif: RAI f (SPF) rats at 0, 100 or 500 mg/kg bw per day. The vehicle was 0.5% aqueous methyl cellulose with 0.1% Tween 80. Animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly. Blood was sampled at termination for haematology and clinical biochemistry. All rats were necropsied, and weights of liver, spleen, kidneys, testes, heart, brain, thyroid, thymus, gonads and adrenals were recorded. These organs (except for the brain and gonads) and the parathyroid glands and lungs were also examined microscopically.

At the high dose, one male and three females were killed in a moribund condition. At 500 mg/kg bw per day in animals treated with batch B, hunch-backed posture, piloerection and laboured breathing were observed. Body weight gain was not affected by treatment. At 500 mg/kg bw per day (batches A and B), feed consumption was reduced by 10–13% in both sexes. A decreased prothrombin time was observed in male and female rats at the low dose (6–10%) and high dose (15–

18%). Slightly higher platelet counts occurred in both sexes at 500 mg/kg bw per day (12–30%), but this was significant in males only. There was an increase in plasma protein concentrations (3–5% at the low dose, 7–9% at the high dose) associated with higher globulin levels and minimally lower albumin to globulin ratios and a decrease (22–28%) in total bilirubin at both doses. At 500 mg/kg bw per day, increased plasma albumin (5–14%, males) and cholesterol levels (25–91%, both sexes) and decreased chloride levels (4–6%, both sexes) were observed. Glucose levels were increased in females at 100 mg/kg bw per day (16–17%) and 500 mg/kg bw per day (39–44%). Urea levels were increased (17–38%) at 500 mg/kg bw per day in both sexes (significant only in the 500 mg/kg bw per day group receiving batch A). Relative liver weights were increased at 100 mg/kg bw per day (8–13%) and 500 mg/kg bw per day (45–60%). Relative kidney weights were increased at 500 mg/kg bw per day (15–22%). Male rats had increased relative thyroid weights at 100 mg/kg bw per day (11–40%) and 500 mg/kg bw per day (34–53%). At the high dose, ovary weights were increased (15–17%). Histopathology showed minimal hypertrophy of the centrilobular hepatocytes in all treated groups in males and at 500 mg/kg bw per day in females, minimal to moderate hepatocellular necrosis at 500 mg/kg bw per day in males and inflammatory cell infiltrations in the liver of both sexes at 500 mg/kg bw per day. At both doses, minimal hypertrophy of the follicle epithelium of the thyroid was seen in both sexes. High-dose females showed cortical atrophy of the adrenal glands and minimal extramedullary haematopoiesis in the spleen. The data showed that both batches induced similar toxicity, although the effects tended to be less accentuated in the rats treated with batch A.

A NOAEL could not be identified. The LOAEL for both batches of penconazole was 100 mg/kg bw per day, based on changes in clinical chemistry and haematology parameters and minimal hypertrophy of the follicle epithelium of the thyroid at all doses (Fankhauser, 1991).

In a 13-week dietary toxicity study, penconazole (purity 91.7%; batch no. P.11-14) was administered to groups of 20 male and 20 female Tif: RAI f (SPF) rats at 0, 30, 300 or 3000 ppm (equal to 0, 2.0, 19 and 202 mg/kg bw per day for males and 0, 2.1, 21 and 209 mg/kg bw per day for females, respectively). Animals were checked daily for mortality and clinical signs (including neurological, oral and behavioural inspection). Ophthalmological examinations and a hearing test were conducted at the beginning and towards the end of the treatment period. Body weights and feed consumption were measured weekly. Water consumption was measured monthly. Blood was sampled for haematology and clinical biochemistry at termination. Urine analysis was not performed. All rats were necropsied, and weights of liver, spleen, kidneys, testes, ovaries, heart, brain, thymus and adrenals were recorded. A wide range of tissues of all rats was examined microscopically.

No mortalities or clinical signs were observed. Ophthalmoscopy and the hearing test revealed no treatment-related effects. Significant reductions in body weight gain (26%) and feed consumption (10%) were found in high-dose females. Occasional changes in haematology and clinical chemistry were small and within the historical control range and were considered not to be toxicologically relevant. Absolute (21–22%) and relative liver weights (27–40%) were increased in both sexes at 3000 ppm. In the absence of other signs of liver toxicity, these increases in liver weight are not considered adverse. In the 3000 ppm males, both the absolute and relative testes weights were slightly higher (5% and 10%, respectively). Necropsy revealed no treatment-related effects. Histopathology revealed minimal hypertrophy of the hepatocytes in both sexes at 3000 ppm.

The NOAEL was 300 ppm (equal to 19 mg/kg bw per day), based on reduced body weight gain and feed consumption in females and increased testes weight observed at 3000 ppm (equal to 202 mg/kg bw per day) (Basler, 1982).

In another 13-week dietary toxicity study, penconazole (purity 91.7%; batch no. P.11-14) was administered to groups of 20 male and 20 female Tif: RAI f (SPF) rats at 0, 10, 30 or 100 ppm (equal to 0, 0.77, 2.1 and 7.1 mg/kg bw per day for males and 0, 0.78, 2.1 and 7.3 mg/kg bw per day for females, respectively). Animals were checked daily for mortality and clinical signs (including neurological, oral and behavioural inspection). Ophthalmological examinations and a hearing test

were conducted at the beginning and towards the end of the treatment period in control and 100 ppm rats. Body weights and feed consumption were measured weekly. Water consumption was measured weekly during the first month and monthly thereafter. Blood was sampled for haematology and clinical biochemistry at termination. Urine analysis was not performed. All rats were necropsied, and weights of liver, kidneys, testes, ovaries, heart, brain, thymus and adrenals were recorded. A wide range of tissues of all rats was examined microscopically.

No mortalities or clinical signs were observed. No treatment-related effects on body weight gain, feed and water consumption, or ophthalmoscopy or in the hearing test were observed. Occasional changes in haematology and clinical chemistry were small and within the historical control range and were considered not to be toxicologically relevant. Absolute and relative liver weights were increased in males at 10 and 30 ppm (up to 23%), but not at 100 ppm. Relative kidney weights were slightly decreased in females at 10 and 30 ppm (11%) and 100 ppm (7%). In the absence of a dose–response relationship and other signs of liver or kidney toxicity, these changes in organ weight were considered not to be adverse. In the 3000 ppm males, both the absolute and relative testes weights were slightly higher (5% and 10%, respectively). Necropsy and histopathology revealed no treatment-related effects.

The NOAEL was 100 ppm (equal to 7.1 mg/kg bw per day), the highest dose tested (Basler, 1983).

In a third 13-week dietary toxicity study, penconazole (purity 98.7%; batch no. FL-840833) was administered to groups of 15 male and 15 female Charles River (Crl:CD(SD)BR) rats at 0, 10, 100, 300, 500, 1000 or 2400 ppm (equal to 0, 0.81, 7.5, 23, 38, 72 and 179 mg/kg bw per day for males and 0, 0.96, 9.1, 28, 45, 86 and 209 mg/kg bw per day for females, respectively). Animals were checked daily for mortality and clinical signs (including neurological, oral and behavioural inspection). Detailed physical examination was performed weekly. Ophthalmological examinations were conducted at the beginning of the study in all rats and towards the end of the treatment period in control and 2400 ppm rats. Body weights and feed consumption were measured weekly. Blood was sampled for haematology and clinical biochemistry at termination. Urine analysis was performed during week 13. All rats were necropsied, and weights of liver, kidneys, testes, ovaries, heart, brain, spleen and adrenals were recorded. A wide range of tissues of all rats was examined microscopically.

No mortalities or clinical signs were observed. Over the entire study duration, decreases in body weight gain (15%) and feed consumption (9%) were observed in females at 2400 ppm. In males at 1000 and 2400 ppm, body weight gain was decreased (10%) during the first week of treatment. Ophthalmoscopy and haematology showed no effects of treatment. Occasional changes in clinical chemistry were small, often not dose related and within the historical control range and were considered not to be toxicologically relevant. In all male treatment groups, urea nitrogen was slightly, but significantly, higher (12–35%) than in the control group. The creatinine level was marginally increased in the 1000 ppm ($P < 0.05$) and 2400 ppm (statistically not significant) males, as well as in the 500, 1000 and 2400 ppm females (not significant). Total protein level was somewhat increased (9%) in males at 2400 ppm, and the albumin to globulin ratio was marginally lower (10%) in the males at 1000 and 2400 ppm. The albumin to globulin ratio was also significantly lower (10%) in females given 2400 ppm because of significantly lower albumin. The changes were not accompanied by other evidence of primary renal toxicity. Absolute and relative liver weights were increased in males at 1000 and 2400 ppm (up to 31%) and in females at 500, 1000 and 2400 ppm (up to 29%). The absolute and relative weights of the left adrenal were significantly lower in males at 100 and 500 ppm, but not at higher doses. No changes in adrenal weights were observed in females. Therefore, the effects on adrenal weights in males were not considered to be related to the treatment. At 2400 ppm, females showed significantly lower body weights, resulting in significantly higher relative weights for the brain, left adrenal, left ovary, and left and right kidney. Histopathological examination showed increased incidences of centrilobular hypertrophy of hepatocytes in the 1000 and 2400 ppm males and females and a slightly increased incidence in males at 500 ppm. Degeneration of the hepatocytes

around the central vein was observed in males and females at 2400 ppm. The incidence of hepatocellular vacuolation was dose-dependently increased in males at 500, 1000 and 2400 ppm.

The NOAEL was 300 ppm (equal to 23 mg/kg bw per day), based on an increased incidence of hepatocellular vacuolation and hypertrophy at 500 ppm (equal to 38 mg/kg bw per day) (Hiles, 1987c).

Dogs

In a 1-year dietary toxicity study, 10 male and 10 female Beagle dogs per dose group received penconazole (purity 91.7%; batch no. P.11-14) at a dietary concentration of 0, 100, 500 or 5000 ppm (equal to 0, 3.1, 16.9 and 133 mg/kg bw per day for males and 0, 3.3, 16.7 and 139 mg/kg bw per day for females, respectively). During week 20, the highest dose was reduced to 2500 ppm (equal to 86 mg/kg bw per day for males and 89 mg/kg bw per day for females) because of excessive reduction in feed consumption and body weight gain for the animals in that group.

Four males and four females per dose group were killed after 13 weeks or 1 year of treatment, whereas two dogs of each sex per dose were killed at the end of a 4-week recovery period following 1 year of treatment. The dogs were checked daily for mortality and clinical signs. Feed consumption was measured daily, and body weights were measured weekly. Ophthalmological and auditory examinations were performed pretreatment, in week 13, in week 52 or 56, and in the week before termination. Haematology, clinical chemistry and urine analysis were performed pretrial and during weeks 13, 26, 52 and 56. All dogs were necropsied, and weights of brain, thyroid, heart, liver, adrenals, kidneys, testes and ovaries were recorded. Histology was performed on a large selection of organs.

None of the dogs died during the study. An increased incidence of vomiting was seen in both sexes at 5000 ppm and in females at 2500 ppm. Ophthalmological and auditory examinations revealed no treatment-related effects.

During the first 13 weeks of treatment, slight reductions in body weight gain (2–27%) were observed at 500 ppm, whereas at 5000 ppm, the dogs lost body weight (10–12%). Feed consumption was drastically reduced during the first 19 weeks of treatment at 5000 ppm (42–53% during week 1). After lowering the dose to 2500 ppm, no difference in feed consumption was observed in males, whereas feed consumption tended to be increased in high-dose females. At 13 weeks, reduced haemoglobin levels (9%) and erythrocyte counts (10%) were observed in males at 5000 ppm. However, similar changes were also observed before treatment started, and therefore these changes are not considered to be toxicologically relevant. At 13 weeks, increased activities of ALP (~400%), GGT (~1000%), aspartate aminotransferase (ASAT) (~150%), ALAT (~800%) and ornithine carbamoyltransferase (OCT, ~450%) were observed in both sexes at 5000 ppm. At 5000 ppm, there was a decrease in glucose levels (8–12%, both sexes), decreased urea nitrogen (females), increased bilirubin (32%, females), increased total globulin (12%, males), increased sodium concentrations (3%, females) and decreased chloride concentration (3%, males). Urine analysis showed no treatment-related changes. Absolute and relative liver weights of males and females were higher at 500 (15–24%) and 5000 ppm (absolute, 30–41%; relative, 75–88%). Kidney weights of both sexes at 5000 ppm were increased (absolute, 16–18%; relative, 54–60%), as was the relative adrenal weight of the 5000 ppm females (38%). Testes weight was reduced at 5000 ppm (absolute, 48%; relative, 27%).

At 13 weeks, all 5000 ppm dogs and one 500 ppm male showed minimal, multifocal changes in the liver in the form of monocellular hepatocyte necrosis associated with minimal inflammatory cell infiltration. Small, circumscribed foci (with loss of hepatocytes, haemorrhage) and inflammatory cell infiltration were also found in some dogs. Two males at 5000 ppm also had vacuolation of the hepatocyte cytoplasm that was considered to be degenerative in nature. All males at 5000 ppm had a moderate to marked reduction in spermatogenic activity, characterized by atrophy of the seminiferous epithelium associated with formation of giant cells and absence of spermatozoa in the epididymis (which contained cellular debris).

After 12 months of treatment, body weight gain over the entire study was not affected at 100 ppm, whereas it was slightly reduced at 500 ppm (−20%) and markedly reduced at 2500 ppm (−40% in males, −60% in females). After reduction of the dose level from 5000 ppm to 2500 ppm in week 20, the high-dose animals gained weight faster than the animals of the other groups, including the controls. After lowering the dose to 2500 ppm, feed consumption returned to normal. No treatment-related changes in haematology were observed at 26 or 52 weeks. After reduction of the highest concentration of 5000 ppm to 2500 ppm, most of the metabolic parameters affected at the 13-week investigation returned to normal (e.g. urea nitrogen, bilirubin, sodium and chloride concentrations). OCT activity remained increased in the 2500 ppm animals (300–1700%, both sexes) until termination of the treatment. Total globulin level was increased (16%) in males at 2500 ppm at weeks 26 and 53. Levels of the electrolytes sodium and chloride showed slight deviations in the 5000 ppm group at 13 weeks, but were comparable with control values after the dietary concentration had been reduced to 2500 ppm. Slight increases in inorganic phosphate levels that were observed in high-dose females throughout the study were within normal biological ranges. At 2500 ppm, increased activities of ALP (~400%), GGT (~300–500%), ASAT (~100%) and ALAT (~400–600%) were observed at 26 and 52 weeks. Liver weights of females were higher at 500 ppm (absolute, 27%; relative, 28%) and in both sexes at 2500 ppm (absolute, 27–46%; relative, 35–63%). Kidney weights of both sexes at 2500 ppm were increased (absolute, 12–25%; relative, 21–39%). In females, increased relative weights of heart and ovaries were observed. In the absence of a dose–response relationship and histopathological changes in these organs, these observations were considered not to be treatment related. Histopathology showed minimal lesions of the liver in all dogs at 2500 ppm and in some dogs at 500 ppm. These lesions were characterized by monocellular hepatocyte necrosis associated with inflammatory cell infiltration. In addition, two 2500 ppm males showed absence of spermatozoa in the epididymis due to atrophy of the seminiferous epithelium.

After 4 weeks of recovery following the 12-month treatment period, most affected clinical chemistry parameters and enzyme activities returned to normal in the high-dose dogs (ALP, GGT, ASAT) or were at least clearly lower than at the end of the treatment (ALAT, OCT). Histopathology in recovery animals showed hepatocyte necrosis associated with inflammatory cell infiltration in one high-dose male. Minimal to moderate bilateral tubular atrophy was still present in both males of the high-dose group after the 4-week recovery period.

The NOAEL was 100 ppm (equal to 3.1 mg/kg bw per day), based on reduced body weight gain, increased absolute and relative liver weights, and slight histopathological changes in the liver (hepatocyte necrosis associated with inflammatory cell infiltration) in males and females at 500 ppm (equivalent to 16.7 mg/kg bw per day) (Gfeller, 1984).

(b) Dermal application

Groups of five male and five female New Zealand White rabbits were dermally exposed to penconazole (purity 91.7%; batch no. P.11-14) moistened with water at a dose of 0, 1000, 1500 or 2000 mg/kg bw per day for 6 hours/day, 5 days/week, for 3 weeks. The test item was applied to the clipped dorsal area (about 10% of the body surface) and held in contact with the skin with gauze patches. Patches were covered with aluminium foil and fastened with adhesive tape. A satellite group of the same size received the 2000 mg/kg bw per day treatment for 3 weeks and was then observed for a further 2-week recovery period without treatment. The rabbits were checked daily for mortality and clinical signs. Body weights were recorded weekly, and feed consumption was recorded twice weekly. Haematology and clinical chemistry were performed at the end of the study. At termination of the study, all animals were killed and necropsied. Weights of heart, spleen, brain, liver, thymus, adrenals, kidneys, testes and ovaries were recorded. Histology was performed on a selection of organs and tissues.

No mortality and no treatment-related clinical signs were observed. No dermal irritation was noted. Body weight gain, feed consumption, haematological and clinical chemistry parameters, and organ weights were not affected. Macroscopic and histopathological examination revealed no effect of treatment with penconazole.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (Seifert, 1983).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 2-year dietary carcinogenicity study, performed according to OECD Test Guideline 453, penconazole (purity 91.7%; batch no. P.11-14) was administered to groups of 80 male and 80 female Tif: MAG f (SPF) mice at 0, 5, 75, 150 or 300 ppm (equal to 0, 0.75, 9.8, 19 and 41 mg/kg bw per day for males and 0, 0.67, 8.8, 17 and 36 mg/kg bw per day for females, respectively). Fifty animals of each sex per dose were used for the evaluation of carcinogenic potential, 20 animals of each sex per dose were used for laboratory investigations up to 24 months and 10 animals of each sex per dose were used for an interim kill after 12 months. The mice were checked daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly during the first 3 months and monthly thereafter. Water consumption was measured at weeks 4, 8, 12, 18 and 23 of the study. A hearing test and eye examination were performed on control and high-dose animals pretreatment and on days 177, 360, 568–569 and 716. Blood and urine from the mice destined for laboratory investigations were sampled for haematology, clinical chemistry and urine analysis at weeks 14 (except blood chemistry), 27, 52, 81 and 105. Animals found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (adrenals, brain, heart, liver, kidneys, lungs, spleen, thyroid, ovaries, testes, prostate and pituitary) were recorded. A wide range of tissues was examined microscopically.

No effect of treatment with penconazole on mortality or clinical signs was observed. The hearing tests and eye examinations revealed no effect of treatment. Body weight gain, feed and water consumption, and feed efficiency were not affected by treatment. Occasionally observed statistically significant differences in haematological, clinical chemistry and urine analysis parameters relative to controls did not show any systematic or dose-dependent pattern and were attributed to normal spontaneous physiological variations. After 53 weeks, relative liver weights were marginally increased in males at 300 ppm (10%). In the absence of related findings on clinical chemistry and histopathology, this increase was not considered to be adverse. Macroscopic and histopathological examination did not reveal an effect of treatment. There were no treatment-related increases in the incidence of neoplastic lesions.

The NOAEL was 300 ppm (equal to 36 mg/kg bw per day), the highest dose tested. Penconazole was not carcinogenic in Tif: MAG f (SPF) mice under the conditions of the study (Basler, 1985a).

In an 80-week dietary carcinogenicity study performed according to OECD Test Guideline 451, penconazole (purity 97.7%; batch no. WS007001[CH]) was administered to groups of 50 male and 50 female C57BL/10J$_f$CD-1 mice at 0, 25, 200 or 1500 ppm (equal to 0, 2.7, 22 and 178 mg/kg bw per day for males and 0, 3.5, 28 and 222 mg/kg bw per day for females, respectively). The mice were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Body weight was recorded weekly during weeks 1–15 and biweekly thereafter. Feed consumption was recorded weekly during weeks 1–13, in week 16 and every fourth week thereafter. Feed utilization efficiency was calculated every month for the first 13 weeks. Blood smears prepared from blood collected after 53 weeks and at termination were examined. Animals found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (adrenals, brain, heart, liver including gallbladder, kidneys, lungs, spleen, ovaries, uterus and cervix, testes, epididymides and prostate) were recorded. A wide range of tissues was examined microscopically.

Mortality at 80 weeks was not affected by treatment. The number of males with thin appearance was increased at 1500 ppm. Body weight gain was decreased at 1500 ppm in males (29%) and females (23%). Feed intake was not affected, but feed utilization was less efficient in both sexes at the high dose. Macroscopic and blood smear examination did not reveal an effect of treatment. In

high-dose males, liver weights were increased (absolute, 11%; relative, 27%). Slight reductions in kidney weights (< 10%) in both sexes at 1500 ppm were not considered treatment related. At 1500 ppm, spleen weights were reduced in males (absolute, 31%; relative, 23%) and females (absolute, 41%; relative, 37%). Histopathological examination showed an increased incidence and severity of hepatocellular vacuolation in both sexes at 1500 ppm. There were no treatment-related increases in the incidence of neoplastic lesions.

The NOAEL was 200 ppm (equal to 22 mg/kg bw per day), based on decreased body weight gain and absolute and relative spleen weights and increased incidence and severity of hepatocellular vacuolation in both sexes and increased absolute and relative liver weights in males at 1500 ppm (equal to 178 mg/kg bw per day). Penconazole was not carcinogenic in C57BL/10J$_f$CD-1 mice under the conditions of the study (Milburn, 2004).

Rats

In a chronic (27-month) dietary toxicity study performed according to OECD Test Guideline 453, penconazole (purity 91.7%; batch no. P.11-14) was administered to groups of 80 male and 80 female Tif: RAI f (SPF) rats at 0, 5, 75, 150 or 300 ppm (equal to 0, 0.30, 3.8, 7.3 and 15 mg/kg bw per day for males and 0, 0.31, 4.0, 8.1 and 17 mg/kg bw per day for females, respectively). Fifty animals of each sex per dose were used for the evaluation of carcinogenic potential, 20 animals of each sex per dose were used for laboratory investigations up to 24 months and 10 animals of each sex per dose were used for an interim kill after 12 months. The rats were checked daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly during the first 3 months and monthly thereafter. Water consumption was measured monthly during the first 5 months. A hearing test and eye examination were performed on control and high-dose animals pretreatment and on days 177, 361, 568–570 and 730–732. Blood and urine from the rats destined for laboratory investigations were sampled for haematology, clinical chemistry and urine analysis at weeks 14 (except blood chemistry), 27, 52, 81, 104 and 116–117. Animals found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (adrenals, brain, heart, liver, kidneys, lungs, spleen, thyroid, ovaries, testes, prostate and pituitary) were recorded. A wide range of tissues was examined microscopically.

There were no effects of treatment on mortality or clinical signs. The hearing tests and eye examinations revealed no effect of treatment. Body weight gain, feed and water consumption, and feed efficiency were not affected by treatment. GGT levels were increased (41–120%) in high-dose females at 27 and 52 weeks. Other occasionally observed statistically significant differences in haematological, clinical chemistry and urine analysis parameters relative to controls did not show any systematic or dose-dependent pattern and were attributed to normal spontaneous physiological variations. Relative liver weights were increased in females at 150 and 300 ppm (13–15%) after 52 weeks, and absolute liver weights were increased in 300 ppm females (13%) after 104 weeks. Absolute and relative pituitary weights were decreased in high-dose males (25–29%) at 1 year, but not after 2 years. A trend (statistically not significant) to higher absolute spleen weights (10–16%) was seen in treated male and female rats at 1 year. As this was not confirmed at the 2-year time point and as relative spleen weights were not affected, this finding is not considered to be toxicologically relevant. There were a number of statistically significant differences in a few other organ weights relative to controls, but as these were not dose related, they are considered to be unrelated to the treatment. Histopathological examination revealed no effect of treatment. The incidence and type of tumours were not affected by treatment with penconazole.

The NOAEL was 150 ppm (equal to 8.1 mg/kg bw per day), based on increased absolute and relative liver weights and an increase in GGT levels at 1 year in females at 300 ppm (equal to 17 mg/kg bw per day). Penconazole was not carcinogenic in Tif: RAI f (SPF) rats under the conditions of the study (Basler, 1985b).

2.4 Genotoxicity

The results of the genotoxicity tests with penconazole are summarized in Table 15. All tests gave negative results.

Table 15. Overview of genotoxicity tests with penconazole[a]

Test	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Gene mutations	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537	10–2 560 µg/plate (±S9)	91.7	Negative	Deparade (1984)[b]
Gene mutations	*S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537; *Escherichia coli* WP2 uvrA	12.5–5 000 µg/plate (±S9)	96.1	Negative	Deparade (1999a)[c]
Gene mutations	V79 Chinese hamster cells, HPRT test	5–80 µg/mL (±S9)	96	Negative Study not fully adherent to OECD guideline	Ogorek (1999a)[d]
Chromosomal aberrations	Chinese hamster ovary cells	6.25–50 µg/mL (−S9) 6.25–25 µg/mL (−S9)	96	Negative	Ogorek (1999b)[e]
Unscheduled DNA synthesis	Male Tif: RAI f (SPF) rat hepatocytes	0.32–40 µg/mL	91.7	Negative	Puri (1984)[f]
In vivo					
Micronucleus formation	ICO:CD-1(CRL) mouse bone marrow	Gavage dose of 200, 400 or 800 mg/kg bw in males and 125, 250 or 500 mg/kg bw in females	96.1	Negative	Deparade (1999b)[g]

bw: body weight; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; OECD: Organisation for Economic Co-operation and Development; S9: 9000 × *g* supernatant fraction from rat liver homogenate

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

[b] Batch no. P.11-14. Study design resembles OECD Test Guideline 471. Without S9, cytotoxicity was observed at 640 and 2560 µg/plate (first assay) or 2560 µg/plate (second assay). With S9 activation, cytotoxicity occurred at 2560 µg/plate.

[c] Batch no. EN 603012. Performed according to OECD Test Guideline 471.

[d] Batch no. EN 602012. Performed according to OECD Test Guideline 476. In preliminary tests, cytotoxicity was complete or excessive at concentrations of 50 µg/mL and above.

[e] Batch no. EN 602012. Performed according to OECD Test Guideline 473. In preliminary tests with and without S9, inhibition of cell growth was complete or almost complete at concentrations of 100 µg/mL and above.

[f] Batch no. P.11-14. Study design resembles OECD Test Guideline 482, which was deleted in 2014. Vehicle was dimethyl sulfoxide. Cytotoxicity data were not reported. In the report, it is stated that "from the results obtained, the highest usable concentration was calculated to be 40 µg/mL".

[g] Batch no. EN 603012. Performed according to OECD Test Guideline 474. Vehicle was 0.5% carboxymethyl cellulose in water. Five male and five female young adult mice were used per group. Groups of animals treated at the highest dose or with the vehicle alone were killed 24 and 48 hours after administration, whereas animals administered the intermediate or lowest dose or the positive control substance were killed 24 hours after administration. The high-dose males showed occasional signs of toxicity (ventral recumbence, hunched posture, reduced locomotor activity). No effect of penconazole on polychromatic erythrocyte/normochromatic erythrocyte ratio was observed. Toxicokinetic studies (Van Dijk, 1987; Hassler, 1999) show that radioactivity does reach the bone marrow, albeit in low concentrations.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation dietary reproductive toxicity study, Tif: RAI f (SPF) albino rats (20 of each sex per group for the F_0 and F_1 generations) were fed penconazole (purity 91.7%; batch no. P.11-14) at a dietary concentration of 0, 80, 400 or 2000 ppm. The average dietary intakes over the premating, gestation and lactation periods were equal to 0, 5.5, 29 and 146 mg/kg bw per day for males and 0, 7.5, 40 and 202 mg/kg bw per day for females of the F_0 generation and 0, 6.5, 31 and 166 mg/kg bw per day for males and 0, 8.5, 43 and 227 mg/kg bw per day for females of the F_1 generation, respectively. F_0 adults were treated over a 9-week premating period and throughout the 3-week mating period, gestation and 21-day lactation of the F_1 pups. At postnatal day (PND) 28, 20 weanling rats of each sex per dose were selected for producing the F_2 generation. The remaining weanlings were killed and checked for macroscopic anomalies. The F_1 parental rats were treated for 9 weeks before mating. Clinical examination was performed daily. Detailed observations were made on maternal F_0 and F_1 animals on PNDs 0, 4, 7, 14 and 21. Body weights of parental rats were recorded weekly. In addition, females were weighed on gestation days (GDs) 0, 7, 14 and 20 and PNDs 0, 4, 7, 14 and 21. Feed consumption was recorded weekly during the premating period. In addition, feed consumption of parental females was measured on GDs 0, 6, 11, 16 and 21 and on PNDs 1, 6, 11, 16 and 21. Fertility and mating indices of males and females, copulatory interval 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. Pups were weighed on PNDs 0, 4, 7, 14 and 21. All pups were checked daily for mortality and clinical signs. The following developmental parameters were assessed in all pups: pinna unfolding (PND 4), onset of hair growth (PND 5), incisor eruption (PND 8), eye opening and righting reflex (PND 14) and photophobotaxis (PNDs 21–23). In pups selected for breeding the F_2 generation, the following parameters were assessed: cliff avoidance, palmar grasp ability, negative geotaxis, exploratory behaviour (PNDs 29–32), vaginal opening (PND 30), pupillary reflex (PNDs 35–40), hearing ability and exploratory behaviour (PNDs 40–45). After weaning, the parental rats were killed and examined macroscopically. In males of the F_0 generation, testes and epididymides were weighed. In the F_1 adult generation and in five F_1 and F_2 weanlings of each sex per dose, weights of adrenals (adults only), brain, kidneys, heart, liver, lungs (weanlings only), ovaries, spleen, testes and thymus were recorded. A wide range of organs from these animals was examined histopathologically.

One control F_1 female, two 400 ppm females (one F_0, one F_1) and six 2000 ppm females (three F_0, three F_1) died during the lactation period. The cause of death was not reported consistently (e.g. for one dam, it is reported that it died during delivery, was killed due to spontaneous death of all pups or was killed in a moribund state). For some dams, it is not clear whether litters were delivered successfully. The interpretation of the reported mortalities is therefore difficult. No clinical signs were reported. During the premating phase, body weight gain was slightly decreased at 2000 ppm (8% in F_0 females, 3% in F_1 males, 7% in F_1 females). During gestation, a slight reduction in body weight gain (16%) was observed in F_1 females at 2000 ppm. Feed consumption was slightly reduced (4%) in females at 2000 ppm. These slight reductions in body weight gain and feed consumption were considered not to be toxicologically relevant. Relative liver weights were increased at 2000 ppm in F_1 parental males (13%) and females (37%). Relative testes weight was slightly increased in F_1 males at 2000 ppm; however, absolute testes weight and testes weight relative to brain weight were not affected. In the liver of adult F_1 rats, slight hypertrophy of the hepatocytes was observed at 400 and 2000 ppm, mainly in the centrilobular region. In 2/16 adult F_1 females at 2000 ppm, slight recent necrosis in the liver was found. In the absence of other histopathological changes, the slight increase in hypertrophy in rats at 400 ppm and in male rats at 2000 ppm is not considered to be toxicologically relevant. A slightly lower gestation index (79–84% versus 95–100% in controls) was observed at 2000 ppm. Gestation duration was slightly increased (from 0.7 to 0.8 day) at 2000 ppm. In both generations, the mean number of implantation losses was slightly increased in all treated groups (not dose related). Reproductive performance, mating and fertility indices were not impaired in F_0 or F_1 parents. In both generations, the survival index at PND 4 was not affected by treatment, but the postnatal mortality rates of the pups were markedly increased in all treated and control groups during

the second week of lactation, particularly for F_1 pups from the control group and to a lesser extent in the 80 and 400 ppm groups. Mean body weight gains of F_1 pups appeared to be lower at weaning at the 2000 ppm level (18% and 13% in male and female pups, respectively, compared with controls; not significant). However, this is likely to be a consequence of the higher litter size at weaning at 2000 ppm (13.2 pups/litter) compared with controls (9.9 pups/litter). The general development and behaviour of the offspring were not affected by treatment. In the F_1 and F_2 weanlings, increased relative liver weights (22–29%) were observed. In F_1 and F_2 weanlings, no histopathological changes were seen in treated or control animals. In the absence of histopathological changes, the increase in relative liver weights in weanlings at 2000 ppm is not considered to be toxicologically relevant.

The NOAEL for parental toxicity was 400 ppm (equal to 43 mg/kg bw per day), based on increased relative liver weights and the observation of hepatocellular necrosis in F_1 parental females at 2000 ppm (equal to 227 mg/kg bw per day).

The NOAEL for offspring toxicity was 2000 ppm (equal to 146 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 400 ppm (equal to 40 mg/kg bw per day), based on a lower gestation index and a longer gestation duration in F_0 and F_1 females at 2000 ppm (equal to 202 mg/kg bw per day) (Fritz, 1983a,b).

In another two-generation dietary reproductive toxicity study, Charles River COBS CD albino rats (30 of each sex per group for the F_0 and F_1 generations) were fed penconazole (purity not specified in the report, but the purity of batch no. FL-840833 in other studies is reported to be 98.7%) at a dietary concentration of 0, 25, 250 or 2500 ppm. The achieved intakes of penconazole by the animals during the premating, gestation and lactation periods are summarized in Table 16.

Table 16. Mean daily test substance intake

	Mean daily test substance intake (mg/kg bw per day)		
Generation	**25 ppm**	**250 ppm**	**2 500 ppm**
Premating period / males			
F_0 parents	2.0	20	191
F_1 parents	2.2	22	219
Premating period / females			
F_0 parents	2.4	24	238
F_1 parents	2.5	25	246
Gestation period / females			
F_0 generation	1.9	18	180
F_1 generation	1.7	17	175
Lactation period / females			
F_0 generation	3.3	34	346
F_1 generation	3.2	33	337

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million
Source: Schardein (1987)

F_0 adults were treated over a 9-week premating period and throughout the 3-week mating period, gestation and 21-day lactation of the F_1 pups. On PND 4, litters were culled to eight pups. Upon completion of weaning for each litter, 30 weaned rats of each sex (one male and one female per

litter, if possible) were randomly selected in each dose group to become F_1 parents for the F_2 offspring. The remaining F_1 weanlings were killed and subjected to a gross external examination and necropsy. Treatment of the selected F_1 rats continued for 15 weeks before mating and during the 3-week mating, gestation and lactation periods. Upon completion of weaning for each litter, all surviving F_2 pups were killed and subjected to a complete gross necropsy. After weaning of the F_1 and F_2 pups, the respective F_0 and F_1 parents were killed and necropsied. Clinical examination was performed daily. Detailed observations were made weekly on all parental rats and on maternal F_0 and F_1 animals on GDs 0, 7, 14 and 21 and PNDs 0, 4, 7, 14 and 21. Body weights of parental rats were recorded weekly. In addition, females were weighed on GDs 0, 7, 14 and 20 and PNDs 0, 4, 7, 14 and 21. Offspring were weighed per sex on PNDs 0, 4, 7 and 14 and individually on PND 21. Parental feed consumption was recorded weekly during the premating period. In addition, feed consumption of parental females was measured on GDs 0, 7, 14 and 20 and on PNDs 0, 7 and 14. Fertility and mating indices of males and females, copulatory interval 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 and clinical signs. The culled pups were examined for cervical, thoracic and abdominal abnormalities. After weaning, the parental rats were killed and necropsied. In all parental animals and 10 pups of each sex per dose of the F_1 and F_2 generations, the testes and ovaries were weighed. The liver, pituitary, ovaries, uterus including cervix, vagina, testes, seminal vesicle, epididymis, coagulation gland and prostate of these animals were examined histopathologically.

One 2500 ppm F_0 male and one 25 ppm F_1 female died. These deaths are not considered to be treatment related. No clinical signs were reported. During the premating phase, body weight gains in F_0 females at 2500 ppm were decreased (18%). In the F_1 parental males and females, body weight gain was slightly reduced at 2500 ppm (7–9%) during the premating phase. Feed consumption was also slightly reduced (7–8%) in F_0 and F_1 females at 2500 ppm. In the F_0 generation, the proportion of rats that mated during the first 4 days was reduced at 2500 ppm (63% versus 93% in controls). The reverse situation was seen in the F_1 generation (73% at 2500 ppm versus 60% in controls). In both generations, the overall mating index after completion of the 3-week mating period was slightly decreased in the 2500 ppm groups (80% versus 90–97% in controls). Values for fertility and gestation indices, parturition and length of gestation were similar to control values in all treated groups of both generations. In both generations, the number of pups that were dead at birth or that died during the first 4 days of lactation (including missing/cannibalized pups) was slightly higher at the 2500 ppm level (n = 14–37) when compared with controls (n = 4–14). After culling, pup survival during lactation was similar to control survival in all treatment groups. The ratio of male to female rats at the 2500 ppm level in the F_1 generation was lower throughout lactation. However, a similar sex ratio was observed in control pups of the F_2 generation, and the sex ratio was not affected at 2500 ppm in the F_2 generation. It is concluded that the sex ratio was not affected by treatment with penconazole. In both generations, slight, but generally significant, decreases in mean pup weights were evident for male and female high-dose pups on PND 14 (6–12%) and PND 21 (6–11%). In both generations, uterine examinations of dams at weaning gave no indication of any treatment-induced deviations in the number of implantation sites, postimplantation loss or litter size. Necropsy and histological examination of the parental animals or the pups revealed no treatment-related effects. In parental F_0 and F_1 females, a slight increase in relative ovary weights was observed. However, as absolute ovary weights were not affected, this is considered not to be toxicologically relevant.

The NOAEL for parental toxicity was 250 ppm (equal to 24 mg/kg bw per day), based on reduced body weight gain and feed consumption during the premating period in F_0 and F_1 females at 2500 ppm (equal to 238 mg/kg bw per day).

The NOAEL for offspring toxicity was 250 ppm (equal to 20 mg/kg bw per day), based on an increased number of pups that were born dead or died during PNDs 0–4 and a decreased body weight gain of pups during lactation at 2500 ppm (equal to 191 mg/kg bw per day).

The NOAEL for reproductive toxicity was 250 ppm (equal to 20 mg/kg bw per day), based on a decreased mating index at 2500 ppm (equal to 191 mg/kg bw per day) (Schardein, 1987).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 25 pregnant female Tif: RAI f (SPF) rats were treated orally, by gavage, with penconazole (purity 88.4%; batch no. P.2+3) in 2% aqueous carboxymethyl cellulose at a dose of 0, 30, 100 or 300 mg/kg bw per day from days 6 through 15 of gestation (day 0 = day on which sperm were detected in the vaginal smear). The selection of the upper dose was based on a preliminary study. In a supplementary study, to further investigate the effect of penconazole on skeletal development, groups of 15 pregnant rats were treated with penconazole at a dose of 0 or 300 mg/kg bw per day from GD 6 to GD 15 or 450 mg/kg bw per day from GD 10 to GD 14. Clinical signs, mortality and weight gain were recorded daily. Feed consumption was measured on GDs 6, 11, 16 and 21. All females were killed on day 21 of gestation and subjected to gross examination. The uterus was examined and weighed, and the numbers of live and dead fetuses, implantations, and early and late resorptions were counted. The number of corpora lutea was not reported. Body weight and sex of the fetuses were recorded. About two thirds of the fetuses from each litter were selected for skeletal examinations, and one third for visceral examinations.

Two high-dose dams died at the end of gestation. Necropsy did not reveal any obvious pathological conditions. No treatment-related clinical signs or abortions were observed. Body weight gain was slightly decreased at 30 and 300 mg/kg bw per day, but was increased at 100 mg/kg bw per day. Feed consumption was not significantly affected by treatment. The number of early resorptions was increased at 300 mg/kg bw per day (9%) compared with controls (5%); however, the increase was not statistically significant. The number of live fetuses was slightly decreased at 100 mg/kg bw per day (5%) and 300 mg/kg bw per day (9%). Fetal body weights and sex ratios were not affected by treatment. The number of skeletal anomalies (mainly irregularly shaped second and fifth sternebrae) was increased at 300 mg/kg bw per day (11/182) compared with the controls (2/187). The number of still unossified phalangeal nuclei of the hindlimbs was slightly increased at 100 mg/kg bw per day (37%) and 300 mg/kg bw per day (25%) compared with controls (15%).

In the supplementary study, four dams at 300 mg/kg bw per day and two dams at 450 mg/kg bw per day died on GD 21. Necropsy did not reveal any obvious pathological conditions. Body weight gain was reduced at 300 mg/kg bw per day, but not at 450 mg/kg bw per day. Body weights of fetuses at 300 and 450 mg/kg bw per day were slightly reduced (6%). Skeletal assessment revealed an increased number of unossified phalangeal nuclei of the forelimbs at 450 mg/kg bw per day and unossified phalangeal nuclei of the hindlimbs and heel bone in both groups treated with penconazole. In 11 fetuses from two litters (mainly in one litter) at 450 mg/kg bw per day, the fronto-parietal region showed “wide sutures”. One skeletal anomaly (irregularly shaped sternebrae) was observed in the vehicle control. The Meeting noted that the 450 mg/kg bw per day group was treated only from GD 10 to GD 14. Therefore, the data from this group are considered to be of limited value.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality and reduced body weight gain observed at the end of gestation at 300 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on delayed ossification observed at 300 mg/kg bw per day (Fritz, 1981).

In another developmental toxicity study, groups of 25 pregnant female Sprague-Dawley rats were treated orally, by gavage, with penconazole (purity 98.7%; batch no. FL.840833) in corn oil at a dose of 0, 5, 100 or 500 mg/kg bw per day from days 6 through 15 of gestation (day 0 = day on which breeding was confirmed). Clinical signs and mortality were recorded daily. Body weight and feed consumption were recorded on GDs 0, 6, 13 and 19. All females were killed on day 20 of gestation and subjected to gross examination. The uterus was examined and weighed, and the numbers 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.

One dam at 5 mg/kg bw per day died as a result of a dosing injury. Two high-dose dams died, on GD 11 and GD 12, respectively. Based on antemortem observations (damp, yellow fur and discharge around the nose and mouth or exudate around eyes, nose, mouth and perianal region, observed early during treatment) and the finding of stomach lesions (multiple black or distended smooth non-glandular mucosa) in both females and distended colon and dark contents in the small intestine in one female, these deaths were considered to be treatment related. At 500 mg/kg bw per day, crusty eye(s), crusty nose and/or muzzle, damp and yellow/brown-stained fur in perianal and/or abdominal region, staggered gait, emaciation, loose stool, weakness and/or lethargy were noted. The females of the highest-dose group showed a clear and statistically significant reduction in net final body weight gain. Net body weight gain was reduced by 41% at 500 mg/kg bw per day. On GD 6, feed consumption was reduced by 19% and 42% at 100 and 500 mg/kg bw per day, respectively. In the dams that survived to termination, no gross pathological changes were observed. Dams at 500 mg/kg bw per day showed an increase in the mean number of early resorptions (2.2 [14.6%] versus 0.3 [2%] in controls, not statistically significant) and late resorption sites (0.6 [4%] versus 0.0 [0%] in controls, statistically significant) and a slight decrease in the mean number of viable fetuses. Although litter weight was not statistically significantly affected by treatment, individual fetal weight data showed significant decreases for male (6%) and female fetuses (3%) at 500 mg/kg bw per day. Fetal weight data at 5 and 100 mg/kg bw per day were not affected. Slight increases in the number of runts (nine versus two in controls) and the number of litters with runts (56% versus 9% in controls) were observed at 500 mg/kg bw per day. Occasionally observed external and visceral abnormalities were within the normal range for this strain of rats and were considered to be spontaneous. Skeletal examinations revealed no treatment-related malformations. At 500 mg/kg bw per day, there was a slight increase in the occurrence of cervical ribs (eight fetuses from five litters, versus one control fetus). The total number of fetuses/litters with abnormal findings at the high dose showed a statistically significant increase compared with the controls.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality observed after 5 and 6 days of treatment, clinical signs observed early during treatment, a reduction in net body weight gain and feed consumption on GD 6, stomach lesions and an increased incidence of late resorptions at 500 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on a slight increase in the occurrence of cervical ribs and an increase in the total number of fetuses/litters with abnormal findings at 500 mg/kg bw per day (Salamon, 1985).

Rabbits

In a developmental toxicity study, groups of 20 mated Chinchilla-type rabbits were treated orally, by gavage, with penconazole (purity 91.7%; batch no. P.11-14) in 0.5% aqueous sodium carboxymethyl cellulose at a dose of 0, 25, 75 or 150 mg/kg bw per day from days 6 through 18 of gestation (the day of mating was designated as GD 0). Mortality and clinical signs were recorded daily. Body weight was measured daily from GD 0 to GD 28. Feed consumption was measured on GDs 0, 6, 11, 15, 19, 24 and 28. All females were killed on day 28 of gestation. All does were examined macroscopically for abnormalities. The reproductive tract was examined, and the numbers 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 subjected to skeletal and visceral examinations.

One control dam and one high-dose dam died. These deaths were not considered to be treatment related. No effect of treatment on the incidence of clinical signs was observed. Body weight gains for the overall pregnancy period (GDs 0–28) were comparable for all groups. However, body weight development of high-dose females (150 mg/kg bw per day) showed a different pattern during the treatment period. At 150 mg/kg bw per day, mean body weight gain was clearly lower (50% compared with controls) during GDs 6–11, whereas it was increased during GDs 11–15 (56% compared with controls) and lowered again during GDs 15–18 (40% compared with controls). After cessation of treatment, body weight gain was increased (43% compared with controls) at the high

dose, so that the overall body weight gain during the gestation period was only 8% below the control value. Mean feed consumption at the high dose was decreased (22%) during treatment and increased (16%) during the post-treatment period. At 150 mg/kg bw per day, the numbers of corpora lutea were about 11–14% higher. In this high-dose group, the incidence of embryonic resorptions was slightly increased (9.7% of implantations versus 4.8% in the controls). These deviations were not statistically significant. Fetal weights were not affected by the treatment. In the high-dose group, one fetus with multiple malformations (right forelimb with first and fifth digits missing, cleft lip, cleft palate, microphthalmia, internal hydrocephaly, brachymelia, sternum poorly ossified), one with microphthalmia and another one with microphthalmia associated with internal hydrocephaly were found. Skeletal examinations revealed no treatment-related effects.

The NOAEL for maternal toxicity was 75 mg/kg bw per day, based on reduction of body weight gain and feed consumption during treatment at 150 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 75 mg/kg bw per day, based on the increased incidence of microphthalmia and hydroencephalus at 150 mg/kg bw per day (Giese, 1982).

In another developmental toxicity study, groups of 20 artificially inseminated New Zealand White rabbits were treated orally, by gavage, with penconazole (purity 98.7%; batch no. FL.840833) in 3% aqueous cornstarch at a dose of 0, 10, 50 or 200 mg/kg bw per day from days 7 through 19 of gestation (the day of artificial insemination was designated as GD 0). Mortality and clinical signs were recorded daily. Body weight was measured on GDs 0, 7, 10, 14, 20, 24 and 29. Feed consumption was measured daily from GD 0 to GD 29. All females were killed on day 29 of gestation. All does were examined macroscopically for abnormalities. The reproductive tract was examined, and the numbers of live and dead fetuses, corpora lutea, implantations, and early and late resorptions were counted. The body weight and sex of the fetuses were recorded. All fetuses were subjected to skeletal and visceral examinations.

One control dam and one high-dose dam died. These deaths were not considered to be treatment related. In the low-, mid- and high-dose groups, two, two and one dam delivered before scheduled caesarean section on GD 29. This was similar to the historical control incidence. In high-dose dams, decreased defecation and urination were seen. Females at 200 mg/kg bw per day lost weight (123 g) during GDs 7–14, whereas control dams gained weight (87 g) over this period. Feed consumption was decreased (by about 50%) in high-dose dams from GD 7 to GD 14 and increased (by about 38%) from GD 20 to GD 29. The reduction in body weight is considered to be a consequence of the reduced feed intake and is not considered to be toxicologically adverse. After the end of treatment, the control, low-dose and mid-dose dams lost weight, whereas the body weights of the high-dose dams remained virtually constant, so that no significant differences in body weight were observed at termination. The numbers of corpora lutea and implantations were slightly decreased at the high dose. This is not considered to be treatment related. At the high dose, the mean number of early resorptions per dam was increased (1 versus 0.5 in controls). As the increase was small, was not statistically significant and remained within the range of the laboratory historical control data for this animal strain, this was not considered to be toxicologically adverse. At the high dose, the mean number of live fetuses per dam was decreased (4.8 versus 6.9 in controls). Macroscopic examination showed that the numbers of fetal malformations were lower in all treated groups than in the vehicle control group. Slightly increased incidences of fetuses with unossified hyoid body and/or arches in the mid- and high-dose groups (3.4% and 7.8%, respectively) and of fetuses with reduced ossification of the skull in the high-dose group were within the range of the historical control data of the testing laboratory and were considered not to be treatment related. Penconazole was not teratogenic under the conditions of the study.

The NOAEL for maternal toxicity was 200 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on the reduced number of live fetuses at 200 mg/kg bw per day (Nemec, 1985).

2.6 Special studies

(a) Study on liver enzyme induction

Groups of six male albino rats (strain RAI) and six male mice (MAG) per treatment group were administered penconazole (purity 98.7%; batch number not reported) at a dose of 0, 10, 80, 160, or 320 mg/kg bw per day by gavage for 14 consecutive days. Control animals (eight per species) received the vehicle alone (10 mL/kg bw). After the 14th application, the animals were fasted for 24 hours, then weighed and killed, and their livers were removed. The phospholipid and cytochrome P450 contents and activities of the ethoxycoumarin *O*-deethylase (ECOD), uridine diphosphate-glucuronosyltransferase (UGT) and epoxide hydrolase enzymes were determined in the microsomal liver fractions. Protein concentrations were determined in homogenates, microsomal fractions and cytosolic fractions. DNA concentration was determined in liver homogenates from the control and high-dose animals. The activity of the microsomal ECOD was also investigated in the presence and absence of monooxygenase inhibitors (tetrahydrofuran, 10 mmol/L; metyrapone, 100 µmol/L) or activator (7,8-benzoflavone, 10 µmol/L). Glutathione *S*-transferase (GST) was determined in the cytosolic fraction. Electron microscopic observations were conducted in the liver from control (two rats + two mice) and high-dose animals (four rats + three mice).

No deaths occurred throughout the treatment period. There was no effect on the body weight gain of either rats or mice, but relative liver weights were statistically significantly and dose-dependently increased in both species at 80 mg/kg bw per day and higher in rats and from 160 mg/kg bw per day upwards in mice. There were no effects on liver weights at 10 mg/kg bw per day. Total liver DNA contents were increased in both species in the high-dose groups (20% and 25% in rats and mice, respectively), but the DNA concentration per gram liver was lower (17–20%) following treatment with penconazole at 320 mg/kg bw per day. In the rat, relative protein contents in homogenates and in cytosolic fractions remained unchanged, whereas they were slightly increased in the mouse (but not dose dependently). In both species, penconazole caused a strong dose-dependent increase in microsomal protein (up to about 60% relative to controls) and phospholipid contents (practically doubled at 320 mg/kg bw per day). Therefore, the phospholipid/protein ratios were higher at 320 mg/kg bw per day than in the respective controls (Table 17).

Activities of xenobiotic-metabolizing liver enzymes (Table 18) were drastically increased. In both species, the effects were highly significant from the 80 mg/kg bw per day level and generally more pronounced in rats than in mice.

The influence of monooxygenase inhibitors on the activity of the rat liver microsomal ECOD activity in treated animals was more sensitive to inhibition by metyrapone, but less sensitive to inhibition by tetrahydrofuran, compared with the ECOD from the untreated control. In contrast, activation by benzoflavone was higher in untreated (+132%) than in treated samples (+49% versus controls) (Table 19). ECOD is known to be catalysed by several cytochrome P450 isozymes, but to various extents: metyrapone inhibits isozymes that are induced by phenobarbital, whereas benzoflavone inhibits the cytochrome P450 forms that respond to polycyclic aromatic hydrocarbon treatment. These results indicate that in rats, penconazole treatment altered the pattern of microsomal cytochrome P450 isozymes in a manner similar to phenobarbital.

No apparent macroscopic changes of organs were observed at necropsy.

At the 320 mg/kg bw per day dose in both species, there was extensive proliferation of smooth endoplasmic reticulum membranes. In 2/4 rats, a few cytoplasmic structures occurred that showed the morphological aspects of membranous whorls or "fingerprints". One treated rat showed numerous vacuoles or "blisters" of variable size in the pericanalicular regions of the hepatocytes (which contained flocculent material), whereas the bile canaliculi of the liver had normal structure. The other hepatocyte organelles of treated animals exhibited the morphological features of the corresponding controls.

Treatment of male rats and mice with penconazole at an oral dose of 80, 160 or 320 mg/kg bw per day for 14 consecutive days caused a marked liver enlargement, a proliferation of smooth endoplasmic reticulum membranes and a pronounced induction of the activity of several hepatic

Table 17. Study on liver enzyme induction: mean protein and DNA contents (and per cent deviation relative to controls)

	0 mg/kg bw per day	10 mg/kg bw per day	80 mg/kg bw per day	160 mg/kg bw per day	320 mg/kg bw per day
Rats					
Liver DNA					
mg	26.0	nd	nd	nd	31.1* (+20)
mg/g liver[a]	2.89	–	–	–	2.32 (−20)
Protein (mg/g liver)					
Homogenate	161	169	171	170	164
Cytosolic	56.8	54.9	57.9	56.3	55.3
Microsomal	9.55	10.6 (+11)	14.0* (+47)	15.3* (+60)	14.8* (+55)
Phospholipid (mg/g liver)					
Microsomal	4.79	5.20 (+9)	7.70* (+61)	9.86* (+106)	9.48* (+98)
Mice					
Liver DNA					
mg	4.52	nd	nd	nd	5.65** (+25)
mg/g liver[a]	3.43	–	–	–	2.86 (−17)
Protein (mg/g liver)					
Homogenate	134	136	150*	146	155** (+16)
Cytosolic	72.9	73.8	87.3* (+20)	79.6 (+9)	79.0 (+8)
Microsomal	12.8	14.2 (+11)	18.8* (+47)	16.8* (+31)	19.4* (+52)
Phospholipid (mg/g liver)					
Microsomal	5.55	6.84 (+23)	7.81* (+41)	7.61* (+37)	10.4* (+87)

bw: body weight; DNA: deoxyribonucleic acid; nd: not determined; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
[a] As calculated from the mean DNA and liver weight values (no statistical evaluation).
Source: Waechter, Bentley & Staeubli (1985)

xenobiotic-metabolizing enzymes as a result of an adaptive response of the liver to an enhanced functional load. The results indicate that penconazole belongs to the phenobarbital class of monooxygenase inducers and that the observed liver growth resulted from a combination of both cell division (hyperplasia) and hypertrophy (Waechter, Bentley & Staeubli, 1985).

(b) Studies with metabolites

1,2,4-Triazole

In a 12-month dietary toxicity study, performed according to OECD Test Guideline 452, 1,2,4-triazole (purity 98.5%; batch no. S4317788) was administered to groups of 30 male and 30 female Wistar: Crl:WI(Han) rats at 0, 125, 375, 1000 or 2000 ppm (equal to 0, 6.9, 21, 58 and 113 mg/kg bw per day for males and 0, 8.3, 26, 71 and 136 mg/kg bw per day for females, respectively). Twenty animals of each sex per dose were used to evaluate the potential for general toxicity, and 10 animals of each sex per dose were used to evaluate the potential for neurotoxicity. The rats were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly.

Table 18. Study on liver enzyme induction: activities of xenobiotic-metabolizing enzymes (and factor increase with respect to controls[a])

	0 mg/kg bw per day	10 mg/kg bw per day	80 mg/kg bw per day	160 mg/kg bw per day	320 mg/kg bw per day
Rats					
Cytochrome P450 (nmol/g liver)	11.6	15.1* [×1.3]	31.5* [×2.7]	42.2* [×3.6]	51.9* [×4.5]
ECOD (nmol/min/g liver)	4.96	6.51 [×1.3]	13.6* [×2.7]	19.9* [×4.0]	21.1* [×4.3]
Epoxide hydrolase (nmol/min/g liver)	141	179* [×1.3]	381* [×2.7]	517* [×3.7]	618* [×4.4]
UGT (μmol/min/g)	1.53	1.68 [×1.1]	2.82* [×1.8]	3.36* [×2.2]	3.42* [×2.2]
GST (μmol/min/g)	100	110 [×1.1]	132* [×1.3]	179* [×1.8]	194* [×1.9]
Mice					
Cytochrome P450 (nmol/g liver)	17.2	20.0 [×1.2]	30.3* [×1.8]	35.2* [×2.1]	43.1* [×2.5]
ECOD (nmol/min/g liver)	30.7	36.7 [×1.2]	67.1* [×2.2]	75.5* [×2.5]	106* [×3.5]
Epoxide hydrolase (nmol/min/g liver)	62.9	57.7 [×0.9]	72.0 [×1.1]	101* [×1.6]	155* [×2.5]
UGT (μmol/min/g)	1 000	1 010	1 420* [×1.4]	1 290* [×1.3]	1 530* [×1.5]
GST (μmol/min/g)	407	317 [×0.8]	440 [×1.1]	473 [×1.2]	517 [×1.3]

bw: body weight; ECOD: ethoxycoumarin-*O*-deethylase; GST: glutathione *S*-transferase; UGT: uridine diphosphate-glucuronosyltransferase; *: $P < 0.05$ (Student's *t*-test)

[a] Increasing factor versus activity of the controls shown in square brackets.

Source: Waechter, Bentley & Staeubli (1985)

Table 19. Liver enzyme induction – Inhibition of ECOD activity (and per cent deviation relative to controls)

		Enzyme activity (nmol/min/g liver)[a]	
Inhibitor	**Concentration**	**Untreated with penconazole**	**Treated with penconazole**
None	0 (control)	4.5	24.3
Tetrahydrofuran	10 mmol/L	2.03 (−55)	16.3 (−33)
Metyrapone	100 μmol/L	2.66 (−41)	5.83 (−76)
7,8-Benzoflavone	10 μmol/L	10.4 (+132)	36.2 (+49)

ECOD: ethoxycoumarin-*O*-deethylase

[a] Per cent deviation relative to controls shown in parentheses.

Source: Waechter, Bentley & Staeubli (1985)

Body weight and feed consumption were recorded weekly during the first 3 months and monthly thereafter. Ophthalmic examinations were performed before the start of the study and prior to termination. Blood and urine for haematology, clinical chemistry and urine analysis were collected from 10 rats of each sex per dose at 3, 6 and 12 months. The estrous cycle was characterized in 10 females per dose over a 23-week period prior to termination. The rats designated for neurotoxicological examination were subjected to functional observational battery and motor activity testing before treatment started and at 3, 6, 9 and 12 months. At termination, sperm were collected from one testis and one epididymis for assessment of the number of sperm cells and their morphology and motility. Animals found dead or killed prematurely were weighed and necropsied. Gross lesions were examined. At termination, 20 rats of each sex per dose were necropsied, and organ weights (adrenals, brain, heart, liver, kidneys, spleen, thyroid, uterus, ovaries, testes, epididymides, prostate and pituitary) were recorded. A wide range of tissues was examined microscopically. The animals of the neurotoxicology group were subjected to a detailed histological examination of the nervous system.

There were no effects of treatment on mortality or clinical signs. In the toxicology group, body weight gain was reduced at 1000 and 2000 ppm in males (up to 8% at both doses) and females (up to 19% at 1000 ppm and up to 20% at 2000 ppm). Similar reductions were observed in the neurotoxicology groups at these doses. Feed consumption was not affected by treatment. The neurological assessments (functional observational battery and motor activity) revealed no treatment-related effects. Landing foot splay was decreased (22–26%) in high-dose females at 3, 6 and 9 months, but not at termination. As these effects were small, were not statistically significant, were not observed after 12 months of treatment and occurred in one sex only, they are considered not to be treatment related. Occasional changes in the motor activity test were small and not statistically significant and were considered not to be related to treatment. There were no effects of treatment on ophthalmoscopy, haematology, clinical chemistry, urine analysis, estrous cycling, sperm parameters, gross pathology or organ weights. Histopathological examination revealed a statistically significant decrease (minimal to marked severity) in the population of Purkinje cells within the cerebellar vermis of the brain (especially in the dorsal region) in 2000 ppm males and females of both the main toxicology and neurotoxicology groups. In minimally affected animals, only a subtle gap or break in the continuity of the Purkinje cell layer located along the internal granular layer was observed. In severely affected animals, the decrease in Purkinje cells was marked with a variable decrease in the width of the molecular layer and density of the internal granular layer. In a few animals, white fibre tract changes with individual nerve fibre or axonal swelling and/or fragmentation, the presence of a phagocytic macrophage or increased numbers of reactive astrocytic cells were found. No other histopathological changes were observed

The NOAEL was 375 ppm (equal to 21 mg/kg bw per day), based on a reduction in body weight gain at 1000 ppm (equal to 58 mg/kg bw per day) (Wahle, 2010a).

Triazole acetic acid

In a 28-day dietary toxicity study in mice, performed according to OECD Test Guideline 407, triazole acetic acid (purity 98.5%; batch no. RDL 211-8-2) was administered to groups of 10 male and 10 female Crl:CD-1 (ICR) mice at 0, 1000, 3000 or 7000 ppm (equal to 0, 159, 483 and 1067 mg/kg bw per day for males and 0, 183, 542 and 1357 mg/kg bw per day for females, respectively). Animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Body weights and feed consumption were recorded before the start of treatment, daily during week 1 of the treatment period and twice weekly during weeks 2–4. Blood was sampled prior to termination for haematology and clinical biochemistry. At termination, all mice were necropsied, and weights of liver, lung, spleen, kidneys, heart, brain, pituitary, thyroid, thymus, epididymides, ovaries, testes, prostate, uterus and adrenals were recorded. An extensive range of organs was examined microscopically

No adverse effects of treatment with triazole acetic acid were observed in any of the test groups.

The NOAEL was 7000 ppm (equal to 1067 mg/kg bw per day), the highest dose tested (Shearer, 2011).

In a 29-day dietary toxicity study in rats, triazole acetic acid (purity 98.4%; batch no. CH-476108) was administered to groups of 10 male and 10 female Wistar: Crl:WI(Han) rats at 0, 3250, 6500 or 13 000 ppm (equal to 0, 243, 483 and 993 mg/kg bw per day for males and 0, 260, 519 and 940 mg/kg bw per day for females, respectively). Animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Five rats of each sex per dose were subjected to a functional observational battery during the fourth week of treatment. Body weights and feed consumption were measured weekly. Ophthalmoscopy was performed before treatment started and prior to termination. Blood and urine were sampled during the fourth week of treatment for haematology, clinical biochemistry and urine analysis. At termination, all rats were necropsied, and weights of liver, lung, spleen, kidneys, testes, heart, brain, thyroid, thymus, epididymides, ovaries, testes, prostate, uterus and adrenals were recorded. An extensive range of organs was examined microscopically.

No adverse effects of treatment with triazole acetic acid were observed in any of the test groups. At 6500 and 13 000 ppm, slightly decreased urinary pH was observed, without any associated histopathological or clinical changes. The decreased urinary pH was attributed to the acidic nature of the test material and was not considered to be toxicologically relevant.

The NOAEL was 13 000 ppm (equal to 940 mg/kg bw per day), the highest dose tested (Wahle, 2010b).

In a 13-week combined dietary toxicity and neurotoxicity study in rats, triazole acetic acid (purity 98.5%; batch no. RDL 211-8-2) was administered to groups of 16 male and 16 female Wistar: Crl:WI(Han) rats. The dietary concentrations were adjusted weekly based on body weight and feed consumption in order to obtain target test substance intakes of 0, 100, 500 and 1000 mg/kg bw per day. Actual mean intakes were 0, 94, 495 and 1002 mg/kg bw per day for males and 0, 119, 627 and 1181 mg/kg bw per day for females, respectively. Ten animals of each sex per dose were designated for general toxicity investigations, and six animals of each sex per dose were designated for the neuropathology investigations. Animals were checked daily for mortality and clinical signs. A detailed physical examination was performed weekly. A functional observational battery and a locomotor activity test were done in 10 rats of each sex per group (overlapping with those assigned for the neuropathology investigations) before the start and during weeks 2, 4, 8 and 13 of treatment. Ophthalmological examinations were conducted at the beginning of the study in all rats and prior to termination. Body weights and feed consumption were measured weekly. At termination, blood and urine were sampled from 10 rats of each sex per dose for haematology, clinical biochemistry and urine analysis. At termination, 10 rats of each sex per dose were necropsied, and weights of liver, lung, spleen, kidneys, heart, brain, thyroid, thymus, epididymides, ovaries, testes, prostate, uterus and adrenals were recorded. An extensive range of organs was examined microscopically. The animals of the neuropathology group were subjected to a detailed histological examination of the nervous system.

No effects of treatment on mortality, clinical signs, body weight, feed consumption, ophthalmology, neurological parameters, locomotor activity, haematology, clinical chemistry, urine analysis, macroscopy, histopathology or organ weights were observed. A slight increase in white blood cell count, accompanied by increases in several absolute differential leukocyte counts, observed in high-dose males was not considered to be treatment related, as the increases were within the historical control range and there were no differences from controls in male relative leukocyte counts or in any haematology parameters in female rats. A very slight decrease in serum potassium concentration in high-dose males was also not considered to be treatment related, as the decrease was small and within the historical control range.

The NOAEL was 1002 mg/kg bw per day, the highest dose tested (Wahle, 2010c).

In a one-generation dietary reproductive toxicity study in rats, performed according to OECD Test Guideline 415, Wistar Crl:WI(Han) rats (25 of each sex per dose group) were fed triazole acetic acid (purity 98.5%; batch no. RDL 211-8-2). The dietary concentrations were adjusted weekly based on body weight and feed consumption in order to obtain target test substance intakes of 0, 100, 300 and 1000 mg/kg bw per day. Actual mean intakes are presented in Table 20.

Table 20. Mean test substance intake in a one-generation reproductive toxicity study in rats

	Mean test substance intake (mg/kg bw per day)		
	100 mg/kg bw per day	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**
Parental males	96	287	959
Parental females (premating)	98	293	976
Parental females			
Gestation period	105	315	1 073
Lactation period	115	353	1 227
F_1 males	93	280	926
F_1 females	78	246	770

bw: body weight; F_1: first filial generation
Source: Schneider (2010)

F_0 adults were treated over at least a 73-day premating period and throughout the 2-week mating period, gestation and 21-day lactation of the F_1 pups. On PND 4, litters were culled to eight pups. Upon completion of weaning for each litter, 25 weaned rats of each sex (one male and one female per litter, if possible) were randomly selected in each dose group. Treatment of the selected pups continued until sexual maturation. The remaining F_1 weanlings were killed and subjected to a gross external examination and necropsy. The rats were checked daily for mortality and clinical signs. Body weights of parental rats were recorded weekly. In addition, females were weighed on GDs 0, 7, 14 and 20 and PNDs 1, 4, 7, 14 and 21. Offspring were weighed on PNDs 1, 4, 7, 14 and 21. Parental feed consumption was recorded on a weekly basis during the premating and gestation periods. In addition, feed consumption of parental females was measured during PNDs 1–4, 4–7, 7–14 and 14–21. Estrous cycle length was evaluated by daily analysis of vaginal smear for all F_0 female parental rats for a minimum of 3 weeks prior to mating. Determination of estrous cycle length was continued throughout the pairing period until the female exhibited evidence of copulation. Fertility and mating indices of males and females, copulatory interval and the length of gestation were determined. At termination of the males, sperm parameters were assessed. 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 and clinical signs. All culled pups and pups killed at PND 21 were subjected to gross examination. After the scheduled kill at weaning on PND 21, the brain, spleen and thymus of one pup of each sex per litter were weighed. All selected female F_1 pups (25 per group) were evaluated daily for vaginal patency beginning on PND 27. On the day of vaginal opening, the body weights of the respective animals were determined. All selected male F_1 pups (25 per group) were evaluated daily for preputial separation beginning on PND 38. On the day of preputial separation, the body weights of the respective animals were determined. After weaning, the parental rats were killed and necropsied. In all parental animals, a wide range of organs was microscopically examined. In addition, most of these organs were weighed. Differential ovarian follicle count was performed in females of the control and high-dose groups.

No mortalities or treatment-related clinical signs were observed in the parental animals throughout the treatment period. Feed consumption of the high-dose males was statistically significantly decreased during weeks 4–6 (up to 5%), weeks 7–8 (about 6%), weeks 9–10 (about 7%)

and weeks 14–16 (up to 7%). Feed consumption of the mid-dose F_0 males was statistically significantly decreased by about 6% during weeks 9–10, weeks 11–12 and weeks 15–16. Body weight gain was statistically significantly decreased in males at 1000 mg/kg bw per day during weeks 1–2 (about 7%), weeks 3–6 (up to 20%) and weeks 14–15 (about 47%). During the entire study, the high-dose males gained about 10% less weight than the controls. Body weight gain of the mid-dose males was slightly below control values throughout the study. Only the decrease during weeks 11–12 (about 32%) was statistically significant. Body weight gain of low-dose males and females of all dose groups was not affected. Estrous cycling, female reproduction and delivery parameters, male fertility and sperm parameters were not affected by treatment. Slight increases in relative kidney weights (6% and 11%, respectively) in mid- and high-dose males and in relative liver weights (8%) in high-dose males were attributed to the observed reduction in body weight gain. As these increases were not accompanied by histopathological changes, they were not considered to be toxicologically relevant. Necropsy and histopathological examination, including a differential ovarian follicle count, revealed no effect of treatment. No effects of treatment on mortality, clinical observations, body weight gain, feed consumption, sexual maturation or gross examination of the pups were observed at any dose.

The NOAEL for parental toxicity was 287 mg/kg bw per day, based on reduced body weight gain and feed consumption in males at 959 mg/kg bw per day.

The NOAEL for offspring toxicity was 770 mg/kg bw per day, the highest dose tested.

The NOAEL for reproductive toxicity was 959 mg/kg bw per day, the highest dose tested (Schneider, 2010).

In a preliminary developmental toxicity study, groups of 20 time-mated female Crl:WI(Han) rats were treated orally, by gavage, with triazole acetic acid (purity 98.5%; batch no. AE C619102-01-03) at a dose of 0, 500, 750 or 1000 mg/kg bw per day from days 6 through 19 of gestation. The rats were checked daily for clinical signs and mortality. A detailed examination was performed once weekly. Body weights were recorded daily from GD 6 to GD 20. Feed consumption was measured over the periods GDs 3–6, 6–9, 9–12, 12–15, 15–18 and 18–20. All females were killed on day 20 of gestation and subjected to gross examination. The gravid uterus was weighed and examined, and the numbers of live and dead fetuses, implantations, and early and late resorptions were counted. The number of corpora lutea, placental weight and body weight, sex and external abnormalities of the fetuses were recorded.

No mortalities or clinical signs were recorded. Although body weight gain in high-dose females was slightly reduced over the treatment period, statistical significance was not observed. This reduction in body weight gain was not considered to be toxicologically adverse. No effect of treatment on feed consumption, pregnancy data or necropsy findings was observed. Examination of the fetuses revealed no effects on litter, fetal and placental weight, fetal sex ratio or external malformations (Mercer, 2011a).

In a developmental toxicity study, performed according to OECD Test Guideline 414, groups of 24 time-mated female Crl:WI(Han) rats were treated orally, by gavage (vehicle was 0.5% carboxymethyl cellulose), with triazole acetic acid (purity 98.5%; batch no. AE C619102-01-03) at a dose of 0, 100, 300 or 1000 mg/kg bw per day from days 6 through 19 of gestation (day of mating = GD 0). The rats were checked daily for clinical signs and mortality. A detailed examination was performed once weekly. Body weights were recorded daily from GD 6 to GD 20. Feed consumption was measured over the periods GDs 6–9, 9–12, 12–15, 15–18 and 18–20. All females were killed on day 20 of gestation and subjected to gross examination. The gravid uterus was weighed and examined, and the numbers of live and dead fetuses, implantations, and early and late resorptions were counted. The number of corpora lutea, placental weight and body weight, sex and external abnormalities of the fetuses were recorded. Half of the fetuses were examined for skeletal abnormalities, whereas the other half was examined for visceral abnormalities.

Three high-dose females were killed on GDs 8–9 owing to a deterioration in their condition. These animals displayed decreased activity, noisy and laboured breathing, hunched posture, piloerection and partially closed eyes. Necropsy revealed gaseous distensions of the gastrointestinal tract. No signs of local irritation of the stomach or gut were reported. Body weight gain in this high-dose group was lower than that of the controls during GDs 8–10. As these effects were observed early during the study, treatment of the remaining animals in this group was discontinued, and these animals were not further examined. No effect of treatment on mortality, clinical signs, body weight gain, feed consumption or necropsy findings was observed at 100 or 300 mg/kg bw per day. There was no effect of treatment on the mean number of corpora lutea, the mean number of implantations or the extent of preimplantation or postimplantation losses at the middle and low doses. The percentage of male fetuses was statistically significantly reduced at 300 mg/kg bw per day. However, values were within the historical control range, and therefore this reduction was considered unrelated to treatment. There was no effect of treatment at 100 or 300 mg/kg bw per day on fetal, litter, placental or gravid uterine weights. External, skeletal and visceral examination of the low- and mid-dose fetuses also showed no effect of treatment. As severe clinical signs in the dams at 1000 mg/kg bw per day necessitated early termination, the effect of triazole acetic acid on fetal development could not be assessed at this dose.

The NOAEL for maternal toxicity was 300 mg/kg bw per day, based on mortality, clinical signs, and reduced body weight gain and feed consumption observed early during treatment at 1000 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, the highest dose tested It is noted that the effect of triazole acetic acid at 1000 mg/kg bw per day on embryo and fetal development could not be assessed due to early termination of the dams at this dose (Mercer, 2011b).

Rabbits

In a developmental toxicity study, performed according to OECD Test Guideline 414, groups of at least 25 time-mated female New Zealand White (Hra:(NZW)SPF) rabbits were treated orally, by gavage, with triazole acetic acid (purity 98.4%; batch no. CH-476108) at a dose of 0, 100, 750 or 1000 mg/kg bw per day from days 6 through 28 of gestation (day 0 = day of mating). The rabbits were checked daily for clinical signs, mortality, abortions and premature deliveries. Body weights and feed consumption were recorded daily. All females were killed on day 29 of gestation and subjected to gross examination. The liver, kidneys and uterus were weighed. The uterus was examined, and the numbers of live and dead fetuses, implantations, and early and late resorptions were counted. The number of corpora lutea and body weight and sex of the fetuses were recorded. Cavitated organs were evaluated in all fetuses by dissection. A single cross-section was made between the parietal and frontal bones of approximately one half of the fetuses per litter, and the brain was examined in situ. The remaining fetuses were examined for soft tissue alterations (including eyes, brain, nasal passages and tongue). All fetuses were examined for skeletal alterations.

One, six and 10 does died or were killed prior to scheduled termination at 100, 750 and 1000 mg/kg bw per day, respectively. The deaths of one and eight does at 750 and 1000 mg/kg bw per day, respectively, were considered to be caused by localized gastrointestinal tract disturbances due to the strong acidic property (pH 1.9–2.0) of the test substance, and not by systemic toxicity. Most of these animals had stomach lesions, generally described as numerous discoloured (black) erosions or ulcerations (pinpoint to 1.0 cm in diameter) on the mucosal surface. These stomach lesions probably led to decreased feed consumption and markedly decreased body weight gain or body weight loss during the period prior to their death. The other deaths are considered unrelated to the test substance (e.g. from intubation errors). One doe at 750 mg/kg bw per day delivered on GD 29, before scheduled termination. The incidences of scant faeces were increased at 750 and 1000 mg/kg bw per day, and the incidence of rales was increased at 750 mg/kg bw per day. These clinical signs were generally observed in does that died or were killed before scheduled termination. No other treatment-related clinical signs were noted. Apart from the gastrointestinal lesions, necropsy revealed no treatment-related changes. Reductions in feed consumption were observed at 750 and 1000 mg/kg bw per day

on the first day of treatment. In the animals that survived to scheduled termination, body weight gain was reduced at 1000 mg/kg bw per day (+450 g in controls, +310 g at 1000 mg/kg bw per day; corrected for gravid uterine weight: −40 g in controls and −130 g at 1000 mg/kg bw per day). In these animals, feed consumption was reduced at 750 mg/kg bw per day (10%) and 1000 mg/kg bw per day (12%).

At 750 and 1000 mg/kg bw per day, fetal weights in both sexes were significantly reduced (by 9–11%) compared with the control group values. No gross external, soft tissue or skeletal fetal alterations (malformations or variations) were caused by doses up to 1000 mg/kg bw per day.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality (first observed on day 4 of treatment), clinical signs, and reduced body weight gain and feed consumption (first observed at/after 1 day of treatment) observed at 750 mg/kg bw per day. These effects are probably caused by a local effect on the gastrointestinal tract.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on decreased fetal weights at 750 mg/kg bw per day (Hoberman, 2010a).

Triazole alanine

In a 12-month dietary toxicity study in rats, performed according to OECD Test Guideline 452, triazole alanine (purity 98%; batch no. MES 133/1) was administered to groups of 30 male and 30 female Wistar: Crl:WI(Han) rats at dietary levels adjusted to achieve target doses of 0, 30, 100, 300 and 1000 mg/kg bw per day. Actual doses were 0, 28, 93, 278 and 916 mg/kg bw per day for males and 0, 36, 120, 375 and 1273 mg/kg bw per day for females, respectively. Twenty animals of each sex per dose were used to evaluate the potential for general toxicity, and 10 animals of each sex per dose were used to evaluate the potential for neurotoxicity. The rats were checked daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Body weight and feed consumption were recorded weekly during the first 3 months and monthly thereafter. Ophthalmic examinations were performed before the start of the study and prior to termination. Blood and urine for haematology, clinical chemistry and urine analysis were collected from 10 rats of each sex per dose at 3, 6 and 12 months. The rats designated for neurotoxicological examinations were subjected to functional observational battery and motor activity testing before treatment started and at 3, 6, 9 and 12 months. Animals found dead or killed prematurely were weighed and necropsied. Gross lesions were examined. At termination, 20 rats of each sex per dose were necropsied, and organ weights (adrenals, brain, heart, liver, kidneys, spleen, thyroid, uterus, ovaries, testes, epididymides, prostate and pituitary) were recorded. A wide range of tissues was examined microscopically. The animals of the neurotoxicology group were subjected to a detailed histological examination of the nervous system.

There were no effects of treatment on mortality, clinical signs, body weight gain, feed consumption, ophthalmology, haematology, urine analysis or clinical chemistry. A decrease in serum potassium level (up to 16%) and an increase in serum glucose level (up to 18%) observed at 6 months in males of the 2000, 6000 and 20 000 ppm groups were not considered to be treatment related, as they occurred in one sex only and were not observed at 3 and 12 months. The neurological assessments (functional observational battery and motor activity) revealed no treatment-related effects. Occasional findings on posture in the home cage, reaction to handling, approach response and body temperature were considered not to be treatment related, as they lacked time and dose dependency. No effects of treatment on gross pathology, organ weights or histopathology were found in the toxicology or neurotoxicology groups. Slightly enhanced incidence and severity of the mineralization of the intestinal mucosa were observed in both sexes at 20 000 ppm, reaching statistical significance for the colon in males. This was considered not to be toxicologically relevant, as the overall incidence of intestinal mineralization was similar between controls (14/20 males, 18/20 females) and high-dose animals (17/20 males, 18/20 females), clinical signs of intestinal disturbance/function were not observed in any animal and this microscopic change is a common background lesion in the ageing rat.

The NOAEL was 20 000 ppm (equal to 916 mg/kg bw per day), the highest dose tested (Wahle, 2012).

In a developmental toxicity study in rabbits, performed according to OECD Test Guideline 414, groups of 25 pregnant female New Zealand White (Hra:(NZW)SPF) rabbits were treated orally, by gavage, with triazole alanine (purity 98%; batch no. MES 133/1) at a dose of 0, 30, 100 or 250 mg/kg bw per day from days 6 through 28 of gestation (day 0 = day of mating). The rabbits were checked daily for clinical signs, mortality, abortions and premature deliveries. Body weights and feed consumption were recorded daily. All females were killed on day 29 of gestation and subjected to gross examination. The liver, kidneys and uterus were weighed. The uterus was examined, and the numbers of live and dead fetuses, implantations, and early and late resorptions were counted. The number of corpora lutea and body weight and sex of the fetuses were recorded. Cavitated organs were evaluated in all fetuses by dissection. A single cross-section was made between the parietal and frontal bones of approximately one half of the fetuses per litter, and the brain was examined in situ. The remaining fetuses were examined for soft tissue alterations (including eyes, brain, nasal passages and tongue). All fetuses were examined for skeletal alterations.

At 250 mg/kg bw per day, a slight increase in the numbers of does with soft or liquid faeces was found. These findings were first observed after 5 days of treatment. No other treatment-related clinical signs, mortalities or abortions were observed. Macroscopic examination did not reveal treatment-related lesions. At 250 mg/kg bw per day, the terminal body weight was slightly lower (3.3%) than the control value. In these high-dose rabbits, significantly lower body weight gains (29%) and feed consumption (11%) were observed from GD 6 to GD 29.

A significant increase in resorptions and postimplantation loss at 30 mg/kg bw per day was not considered to be treatment related, as the increase did not occur at higher doses and the values were within the historical control ranges. Fetal weights of both sexes were significantly reduced at 250 mg/kg bw per day (10.4% in males, 11.6% in females). Although the fetal weights at the high dose were within the historical control range, the observed reductions in body weight were considered to be treatment related by the study authors. No treatment-related malformations were observed in the fetuses. The litter incidence of hyoid, angulated ala (52%) and ribs thickened (12%) at 250 mg/kg bw per day were just outside the historical control range (0–50% and 0–10%, respectively) and are considered to be related to treatment.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased incidences of soft or liquid faeces (first observed after 5 days of treatment) and decreased body weight gain and feed consumption observed at 250 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on decreased fetal weight and increased incidences of hyoid, angulated ala and thickened ribs observed at 250 mg/kg bw per day (Hoberman, 2010b).

3. Observations in humans

No records of adverse health effects were reported during the manufacture or formulation of penconazole-containing products over a 20-year period. Exposure incidences of penconazole formulations reported between 2004 and 2014 were related to intentional misuse (15 cases), occupational exposure (five cases) and accidental exposure (15 cases). In one case, ingestion of penconazole induced effects of moderate severity (not further specified); in the other cases, exposure (oral, dermal, inhalation or eye) produced no or only minor symptoms (information provided by sponsor).

Comments

Biochemical aspects

Absorption was rapid and extensive following the administration of a single oral dose (0.5 or 25 mg/kg bw) of [^{14}C]penconazole to rats (Hamböck, 1980, 1985). At 50 mg/kg bw, maximum blood concentrations were reached in 4 hours in males and 6 hours in females (Hassler, 1999). At this dose, peak tissue concentrations, observed at about 6 and 4 hours after dosing in males and females, respectively, were generally higher in males; the half-life of elimination was also longer in males than in females. Highest tissue concentrations of radioactivity were found in penis (probably related to contamination with urinary radioactivity), liver, lungs and kidneys (Hassler, 1999). A sex difference was apparent in excretion profiles, with females excreting 73–85% of a 0.5 or 25 mg/kg bw dose of penconazole in urine and 14–32% in faeces over a 6-day period, whereas males excreted 62% of the same dose levels in urine and 37–39% in faeces over the same period (Hamböck, 1980, 1985). Excretion was more rapid in females, irrespective of dose level or position of radiolabel. Biliary elimination was greater in males than in females (55% and 40% of the administered dose, respectively). Less than 5% of the dose was excreted in faeces in bile duct–cannulated rats, indicating enterohepatic circulation of biliary metabolites (Van Dijk, 1987). The excretion profiles in males and females were not affected by dose or predosing the rats with unlabelled penconazole for 14 or 90 days (Hiles, 1987b; Van Dijk, 1987).

Primary metabolic reactions involved in the biotransformation of penconazole included cleavage of the triazole ring (estimated 15% of the dose), oxidation of the ω-position of the alkane chain to form the respective carboxylic acid (30% of the dose), oxidation of the 3- or 4-position of the alkane chain to form monohydroxy and dihydroxy derivatives (2.5% of the dose) and oxidation of the triazole ring in the 3- or 5-position (0.7% of the dose). Cleavage of the penconazole molecule to free triazole was more extensive in males than in females (Hamböck, 1980, 1982, 1984, 1985). Secondary metabolic reactions include α-oxidation of the carboxylic acids to form α-hydroxy carboxylic acids (4.4% of the dose), decarboxylation following oxidation to α-ketocarboxylic derivative (9% of the dose), oxidation of the 3,4-dihydroxy derivatives to produce the corresponding 3- or 4-keto derivatives (0.5% of the dose) and conjugation of all alkanol derivatives with glucuronic acid (2.5% of the dose). A small amount of parent penconazole was identified in faeces and was considered to represent unabsorbed dose (Hamböck, 1982, 1984).

Toxicological data

The acute toxicity of penconazole is low (rat: oral LD_{50} > 2000 mg/kg bw; dermal LD_{50} > 3000 mg/kg bw; inhalation LC_{50} > 4.0 mg/L) (Bathe, 1980a,c; Hartmann, 1987). Penconazole was not irritating to the skin or the eyes of rabbits (Ullmann, 1980; Kuhn, 1988). Penconazole was not a skin sensitizer in a Magnusson and Kligman test in guinea-pigs (Cantoreggi, 1998).

In repeated-dose oral toxicity studies with penconazole in mice, rats and dogs, the main adverse effects were body weight changes and liver toxicity.

In a 90-day study in mice using dietary penconazole concentrations of 0, 10, 100, 300, 500, 1000 and 2400 ppm (equal to 0, 1.7, 17, 52, 85, 163 and 423 mg/kg bw per day for males and 0, 2.5, 24, 72, 116, 237 and 614 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 85 mg/kg bw per day), based on lower total protein and cholesterol levels and focal coagulative necrosis in the liver of both sexes at 1000 ppm (equal to 163 mg/kg bw per day) (Hiles, 1987a).

In a second 90-day study in mice using dietary concentrations of 0, 100, 500, 1500, 3000 and 5000 ppm (equal to 0, 14, 69, 229, 437 and 837 mg/kg bw per day for males and 0, 18, 87, 274, 545 and 983 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 69 mg/kg bw per day), based on reductions in cholesterol levels in both sexes, a reduction in total protein and albumin levels in females and a reduction in body weight gain and increased nuclear pleomorphism in hepatocytes in males at 1500 ppm (equal to 229 mg/kg bw per day) (Milburn, 2002).

In a 28-day gavage study in rats using penconazole doses of 0, 100 and 500 mg/kg bw per day, a NOAEL could not be identified. The LOAEL was 100 mg/kg bw per day, based on changes in clinical chemistry and haematology parameters and minimal hypertrophy of the follicle epithelium of the thyroid (Fankhauser, 1991).

In a 13-week study in rats using dietary penconazole concentrations of 0, 30, 300 and 3000 ppm (equal to 0, 2.0, 19 and 202 mg/kg bw per day for males and 0, 2.1, 21 and 209 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 19 mg/kg bw per day), based on reduced body weight gain and feed consumption in females and increased testes weight observed at 3000 ppm (equal to 202 mg/kg bw per day) (Basler, 1982).

In a second 13-week dietary study in rats using penconazole concentrations of 0, 10, 30 and 100 ppm (equal to 0, 0.77, 2.1 and 7.1 mg/kg bw per day for males and 0, 0.78, 2.1 and 7.3 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 7.1 mg/kg bw per day), the highest dose tested (Basler, 1983).

In a third 13-week dietary study in rats using penconazole concentrations of 0, 10, 100, 300, 500, 1000 and 2400 ppm (equal to 0, 0.81, 7.5, 23, 38, 72 and 179 mg/kg bw per day for males and 0, 0.96, 9.1, 28, 45, 86 and 209 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 23 mg/kg bw per day), based on an increased incidence of hepatocellular vacuolation and hypertrophy at 500 ppm (equal to 38 mg/kg bw per day) (Hiles, 1987c).

In a 1-year study, dogs received a dietary penconazole concentration of 0, 100, 500 or 5000 ppm (equal to 0, 3.1, 16.9 and 133 mg/kg bw per day for males and 0, 3.3, 16.7 and 139 mg/kg bw per day for females, respectively). During week 20, the highest dose was reduced to 2500 ppm (equal to 86 mg/kg bw per day for males and 89 mg/kg bw per day for females), because of excessive reduction in feed consumption and body weight gain. The NOAEL was 100 ppm (equal to 3.1 mg/kg bw per day), based on reduced body weight gain, increased absolute and relative liver weights, and slight histopathological changes in the liver (hepatocyte necrosis associated with inflammatory cell infiltration) in males and females at 500 ppm (equal to 16.7 mg/kg bw per day) (Gfeller, 1984).

In a 2-year carcinogenicity study in mice using dietary concentrations of 0, 5, 75, 150 and 300 ppm (equal to 0, 0.75, 9.8, 19 and 41 mg/kg bw per day for males and 0, 0.67, 8.8, 17 and 36 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 36 mg/kg bw per day), the highest dose tested. No treatment-related tumours were observed in mice in this study (Basler, 1985a).

In an 80-week carcinogenicity study in mice using dietary concentrations of 0, 25, 200 and 1500 ppm (equal to 0, 2.7, 22 and 178 mg/kg bw per day for males and 0, 3.5, 28 and 222 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 22 mg/kg bw per day), based on decreased body weight gain and absolute and relative spleen weights and increased incidence and severity of hepatocellular vacuolation in both sexes and increased absolute and relative liver weights in males at 1500 ppm (equal to 178 mg/kg bw per day). No treatment-related tumours were observed in mice in this study (Milburn, 2004).

The overall NOAEL for the long-term toxicity studies in mice was 300 ppm (equal to 36 mg/kg bw per day), and the overall LOAEL was 1500 ppm (equal to 178 mg/kg bw per day).

In a 27-month toxicity and carcinogenicity study in rats using dietary concentrations of 0, 5, 75, 150 and 300 ppm (equal to 0, 0.30, 3.8, 7.3 and 15 mg/kg bw per day for males and 0, 0.31, 4.0, 8.1 and 17 mg/kg bw per day for females, respectively), the NOAEL was 150 ppm (equal to 8.1 mg/kg bw per day), based on increased absolute and relative liver weights and an increase in GGT levels at 1 year in females at 300 ppm (equal to 17 mg/kg bw per day). No treatment-related tumours were observed in rats in this study (Basler, 1985b).

The Meeting concluded that penconazole is not carcinogenic in mice or rats.

Penconazole was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. There was no evidence of genotoxicity (Deparade, 1984, 1999a,b; Puri, 1984; Ogorek, 1999a,b).

The Meeting concluded that penconazole 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 penconazole is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats using penconazole at dietary concentrations of 0, 80, 400 and 2000 ppm (equal to 0, 5.5, 29 and 146 mg/kg bw per day for males and 0, 7.5, 40 and 202 mg/kg bw per day for females of the F_0 generation and 0, 6.5, 31 and 166 mg/kg bw per day for males and 0, 8.5, 43 and 227 mg/kg bw per day for females of the F_1 generation, respectively), the NOAEL for parental toxicity was 400 ppm (equal to 43 mg/kg bw per day), based on increased relative liver weights and the observation of hepatocellular necrosis in F_1 parental females at 2000 ppm (equal to 227 mg/kg bw per day). The NOAEL for offspring toxicity was 2000 ppm (equal to 146 mg/kg bw per day), the highest dose tested. The NOAEL for reproductive toxicity was 400 ppm (equal to 40 mg/kg bw per day), based on a lower gestation index and a longer gestation duration in F_0 and F_1 females at 2000 ppm (equal to 202 mg/kg bw per day) (Fritz, 1983a,b).

In a second two-generation reproductive toxicity study in rats using dietary penconazole concentrations of 0, 25, 250 and 2500 ppm, premating dietary intakes were equal to 0, 2.0, 20 and 191 mg/kg bw per day for males and 0, 2.4, 24 and 238 mg/kg bw per day for females of the F_0 generation and 0, 2.2, 22 and 219 mg/kg bw per day for males and 0, 2.5, 25 and 246 mg/kg bw per day for females of the F_1 generation, respectively. The NOAEL for parental toxicity was 250 ppm (equal to 24 mg/kg bw per day), based on reduced body weight gain and feed consumption during the premating period in F_0 and F_1 females at 2500 ppm (equal to 238 mg/kg bw per day). The NOAEL for offspring toxicity was 250 ppm (equal to 20 mg/kg bw per day), based on an increased number of pups that were born dead or died during PNDs 0–4 and a decreased body weight gain of pups during lactation at 2500 ppm (equal to 191 mg/kg bw per day). The NOAEL for reproductive toxicity was 250 ppm (equal to 20 mg/kg bw per day), based on a decreased mating index at 2500 ppm (equal to 191 mg/kg bw per day) (Schardein, 1987).

In a developmental toxicity study in rats using gavage penconazole doses of 0, 30, 100 and 300 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality and reduced body weight gain observed at the end of gestation at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on delayed ossification observed at 300 mg/kg bw per day (Fritz, 1981).

In a second developmental toxicity study in rats using gavage penconazole doses of 0, 5, 100 and 500 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality observed after 5 and 6 days of treatment, clinical signs observed early during treatment, a reduction in net body weight gain and feed consumption on GD 6, stomach lesions and an increased incidence of late resorptions at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on a slight increase in the occurrence of cervical ribs and an increase in the total number of fetuses/litters with abnormal findings at 500 mg/kg bw per day (Salamon, 1985).

The overall NOAEL for maternal and embryo and fetal toxicity in the two developmental toxicity studies in rats was 100 mg/kg bw per day, and the overall LOAEL was 300 mg/kg bw per day.

In a developmental toxicity study in Chinchilla-type rabbits administered penconazole doses of 0, 25, 75 and 150 mg/kg bw per day by gavage (vehicle was 0.5% aqueous sodium carboxymethyl cellulose), the NOAEL for maternal toxicity was 75 mg/kg bw per day, based on reduction of body weight gain and feed consumption during treatment at 150 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 75 mg/kg bw per day, based on the increased incidences of microphthalmia and hydroencephalus at 150 mg/kg bw per day (Giese, 1982).

In a second developmental toxicity study in New Zealand White rabbits administered penconazole doses of 0, 10, 50 and 200 mg/kg bw per day by gavage (vehicle was 3% aqueous cornstarch), the NOAEL for maternal toxicity was 200 mg/kg bw per day, the highest dose tested. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on the reduced number of live fetuses at 200 mg/kg bw per day (Nemec, 1985).

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

No neurotoxicity studies with penconazole were provided. In view of the absence of evidence of neurotoxicity in other acute and repeated-dose toxicity studies, the Meeting concluded that penconazole is unlikely to be neurotoxic.

A special study in rats and mice indicates that penconazole at gavage doses of 10, 80, 160 and 320 mg/kg bw per day for 14 days induces liver enzyme induction, liver enlargement and proliferation of smooth endoplasmic reticulum and shares some characteristics with a phenobarbital class of monooxygenase inducers (Waechter, Bentley & Staeubli, 1985).

Toxicological data on metabolites and/or degradates

1,2,4-Triazole

In a 12-month toxicity study in rats using dietary 1,2,4-triazole concentrations of 0, 125, 375, 1000 and 2000 ppm (equal to 0, 6.9, 21, 58 and 113 mg/kg bw per day for males and 0, 8.3, 26, 71 and 136 mg/kg bw per day for females, respectively), the NOAEL was 375 ppm (equal to 21 mg/kg bw per day), based on a reduction in body weight gain at 1000 ppm (equal to 58 mg/kg bw per day) (Wahle, 2010a).

Triazole acetic acid

In a 28-day toxicity study in mice using dietary triazole acetic acid concentrations of 0, 1000, 3000 and 7000 ppm (equal to 0, 159, 483 and 1067 mg/kg bw per day for males and 0, 183, 542 and 1357 mg/kg bw per day for females, respectively), the NOAEL was 7000 ppm (equal to 1067 mg/kg bw per day), the highest dose tested (Shearer, 2011).

In a 29-day toxicity study in rats using triazole acetic acid at dietary concentrations of 0, 3250, 6500 and 13 000 ppm (equal to 0, 243, 483 and 993 mg/kg bw per day for males and 0, 260, 519 and 940 mg/kg bw per day for females, respectively), the NOAEL was 13 000 ppm (equal to 940 mg/kg bw per day), the highest dose tested (Wahle, 2010b).

In a 13-week combined toxicity and neurotoxicity study in rats, dietary triazole acetic acid concentrations were adjusted weekly based on body weight and feed consumption in order to obtain target test substance intakes of 0, 100, 500 and 1000 mg/kg bw per day. Actual mean intakes were 0, 94, 495 and 1002 mg/kg bw per day for males and 0, 119, 627 and 1181 mg/kg bw per day for females, respectively. The NOAEL was 1002 mg/kg bw per day, the highest dose tested (Wahle, 2010c).

In a one-generation reproductive toxicity study in rats, dietary triazole acetic acid concentrations were adjusted weekly based on body weight and feed consumption in order to obtain target test substance intakes of 0, 100, 300 and 1000 mg/kg bw per day. Actual premating test substance intakes were 0, 96, 287 and 959 mg/kg bw per day for males and 0, 98, 293 and 976 mg/kg bw per day for females of the F_0 generation and 0, 93, 280 and 926 mg/kg bw per day for males and 0, 78, 246 and 770 mg/kg bw per day for females of the F_1 generation, respectively. The NOAEL for parental toxicity was 287 mg/kg bw per day, based on reduced body weight gain and feed consumption in males at 959 mg/kg bw per day. The NOAEL for offspring toxicity was 770 mg/kg bw per day, the highest dose tested. The NOAEL for reproductive toxicity was 959 mg/kg bw per day, the highest dose tested (Schneider, 2010).

In a developmental toxicity study in rats administered triazole acetic acid at a dose of 0, 100, 300 or 1000 mg/kg bw per day by gavage (vehicle was 0.5% carboxymethyl cellulose), the NOAEL for maternal toxicity was 300 mg/kg bw per day, based on mortality, clinical signs, and reduced body weight gain and feed consumption observed early during treatment at 1000 mg/kg bw per day. Although there are indications that the findings may be due to a local effect on the gastrointestinal tract, no signs of local irritation of the stomach or gut were reported. Therefore, the Meeting could not

discount the possibility that the findings were due to a systemic effect of the compound. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day. As severe clinical signs in the dams at 1000 mg/kg bw per day necessitated early termination, the effect of triazole acetic acid on fetal development could not be assessed at this dose (Mercer, 2011b).

In a developmental toxicity study in rabbits using gavage triazole acetic acid doses of 0, 100, 750 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on mortality (first observed on day 4 of treatment), clinical signs, and reduced body weight gain and feed consumption (first observed at/after 1 day of treatment) at 750 mg/kg bw per day. As most of these animals had stomach lesions, generally described as numerous discoloured (black) erosions/ulcerations (pinpoint to 1.0 cm in diameter) on the mucosal surface, these effects are probably caused by a local effect on the gastrointestinal tract. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on decreased fetal weights at 750 mg/kg bw per day (Hoberman, 2010a).

Triazole alanine

In a 12-month toxicity study in rats in which dietary triazole alanine concentrations were adjusted to achieve doses of 0, 28, 93, 278 and 916 mg/kg bw per day for males and 0, 36, 120, 375 and 1273 mg/kg bw per day for females, the NOAEL was 916 mg/kg bw per day, the highest dose tested (Wahle, 2012).

In a developmental toxicity study in rabbits using gavage triazole alanine doses of 0, 30, 100 and 250 mg/kg bw per day, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased incidences of soft or liquid faeces (first observed after 5 days of treatment) and decreased body weight gain and feed consumption observed at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on decreased fetal weight and increased incidences of hyoid, angulated ala and thickened ribs observed at 250 mg/kg bw per day (Hoberman, 2010b).

Human data

No adverse health effects in plant personnel during the manufacture or formulation of penconazole-containing products over a 20-year period were reported. In incidents related to intentional misuse, occupational exposure and accidental exposure, generally no or only minor symptoms were reported.

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

Toxicological evaluation

Penconazole

The Meeting reaffirmed the ADI of 0–0.03 mg/kg bw for penconazole on the basis of a NOAEL of 3.1 mg/kg bw per day for reduced body weight gain, increased absolute and relative liver weights, and slight histopathological changes in the liver (hepatocyte necrosis associated with inflammatory cell infiltration) in a 1-year study in dogs, using a safety factor of 100.

The Meeting established an ARfD of 0.8 mg/kg bw for penconazole, on the basis of a NOAEL of 75 mg/kg bw per day for increased incidences of microphthalmia and hydrocephalus in a developmental toxicity study in rabbits. The Meeting concluded that this ARfD applies to the general population on the basis of the NOAEL of 100 mg/kg bw per day for early clinical signs, reduced body

weight gain and mortality, which might be due to systemic effects, observed in dams in a developmental toxicity study in rats. A safety factor of 100 was applied.

1,2,4-Triazole

The present Meeting reaffirmed the ADI of 0–0.2 mg/kg bw, established by JMPR in 2008, based on a NOAEL of 16 mg/kg bw per day for testicular effects (sperm abnormalities, sperm counts) observed at 30.9 mg/kg bw per day in a two-generation study of reproductive toxicity in rats, using a safety factor of 100. This ADI is supported by a new 12-month dietary toxicity study in rats with a NOAEL of 21 mg/kg bw per day, based on a reduction in body weight gain at 58 mg/kg bw per day.

The present Meeting reaffirmed the previously established ARfD of 0.3 mg/kg bw for 1,2,4-triazole, based on a NOAEL of 30 mg/kg bw per day for alterations of the urogenital system that occurred in several fetuses at 45 mg/kg bw per day and clinical signs of neurotoxicity in the dams in a study of developmental toxicity in rabbits, and using a safety factor of 100.

Triazole alanine and triazole acetic acid

The present Meeting reaffirmed the group ADI for triazole alanine and triazole acetic acid (alone or in combination) of 0–1 mg/kg bw, established by JMPR in 2008, based on a NOAEL of 100 mg/kg bw per day for delayed ossification in a developmental toxicity study in rats given triazole alanine, a NOAEL of 100 mg/kg bw per day for increased incidences of soft or liquid faeces and decreased body weight gain and feed consumption in a new developmental toxicity study with triazole alanine in rabbits, a NOAEL of 100 mg/kg bw per day for decreased fetal weight and an increase in hyoid, angulated ala and thickened ribs in a new developmental toxicity study with triazole alanine in rabbits, a NOAEL of 100 mg/kg bw per day for mortality, clinical signs, and reduced body weight gain and feed consumption in a new developmental toxicity study in rabbits with triazole acetic acid, and a NOAEL of 100 mg/kg bw per day based on decreased fetal weights in a new developmental toxicity study in rabbits with triazole acetic acid. A safety factor of 100 was used. This group ADI is expressed as triazole alanine.

The present Meeting established an ARfD of 3 mg/kg bw for triazole alanine and triazole acetic acid, based on a NOAEL of 300 mg/kg bw per day on the basis of mortality, clinical signs, and reduced body weight gain and feed consumption observed early during treatment at 1000 mg/kg bw per day in a new developmental toxicity study with triazole acetic acid in rats. A safety factor of 100 was used.

Levels relevant to risk assessment of penconazole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year and 80-week studies of toxicity and carcinogenicity[a,b]	Toxicity	300 ppm, equal to 36 mg/kg bw per day	1 500 ppm, equal to 178 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 178 mg/kg bw per day[c]	–
Rat	Twenty-seven-month study of toxicity and carcinogenicity[a]	Toxicity	150 ppm, equal to 8.1 mg/kg bw per day	300 ppm, equal to 17 mg/kg bw per day
		Carcinogenicity	300 ppm, equal to 15 mg/kg bw per day[c]	–
	Two-generation study of	Reproductive toxicity	250 ppm, equal to 20 mg/kg bw per day	2 500 ppm, equal to 191 mg/kg bw per

Species	Study	Effect	NOAEL	LOAEL
	reproductive toxicity[a]			day
		Parental toxicity	250 ppm, equal to 24 mg/kg bw per day	2 500 ppm, equal to 238 mg/kg bw per day
		Offspring toxicity	250 ppm, equal to 20 mg/kg bw per day	2 500 ppm, equal to 191 mg/kg bw per day
	Developmental toxicity studies[b,d]	Maternal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	75 mg/kg bw per day	150 mg/kg bw per day
		Embryo and fetal toxicity	75 mg/kg bw per day	150 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	200 mg/kg bw per day[c]	–
		Embryo and fetal toxicity	50 mg/kg bw per day	200 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	100 ppm, equal to 3.1 mg/kg bw per day	500 ppm, equal to 16.7 mg/kg bw per day

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

Levels relevant to risk assessment of 1,2,4-triazole[a]

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day study of toxicity[b]	Toxicity	1 000 ppm, equal to 161 mg/kg bw per day	3 000 ppm, equal to 487 mg/kg bw per day
Rat	**One-year study of toxicity[b]**	**Toxicity**	**375 ppm, equal to 21 mg/kg bw per day**	**1 000 ppm, equal to 58 mg/kg bw per day**
	Multigeneration study of reproductive toxicity[b]	Parental toxicity	250 ppm, equal to 16 mg/kg bw per day	500 ppm, equal to 31 mg/kg bw per day
		Offspring toxicity	500 ppm, equal to 31 mg/kg bw per day[c]	–
	Developmental toxicity[d]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
Rabbit	Developmental toxicity[d]	Maternal toxicity	30 mg/kg bw per day	45 mg/kg bw per day
		Embryo and fetal toxicity	30 mg/kg bw per day	45 mg/kg bw per day

[a] Studies in bold are new studies. All other studies are derived from the 2008 JMPR evaluation.
[b] Dietary administration.
[c] Highest dose tested.
[d] Gavage administration.

Levels relevant to risk assessment of triazole acetic acid[a]

Species	Study	Effect	NOAEL	LOAEL
Rat	**Thirteen-week study of toxicity and neurotoxicity[b]**	**Toxicity**	**1 002 mg/kg bw per day[c]**	**–**
		Neurotoxicity	**1 002 mg/kg bw per day[c]**	**–**
	One-generation study of reproductive toxicity[b]	**Parental toxicity**	**287 mg/kg bw per day**	**959 mg/kg bw per day**
		Offspring toxicity	**770 mg/kg bw per day[c]**	**–**
		Reproductive toxicity	**959 mg/kg bw per day[c]**	**–**
	Developmental toxicity study[d]	**Maternal toxicity**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**
		Embryo and fetal toxicity	**300 mg/kg bw per day[c]**	**–[e]**
Rabbit	**Developmental toxicity study[d]**	**Maternal toxicity**	**100 mg/kg bw per day**	**750 mg/kg bw per day**
		Embryo and fetal toxicity	**100 mg/kg bw per day**	**750 mg/kg bw per day**

[a] Studies in bold are new studies.
[b] Dietary administration.
[c] Highest dose tested.
[d] Gavage administration
[e] The effect of triazole acetic acid at 1000 mg/kg bw per day on embryo and fetal development could not be assessed due to early termination of the dams at this dose

Levels relevant to risk assessment of triazole alanine[a]

Species	Study	Effect	NOAEL	LOAEL
Rat	**Twelve-month study of toxicity[b]**	**Toxicity**	**20 000 ppm, equal to 916 mg/kg bw per day[c]**	**–**
	Multigeneration study of reproductive toxicity[b]	Parental toxicity	10 000 ppm, equal to 929 mg/kg bw per day[c]	–
		Offspring toxicity	2 000 ppm, equal to 192 mg/kg bw per day	10 000 ppm, equal to 929 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	1 000 mg/kg bw per day[c]	–
		Embryo and fetal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
Rabbit	**Developmental toxicity study[d]**	**Maternal toxicity**	**100 mg/kg bw per day**	**250 mg/kg bw per day**
		Embryo and fetal toxicity	**100 mg/kg bw per day**	**250 mg/kg bw per day**
Dog	Ninety-day study of toxicity[d]	Toxicity	8 000 ppm, equal to 345 mg/kg bw per day	20 000 ppm, equal to 850 mg/kg bw per day

[a] Studies in bold are new studies. All other studies are derived from the 2008 JMPR evaluation.
[b] Dietary administration.
[c] Highest dose tested.
[d] Gavage administration.

Penconazole

Estimate of acceptable daily intake (ADI)

0–0.03 mg/kg bw

Estimate of acute reference dose (ARfD)

0.8 mg/kg bw

1,2,4-Triazole

Estimate of acceptable daily intake (ADI)

0–0.2 mg/kg bw

Estimate of acute reference dose (ARfD)

0.3 mg/kg bw

Triazole alanine and triazole acetic acid

Estimate of acceptable daily intake (group ADI), expressed as triazole alanine

0–1 mg/kg bw

Estimate of acute reference dose (ARfD)

3 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rats: Rapid; > 95% in both sexes at 0.5 mg/kg bw
Dermal absorption	No data
Distribution	Rats: Widespread distribution, highest concentrations found in liver and kidney
Potential for accumulation	Low potential for accumulation
Rate and extent of excretion	Rapid; 67% and 94% in male and female rats, respectively, in 24 h. Higher urinary excretion in females (73–85%) than in males (62%). Higher biliary excretion in males (55%) than in females (40%).
Metabolism in animals	Extensively metabolized (14 metabolites identified)
Toxicologically significant compounds in animals and plants	Penconazole, 1,2,4-triazole, triazole acetic acid, triazole alanine

Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 3 000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.0 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Reduced body weight gain, liver
Lowest relevant oral NOAEL	3.1 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	2 000 mg/kg bw per day (rabbit; highest dose tested)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver
Lowest relevant NOAEL	8.1 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice or rats[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	Decreased mating index
Lowest relevant parental NOAEL	24 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	20 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	20 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Reduced number of live fetuses
Lowest relevant maternal NOAEL	75 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	50 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Studies on toxicologically relevant metabolites	*1,2,4-Triazole* Reproductive toxicity: NOAEL 16 mg/kg bw per day (rat) Developmental toxicity: NOAEL 30 mg/kg bw per day (rabbit) One-year toxicity: NOAEL 21 mg/kg bw per day (rat)

	Triazole acetic acid No toxicity up to 1 002 mg/kg bw per day in a 13-week study of toxicity and neurotoxicity in rats Acute toxicity: NOAEL 300 mg/kg bw per day for mortality, clinical signs, and reduced body weight gain and feed consumption observed early during treatment at 1 000 mg/kg bw per day in a developmental toxicity study in rats No evidence of reproductive or offspring toxicity in rats at highest doses tested (959 and 770 mg/kg bw per day, respectively) No evidence of developmental toxicity in rats at 300 mg/kg bw per day *Triazole alanine* No toxicity up to 916 mg/kg bw per day in a 12-month study of toxicity in rats Embryo and fetal toxicity: NOAEL 100 mg/kg bw per day (rat, rabbit)
Medical data	Generally no or only minor symptoms after intentional, accidental or occupational exposure incidents

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

Summary

	Value	Study	Safety factor
Penconazole			
ADI	0–0.03 mg/kg bw	One-year study of toxicity (dog)	100
ARfD	0.8 mg/kg bw	Developmental toxicity study (rabbit)	100
1,2,4-Triazole			
ADI	0–0.2 mg/kg bw	Multigeneration reproduction toxicity study (rat), one-year study of toxicity (rat)	100
ARfD	0.3 mg/kg bw	Developmental toxicity study (rabbit)	100
Triazole alanine and triazole acetic acid			
Group ADI	0–1 mg/kg bw	Developmental toxicity studies (rat, rabbit)	100
ARfD	3 mg/kg bw	Developmental toxicity study (rat)	100

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Explanation

Quinclorac is the International Organization for Standardization (ISO)–approved common name for 3,7-dichloroquinoline-8-carboxylic acid (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 84087-01-4. It is a herbicide of the quinoline carboxylic acid class. The pesticidal mode of action is as a mimic of the plant hormone auxin.

Quinclorac 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.

All critical studies contained statements of compliance with good laboratory practice (GLP).

Initial production batches of quinclorac contained cinnoline impurities that were associated with positive results in genotoxicity studies. Improved production methods have reduced the levels of these impurities, and current batches are reported to contain cinnolines at concentrations below 1 part per million (ppm). The sponsor has confirmed that current technical quinclorac has a purity of greater

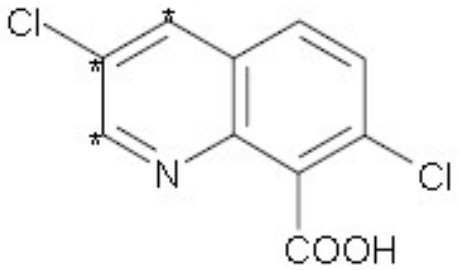
than 99% and that the material tested in the submitted toxicity studies adequately covers the impurities in current production material.

Evaluation for acceptable intake

1. Biochemical aspects

The structure of quinclorac and the position of the radiolabel used in the absorption, distribution, metabolism and excretion studies are shown in Fig. 1.

Fig. 1. Structure of quinclorac with position of radiolabel



* denotes the position of the radiolabel

The absorption, distribution, metabolism and excretion of quinclorac were investigated in a multicomponent study in CD rats (Hawkins et al., 1986). Groups of rats received [2,3,4-^{14}C]quinclorac (radiochemical purity 96.3%; specific activity 360.4 GBq/mol) by gavage in 1.5% aqueous methyl cellulose or in the diet. The dosing and investigation schedule is presented in Table 1. Samples of urine, faeces, bile, blood/plasma and tissues were obtained, processed and analysed by liquid scintillation counting (LSC) or whole-body autoradiography.

Table 1. Dose groups and investigations performed on rats receiving ^{14}C-labelled quinclorac

Group	Phase	No. of males	No. of females	Dose	Analyses performed
Gavage administration[a]					
1A	SOLD	5	5	15 mg/kg bw	Routes and rates of excretion and metabolite analysis in urine, faeces and carcass. Urine samples collected at 8, 24, 48, 72, 96 and 120 h. Faeces collected at 24, 48, 72, 96 and 120 h.
1B	SOHD	5	5	600 mg/kg bw	
1C	ROLD	5	5	14 daily cold doses of 15 mg/kg bw per day + a final radiolabelled dose of 15 mg/kg bw	
2A	SOLD Bile duct cannulated	3	3	15 mg/kg bw	Routes and rates of excretion and metabolite analysis in urine, bile, faeces and carcass. Urine and faeces collected at 24 and 48 h. Bile collected at 3 h intervals up to 48 h.
2B	SOHD Bile duct cannulated	3	3	600 mg/kg bw	
3A	Plasma TK	5	5	15 mg/kg bw	Plasma concentrations determined in blood

Group	Phase	No. of males	No. of females	Dose	Analyses performed
	(SOLD)				drawn at pre-dosing, 0.25, 0.5, 1, 2, 3, 5, 7, 24, (31, 3D only), 48, 72, 96, 120, 168 (not 3A) and 240 (not 3A) h post-dosing.
3B	Plasma TK	5	5	100 mg/kg bw	
3C	Plasma TK (SOHD)	5	5	600 mg/kg bw	
3D	Plasma TK	5	5	1 200 mg/kg bw	
3E	Plasma TK (ROLD)	5	5	7 daily radiolabelled doses of 15 mg/kg bw per day	Blood was drawn prior to the first and the last dose and at 0.25, 0.5, 1, 2, 3, 5, 7, 24, 48, 72, 96, 120, 168 and 240 h (+336 and 432 h for 3F) after the last dose.
3F	Plasma TK (ROHD)	5	5	7 daily radiolabelled doses of 600 mg/kg bw per day	
4A	Tissue accumulation	5	5	7 daily radiolabelled doses of 15 mg/kg bw per day	Pairs of rats (one of each sex) were killed at 0.5, 6, 24, 72 and 120 h after the final dose, and the liver, kidneys, heart, lungs, brain, eyes, testes, ovaries, spleen, pancreas, adrenals, thyroid, uterus, gastrointestinal tract and contents, bone marrow, muscle, fat, blood and plasma were analysed for radioactivity.
4B	Quantitative whole-body autoradiography	5	–	7 daily radiolabelled doses of 15 mg/kg bw per day	One animal was killed 24 h after the first dose, and additional single animals were sacrificed at 0.5, 6, 24, 72 and 120 h after the final dose. They were deep frozen, sectioned sagittally, dried and placed on photographic film.
Dietary administration					
5A	Plasma concentrations	3	3	Rats were offered diet containing 15 000 ppm radiolabelled material (~1 200 mg/kg bw) after 8 h of fasting	Blood was sampled pre-dosing and at 2, 9, 24, 42 and 66 h after dosing.
		3	3		Blood was sampled pre-dosing and at 4, 12, 24, 42 and 66 h after dosing.
		3	3		Blood was sampled pre-dosing and at 6, 18, 24, 42 and 66 h after dosing.
5B	Tissue accumulation	6	6	Rats were offered diet containing 15 000 ppm radiolabelled material (~1 200 mg/kg bw) for 7 days, after which it was replaced with untreated diet	Pairs of rats (one of each sex) were killed at 0.5, 6, 24, 72 and 120 h after withdrawal of the treated diet (one rat of each sex), and the liver, kidneys, heart, lungs, brain, eyes, testes, ovaries, spleen, pancreas, adrenals, thyroid, uterus, gastrointestinal tract and contents, bone marrow, muscle, fat, blood and plasma were analysed for radioactivity.

bw: body weight; ppm: parts per million; ROHD: repeated oral high dose; ROLD: repeated oral low dose; SOHD: single oral high dose; SOLD: single oral low dose; TK: toxicokinetics

[a] Gavage in 1.5% carboxymethyl cellulose.

Source: Hawkins et al. (1986, 1987)

1.1 Absorption, distribution and excretion

The extent of oral absorption was high (> 90%), based on urinary and biliary data, with most of the biliary component reabsorbed and excreted in urine (Table 2). The biliary component increased disproportionately with increasing dose from 15 to 600 mg/kg bw. Absorption of radiolabel was rapid,

Table 2. Excretion pattern in rats exposed to quinclorac

	Excretion after oral administration (% of dose)									
	SOLD		**SOHD**		**ROLD**		**SOLD bile**		**SOHD bile**	
	15 mg/kg bw		**600 mg/kg bw**		**14 × 15 mg/kg bw**		**15 mg/kg bw**		**600 mg/kg bw**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Bile 0–48 h	–	–	–	–	–	–	3	1	14	11
Urine 0–24 h	90	88	85	79	93	89	81	92	69	51
Urine (total)	94	94	96	98	95	91	85	94	80	63
Faeces 0–24 h	1	1	3	0.4	2	0.5	2	4	1	2
Faeces (total)	1	1	4	1	2	1	3	4	2	2
Total excretion	95	95	100	100	98	93	93	100	97	97

bw: body weight; F: females; M: males; ROLD: repeated oral low dose; SOHD: single oral high dose; SOLD: single oral low dose
Source: Hawkins et al. (1986)

with maximal blood concentrations achieved between 0.25 and 1 hour for single doses of 600 mg/kg bw and below (Table 3). Quinclorac was widely distributed in the body, with highest concentrations present in the blood, plasma and kidneys (Table 4). Tissue levels were generally higher (< 2-fold) in females than in males. The labelled material was rapidly excreted, primarily via urine (50–90% in 24 hours) (Table 2). Initial plasma half-lives were calculated to be approximately 3–4 hours. Clearance from the blood was slower following repeated dosing with 600 mg/kg bw and with single doses of 1200 mg/kg bw, resulting in non-proportionate increases in the area under the concentration–time curve (AUC) (Table 3). The excretion pattern and tissue distribution of radioactivity were similar across administered dose levels and when the administration of radiolabelled quinclorac was preceded by 7 or 14 days of administration of the labelled or unlabelled material (Hawkins et al., 1986, 1987).

1.2 Biotransformation

Samples obtained from the study of Hawkins et al. (1986) (described in section 1.1 above) were extracted and analysed for the presence of metabolites using techniques including thin-layer chromatography and mass spectroscopy. Absorbed quinclorac was metabolized to only a limited extent, with unchanged parent compound representing approximately 80% of the excreted radiolabel. The major biotransformation product was quinclorac–glucuronide conjugate, representing approximately 5% of the administered dose. The pattern of metabolism was similar across sexes, dose levels and administration of repeated doses. A number of metabolites each representing less than 5% of the administered dose were not identified (Hawkins et al., 1986, 1987).

The metabolism of quinclorac is so limited that a metabolic pathway is considered unnecessary.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Quinclorac was of low acute toxicity by the oral, dermal and inhalation routes (Table 5). In the acute oral toxicity studies, clinical signs (including dyspnoea, piloerection and poor general state) were seen at 1780 mg/kg bw and above.

Table 3. Plasma radioactivity levels and AUC in rats receiving ^{14}C-labelled quinclorac

	Plasma radioactivity (µg equiv/g)					
Time (h)	**SOLD 15 mg/kg bw**	**ROLD 15 mg/kg bw (7 days)**	**Single oral mid dose 100 mg/kg bw**	**SOHD 600 mg/kg bw**	**ROHD 600 mg/kg bw (7 days)**	**Single oral dose 1 200 mg/kg bw**
Males						
0.25	29	29	171	192	249	263
0.5	33	34	181	235	256	320
1	27	28	125	201	297	347
2	15	21	75	187	293	368
7	6	12	30	201	255	327
24	0.1	0.6	1.4	78	164	505
48	0.1	0.4	0.3	6	27	140
72	0.1	0.3	0.2	1.6	10	6
168	0.1	0.2	0.2	0.8	3	1
AUC (h·µg/mL)	*141*	*297*	*803*	*4 958*	*12 359*	*21 256*
Females						
0.25	32	41	134	188	194	274
0.5	33	39	168	247	239	320
1	24	27	142	224	226	328
2	11	13	514	201	293	368
7	2	3	28	181	210	198
24	0.2	0.3	2.1	73	110	268
48	0.1	0.3	1.6	11	31	319
72	0.1	0.4	3	1.7	28	11
168	0.1	0.2	0.7	2	21	8
AUC (h·µg/mL)	*99*	*163*	*1 003*	*5 113*	*13 613*	*18 588*

AUC: area under the plasma concentration–time curve; bw: body weight; equiv: equivalents; ROHD: repeated oral high dose; ROLD: repeated oral low dose; SOHD: single oral high dose; SOLD: single oral low dose
Source: Hawkins et al. (1986)

(b) Dermal irritation

Quinclorac (batch no. 83/117; purity not stated) produced no erythema or oedema of the skin when tested in Vienna White rabbits (Grundler & Kirsch, 1983c). Similar results were reported in a more recent study (batch no. COD-000475; purity 99.4%) in New Zealand White rabbits (Gamer & Leibold, 2005c).

(c) Ocular irritation

Quinclorac (batch no. 83/117; purity not stated) produced transient, mild conjunctival effects (scores 0–2) when tested in Vienna White rabbits (Grundler & Kirsch, 1983d). Similar results were reported in a more recent study (batch no. COD-000475; purity 99.4%) in New Zealand White rabbits (Remmele & Leibold, 2005).

Table 4. Tissue distribution of radiolabel in rats administered ^{14}C-labelled quinclorac at 15 mg/kg bw per day for 7 days

	Tissue level of radioactivity 0.5 h after the last dose (µg equiv/g)	
Tissue	**Males**	**Females**
Adrenals	4.3	6.7
Bone marrow	3.4	6.8
Brain	0.7	1.3
Kidney	24	42
Liver	6.3	9.1
Pancreas	4.8	6.5
Plasma	35	62
Thyroid	6.7	11
Whole blood	17	23

bw: body weight; equiv: equivalents
Source: Hawkins et al. (1986)

Table 5. Summary of acute toxicity studies with quinclorac

Species	Strain	Sex	Route	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	Wistar	M & F	Oral gavage	Not stated	2 680 mg/kg bw	Grundler & Kirsch (1983a)
Rat	Wistar	F	Oral gavage	99.4	> 2 000 mg/kg bw	Gamer & Leibold (2005a)
Rat	Wistar	M & F	Dermal	Not stated	> 2 000 mg/kg bw	Grundler & Kirsch (1983b)
Rat	Wistar	M & F	Dermal	99.4	> 2 000 mg/kg bw	Gamer & Leibold (2005b)
Rat	Tif:RAIf	M & F	Inhalation (aerosol[a])	50 (formulated product)	> 5.15 mg/L	Klimisch (1986)

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male
[a] Mass median aerodynamic diameter 3.15 µm.

(d) Dermal sensitization

Quinclorac (batch no. COD-000475; purity 99.4%) produced no reactions in a guinea-pig maximization test with a challenge concentration of 25% weight per weight (w/w) (Gamer & Leibold, 2005d). A positive result was reported in an earlier maximization study using quinclorac of a lower purity (batch no. N55; purity 97.4%) and a 25% challenge concentration (Kieczka, 1986).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Groups of 10 male and 10 female B6C3F1/CrlBR mice were given diets containing quinclorac (batch no. N57 III/2; purity 98.3%) at 0, 4000, 8000 or 16 000 ppm for 3 months.

Achieved test article intakes were 0, 1001, 1992 and 4555 mg/kg bw per day for males and 0, 1466, 2735 and 5953 mg/kg bw per day for females, respectively. Clinical signs, body weight development, and feed and water consumption were monitored regularly. Samples for clinical chemistry and haematology investigations were taken prior to sacrifice. All animals were subjected to gross pathological examination followed by a microscopic examination. Control and top-dose animals received a full microscopic examination; for the low- and intermediate-dose groups, only lungs, liver, kidneys and gross lesions were examined.

There were no deaths or clinical signs associated with quinclorac administration. Body weights were significantly reduced in all treated groups, but the magnitude at the middle and low dose levels was less than 10% (Table 6). Water consumption was increased consistently at the top dose level, with an increase in blood urea levels in males at the middle and top doses. There was a reduction in relative kidney weights in top-dose males, but there was no associated pathology. Monocyte and eosinophil counts were reduced in a dose-related manner in males, but without statistical significance (Table 6). The pattern of lesions reported at microscopic examination was similar in test and control animals.

Table 6. Findings (means) in the 3-month study in mice receiving quinclorac in the diet

	Males				**Females**			
Parameter	**0 ppm**	**4 000 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**4 000 ppm**	**8 000 ppm**	**16 000 ppm**
Body weight (g)								
Day 0	22.6	22.6	22.4	22.4	19.1	19.1	19.2	18.9
Day 7	23.9	23.9	23.4	22.8*	20.1	19.8	19.9	19.0**
Day 84	29.0	28.3	27.0*	27.0*	26.2	24.8**	24.2**	23.4**
Day 91	31.1	29.6	26.7**	28.0**	26.6	25.2*	24.9**	24.4**
% body weight deficit (day 91)	–	−5%	−8%	−10%	–	−5%	−6%	−8%
Water consumption (g/animal per day)								
Day 7	6.9	6.8	6.9	7.1	5.8	5.9	6.0	6.6
Day 49	5.0	5.0	5.6	6.0	5.2	5.0	5.6	6.1
Day 84	5.0	5.3	5.5	6.7	5.5	5.2	5.9	6.1
Day 91	5.7	5.8	6.5	7.1	5.3	5.7	6.2	6.7
Urea (mmol/L)	8.28	8.98	9.19*	9.25*	7.52	8.16	8.07	8.36
Monocyte (%)	3.90	3.00	2.10	0.20	1.30	0.70	1.40	1.10
Eosinophils (%)	2.00	0.90	0.80	0.50	1.60	0.80	1.00	1.20
Mean cell volume (fL)	43.50	42.70**	42.54**	42.41**	43.34	42.92	42.84	42.85

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Kuehborth et al. (1988)

The no-observed-adverse-effect level (NOAEL) was 4000 ppm (equal to 1001 mg/kg bw per day), based on increased water consumption and blood urea levels in males at 8000 ppm (equal to 1992 mg/kg bw per day). The effects on body weight and mean cell volume at 4000 ppm are small in magnitude and not considered to be adverse (Kuehborth et al., 1988).

A second 3-month mouse study was performed using a single dietary level of quinclorac. Groups of 10 male and 10 female B6C3F1/CrlBR mice were given diets containing quinclorac (batch no. N57 III/2; purity 98.3%) at 0 or 500 ppm for 3 months. Achieved test article intakes were 0 and 85 mg/kg bw per day for males and 0 and 130 mg/kg bw per day for females, respectively. Clinical signs, body weight development, and feed and water consumption were monitored regularly. Samples for clinical chemistry and haematology investigations were taken prior to sacrifice. All animals were subject to gross pathological examination; there was no microscopic examination.

There were no deaths, clinical signs or clinical chemistry findings associated with treatment. Body weight was lower (6%) in females at day 98 only, but this appeared to be associated with a large increase in control values between days 90 and 98 (Table 7). Eosinophil and monocyte counts were reduced, but the reductions showed large intra-animal variation and were not statistically significant. Relative kidney weight was increased in females, but the magnitude was small (< 10%). None of the findings is considered to be adverse.

Table 7. Body and kidney weights (means) in mice receiving quinclorac in the diet for 90 days

	Males		**Females**	
	0 ppm (control)	**500 ppm**	**0 ppm (control)**	**500 ppm**
Body weight (g)				
Day 0	23.2	23.3	19.5	19.4
Day 90	–	–	25.2	24.7
Day 98	33.2	33.0	27.1	25.5*
Relative organ weights (group mean, g)				
Kidneys	1.644	1.584	1.495	1.605**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Schilling et al. (1988a)

The NOAEL was 500 ppm (equal to 85 mg/kg bw per day), the highest dose tested (Schilling et al., 1988a).

Rats

Groups of 10 male and 10 female Wistar (Chbb=Thom) rats were given diets containing quinclorac (batch no. N32; purity 96.5%) at a dose of 0, 1000, 4000 or 12 000 ppm for 3 months. Achieved test article intakes were 0, 77, 302 and 930 mg/kg bw per day for males and 0, 87, 358 and 1035 mg/kg bw per day for females, respectively. Clinical signs, body weight, and feed and water consumption were monitored regularly. Samples for clinical chemistry and haematology were taken on day 86. Urine analysis samples were obtained on day 80. Ophthalmoscopy was performed on animals in the control and top-dose groups, pretest and prior to sacrifice. All animals were subjected to a gross pathological examination followed by a microscopic examination. Control and top-dose animals received a full microscopic examination; for the low- and intermediate-dose groups, only lungs, liver, kidneys and gross lesions were examined.

An initial deficit in body weight in top-dose males was associated with reduced feed consumption and, although statistically significant, was less than 10% (Table 8). A number of clinical chemistry parameters and water consumption were altered in top-dose animals (Table 8), indicative of potential liver and kidney toxicity. Erythrocyte parameters were reduced in top-dose females. The only pathological findings of note were chronic interstitial nephritis and focal urothelial hyperplasia in top-dose males (Table 8). There were no treatment-related adverse effects in the animals of the low-

Table 8. Findings (means) in rats receiving quinclorac in the diet for 90 days

	Males				Females			
	0 ppm	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**
Body weight (g)								
Day 0	160.1	160.6	161.0	159.8	126.3	125.9	126.3	125.6
Day 7	206.6	210.2	209.8	193.0*	149.8	150.9	148.8	142.9
Day 14	256.5	262.5	258.7	240.9*	171.9	172.7	166.0	162.4
Day 49	391.5	400.8	399.7	357.7*	225.6	226.7	221.1	210.2
Day 91	458.8	477.2	477.2	430.9	252.5	259.7	247.7	235.4
Food consumption (g/animal per day)								
Day 7	23.1	23.8	22.9	19.7	17.2	17.5	17.3	15.6
Day 14	25.8	26.9	26.0	24.2	17.4	17.7	17.3	16.5
Day 49	27.4	26.9	26.7	25.5	18.0	18.2	18.8	17.1
Day 91	25.4	25.9	25.4	23.5	17.4	18.1	17.9	16.1
Water consumption (g/day)								
Days 15–16	27.20	27.30	27.40	36.33	18.60	15.90	15.22	19.50
Days 56–57	31.20	25.80	28.90	37.38	21.44	20.70	21.60	24.00
Days 87–88	27.30	29.30	29.70	34.30	23.80	21.90	24.60	32.00
Clinical chemistry								
Bilirubin (µmol/L)	1.842	0.965*	0.608**	0.572**	1.558	1.258	1.359	0.993*
Triglycerides (mmol/L)	2.690	3.434	3.618	2.013*	1.576	2.637*	1.621	1.397
Urea (mmol/L)	7.306	7.278	7.175	7.064	6.976	7.893*	7.029	7.394
ALAT (µkat/L)	0.832	0.883	0.937	1.050*	0.900	0.864	0.846	0.791
ASAT (µkat/L)	1.488	1.939*	2.092	2.599*	2.034	1.677	2.351	1.890
Haematology								
Haemoglobin (mmol/L)	9.246	8.922	9.034	9.036	9.294	9.217	9.200	8.449*
Haematocrit (L/L)	0.411	0.407	0.400	0.390*	0.405	0.402	0.395	0.374*
HBE (fmol)	1.124	1.129	1.107	1.109	1.140	1.135	1.134	1.109**
POLY (%)	9.10	9.90	9.70	11.90	10.90	16.50	13.90	19.80
Lymphocytes (%)	83.30	84.40	82.90	81.40	81.10	74.80	77.20	68.80
Monocytes (%)	5.30	4.50	6.20	5.20	5.70	6.80	7.40	9.50
Histopathology (no. of animals affected/10)								
Chronic interstitial nephritis	0	1	0	4	0	1	0	1
Focal urothelial hyperplasia	0	0	0	2	0	0	0	0
Liver, focal fatty infiltration	0	0	0	1	0	1	1	1

ALAT: alanine aminotransferase: ASAT: aspartate aminotransferase; HBE: haemoglobin per erythrocyte; POLY: neutrophilic segmented granulocytes; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Kuehborth, Deckardt & Hildebrand (1986)

and mid-dose groups. Plasma bilirubin was decreased dose relatedly in all male groups (Table 8), but this is not considered to be adverse in isolation.

The NOAEL was 4000 ppm (equal to 302 mg/kg bw per day), based on a range of clinical chemistry and haematological changes in both sexes and interstitial nephritis and urothelial hyperplasia in males at 12 000 ppm (equal to 930 mg/kg bw per day) (Kuehborth, Deckardt & Hildebrand, 1986).

Dogs

Quinclorac (batch no. N15; purity 93.6%) was offered to groups of Beagle dogs (two of each sex) in the diet at a dose of 0, 1000, 3000, 9000 or 27 000 ppm for 28 days. Achieved intakes of quinclorac were 0, 31, 95, 278 and 912 mg/kg bw per day for males and 0, 36, 108, 315 and 956 mg/kg bw per day for females, respectively. The animals were observed each day for any evident signs of toxicity. Body weights and feed consumption were determined weekly. Blood samples were taken from all animals pretest and on day 25 for examination of haematology and clinical chemistry parameters. Urine samples were taken pretest and on day 22. At the end of the treatment period, all animals were sacrificed and subjected to gross and limited histopathological examination (six organs plus any gross lesions).

At 27 000 ppm, feed consumption was reduced in females, and there was body weight loss over the duration of the study in both sexes. Kidney lesions were present at the top dose level (Table 9). Plasma alkaline phosphatase activity was reduced significantly at 9000 and 27 000 ppm, but this finding is not considered to be adverse in isolation (Table 9).

Table 9. Findings in dogs fed quinclorac in the diet for 28 days

	Males					**Females**				
	0 ppm	**1 000 ppm**	**3 000 ppm**	**9 000 ppm**	**27 000 ppm**	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**9 000 ppm**	**27 000 ppm**
Body weight (mean), kg										
Day 0	10.95	11.45	11.05	11.40	10.60	8.90	9.85	9.75	10.05	9.45
Day 7	10.95	11.40	10.90	11.35	10.05	8.85	9.75	9.65	9.95	8.85
Day 28	11.20	11.65	11.15	11.35	9.75	8.90	9.85	9.70	10.05	8.25
Parameter										
Alkaline phosphatase (day 25 means), µkat/L	5.05	3.72	4.13	2.58	2.19	3.86	2.33	2.87	2.38	1.41
Kidney tubules dilated	0	0	0	0	1	0	0	0	0	2
Kidney interstitial nephritis	0	0	0	0	1	0	0	0	0	2

ppm: parts per million
Source: Hellwig et al. (1985)

The NOAEL was 9000 ppm (equal to 278 mg/kg bw per day), based on body weight loss and kidney lesions at 27 000 ppm (equal to 912 mg/kg bw per day) (Hellwig et al., 1985).

In a 12-month dog study (six of each sex per group), dose levels of quinclorac (batch no. N32, purity 96.5%; and batch no. N55, purity 97.4%) were 0, 1000, 4000 and 12 000 ppm (equal to 0, 35, 139 and 490 mg/kg bw per day for males and 0, 35, 141 and 472 mg/kg bw per day for females, respectively). Clinical signs, body weight development, and feed and water consumption were monitored regularly. Ophthalmoscopic examinations and clinical pathology investigations (haematology, biochemistry and urine analysis) were completed once before and 3 times during the administration period. At the end of the treatment period, all animals were subjected to gross pathological examination followed by an extensive microscopic examination.

Top-dose animals suffered body weight loss at the start of the study, with no subsequent recovery (Table 10); body weights in other treated groups were lower than control values, but within normal variation for Beagle dogs. Feed efficiency was reduced at the top dose. A range of clinical chemistry changes was seen in top-dose animals; some of these, including reductions in creatinine, urea, calcium and bilirubin levels and alkaline phosphatase activity, were also observed at lower dose levels (Table 10). Haemoglobin, mean cell volume and erythrocyte counts were consistently lower in the top-dose group; changes at lower dose levels were mainly sporadic. Increases in relative brain, adrenal and thyroid weights appear to be secondary to the body weight deficits at the top dose level. Kidney and liver weights were increased in absolute and relative terms (Table 10). The increased relative liver weights at the low and middle doses did not exhibit a dose–response relationship and are not considered to be adverse. The increased relative kidney weights in males at 4000 ppm were without any related functional or histopathological changes, but the magnitude (20%) is such that there could be associated adverse effects. The only organs exhibiting treatment-related effects during histopathological examination were liver and kidneys in top-dose animals (Table 10).

The NOAEL was 1000 ppm (equal to 35 mg/kg bw per day), on the basis of increased relative kidney weights (~20%) in males at 4000 ppm (equal to 139 mg/kg bw per day). The changes in clinical chemistry findings at the low dose level are not considered to be adverse (Hellwig et al., 1988a).

(b) Dermal application

Quinclorac (batch no. III/2 N 57; purity 98.29%) was applied to the shaved skin of groups of New Zealand White rabbits at 40 or 200 mg/kg bw per day (five of each sex) or 0 or 1000 mg/kg bw per day (10 of each sex) for 6 hours/day, 7 days/week, for 21 applications. Animals were observed for clinical signs, local irritation, and feed consumption and body weight changes. Ophthalmoscopy and blood sampling were performed during week 3. At necropsy, a range of tissues was examined, and a limited number of organs were weighed and examined microscopically.

There were no deaths or clinical signs of toxicity. Animals of the high-dose group showed partly yellowish discoloured skin, which was attributed to the colour of the applied test material. A significant increase in absolute kidney weight in top-dose males was related to the higher body weight in this group. A dose-related reduction in uric acid was seen in males, but is not considered adverse. No other treatment-related effects were noted during the entire study period.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Ullmann et al., 1990).

(c) Exposure by inhalation

No data were submitted. Quinclorac has a low vapour pressure ($< 10^{-8}$ Pa), and respirable exposures are expected to be very low.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of mice (B6C3F1/CrlBr), 50 of each sex per dose level, received quinclorac (batch no. N55, purity 97.4%; and batch no. N57, purity 98.3%) at a dietary level of 0, 1000, 4000 or 8000 ppm for 78 weeks. The average compound intakes were 0, 170, 711 and 1444 mg/kg bw per day for males

Table 10. Findings in dogs fed quinclorac in the diet for 12 months

	Males				Females			
	0 ppm	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**
Body weight (group mean values, kg)								
Day 0	8.8	8.7	8.7	8.6	9.0	9.0	8.8	8.9
Day 70	10.3	9.5	9.4	8.2*	9.8	9.7	9.6	8.5
Day 91	10.5	9.6	9.8	8.5**	10.1	10.0	9.9	8.7
Day 182	11.3	10.7	10.8	9.0**	10.8	10.5	10.7	9.3
Day 273	11.3	10.6	10.4	8.7**	10.8	10.5	10.5	9.0
Day 364	11.2	10.4	10.0	8.3**	10.9	10.4	10.3	9.1
Feed efficiency (group means)								
Weeks 1–52	1.9	1.3	1.0	−0.3	1.2	1.1	1.2	−0.1
Creatinine (group means, µmol/L)								
Pre-dosing	76.0	75.7	71.7	70.3	83.8	72.2	78. 8	79.4
13 weeks	86.4*	80.0	77.8*	66.4**	84.1	85.4*	81.2	71.6**
26 weeks	86.3	80.7	75.4	58.6**	86.4	79.1*	78.0*	65.8**
52 weeks	86.2	78.3	69.7**	53.1**	85.5	80.3	74.6*	61.4**
Urea (group means, mmol/L)								
Pre-dosing	4.16	4.58	3.99	4.12	5.25	4.30	4.42	4.43
52 weeks	8.45*	4.49	4.35*	4.24*	5.55*	5.36	3.73**	4.19*
Calcium (group means, mmol/L)								
Pre-dosing	2.88	2.87	2.86	2.77	2.86	2.87	2.85	2.82
13 weeks	2.82	2.75*	2.75	2.66*	2.80	2.70**	2.73*	2.68**
26 weeks	2.77	2.72*	2.73	2.61*	2.76*	2.78	2.71*	2.64**
52 weeks	2.64**	2.61**	2.55**	2.49**	2.80	2.79	2.71**	2.66**
Total bilirubin (group means, µmol/L)								
Pre-dosing	2.63	2.70	2.27	3.12	3.29	2.98	3.17	2.93
13 weeks	3.55	3.74*	2.09*	2.40	3.46	3.59	3.14	2.44
26 weeks	2.49	2.71	1.72	1.22**	2.22	2.39	1.95	1.21**
52 weeks	2.81	2.29	1.25*	1.18*	2.44	2.42	2.30	1.48**
Alkaline phosphatase (group means, µkat/L)								

	Males				Females			
	0 ppm	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**
Pre-dosing	6.42	6.05	5.31	4.90*	4.37	5.91	6.19*	5.37
13 weeks	4.88	4.57	2.82**	2.36**	3.34	4.17*	3.06**	2.20**
26 weeks	3.71*	2.99**	2.13**	2.28**	2.92*	4.32	3.18**	2.31**
52 weeks	4.23	3.93	2.28**	3.04*	3.51	4.89	3.90*	3.18*
Haemoglobin (group means, mmol/L)								
Pre-dosing	8.80	9.01	8.95	8.31	9.21	8.57*	9.23	8.73
13 weeks	9.67	9.33	9.16	7.41**	9.79	9.40	10.1*	8.52*
26 weeks	10.1	10.0	10.0	8.25**	10.5	9.77*	9.49*	8.73**
52 weeks	10.4	10.2	9.97	8.63**	10.2	9.79	10.3	8.74*
Erythrocytes (group means, 10^{12}/L)								
Pre-dosing	6.16	6.30	6.20	5.83	6.42	6.00	6.50	6.13
13 weeks	6.60	6.30	6.29	8.28**	6.62	6.38	6.88	6.11
26 weeks	6.79	6.75	6.83	5.82**	7.10**	6.64	6.47*	6.22**
52 weeks	6.96	6.89*	6.81	6.04**	6.83	6.65	6.93	6.22
MCV (group means, fL)								
Pre-dosing	69.8	69.6	69.7	69.1	71.4	71.0	70.7	71.2
13 weeks	69.7	69.8	69.3	67.3**	70.2	70.2	69.5	67.1**
26 weeks	70.5	70.4	69.6	67.9**	71.5	70.5	70.0	67.1**
52 weeks	70.9	70.7	69.9	68.8	71.6	70.2	70.9	68.0**
Organ weights (group mean values)								
Liver (absolute; g)	365.3	383.3	350.2	406.1	360.0	411.9	402.7	472.7**
Liver (relative; % body wt)	3.258	3.692	3.522	4.902**	3.319	3.987*	3.964*	5.220**
Kidney (absolute; g)	52.3	51.6	56.5	60.1	52.5	54.2	55.6	60.3
Kidneys (relative; % body wt)	0.469	0.499	0.563**	0.726**	0.485	0.527	0.548	0.666**
Brain (relative; % body wt)	0.723	0.762	0.810	1.028**	0.695	0.755	0.758	0.820*
Adrenal (relative; % body wt)	0.009 7	0.010 3	0.011 6	0.013 5**	0.011 6	0.014 8	0.014 8	0.014 8

	Males				Females			
	0 ppm	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**
Thyroid (relative; % body wt)	0.007 4	0.008 2	0.008 4	0.010 3*	0.007 8	0.009 7	0.008 5	0.012 3**
Histopathology findings								
Liver congestion	0	0	0	1	0	0	0	0
Liver single-cell necrosis	0	0	0	2	0	0	0	2
Kidney hydropic degeneration	0	0	0	2	0	0	0	2

MCV: mean cell volume; ppm: parts per million; wt: weight; *: $P < 0.05$; **: $P < 0.01$
Source: Hellwig et al. (1988a)

and 0, 213, 869 and 1828 mg/kg bw per day for females, respectively. Blood samples were taken during week 78 from 10 animals of each sex per group for evaluation of haematological parameters. All animals received a gross examination. An extensive microscopic examination was performed on all control and top-dose animals, with only gross lesions, lung, liver, kidney and gallbladder examined in the mid- and low-dose groups.

There were no effects on mortality, appearance or behaviour. Survival at week 78 was greater than 90% in all groups. The only notable finding was a progressive and dose-related reduction in body weight in all groups exposed to quinclorac (Table 11). The body weight deficit in males from the 1000 ppm group was less than 10% and is not considered to be adverse in isolation. There were no effects on haematological parameters and no increases in non-neoplastic or neoplastic lesions.

Table 11. Body weights of mice receiving quinclorac in the diet for 78 weeks

	Mean body weight (g)							
	Males				Females			
Day	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**8 000 ppm**	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**8 000 ppm**
0	21.3	21.2	21.1	21.0	17.7	17.5	17.4	17.4
7	22.2	22.0	21.4**	21.3**	18.7	18.2*	17.9**	17.8**
182	32.0	31.9	29.4**	28.8**	28.6	27.6	26.9**	26.2**
350	35.4	34.0*	31.5**	30.1**	34.8	31.0**	30.1**	28.9**
546	34.0	31.3**	29.4**	28.6**	33.6	28.8**	28.5**	27.6**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Schilling et al. (1988c)

No NOAEL for non-neoplastic toxicity could be identified. The lowest-observed-adverse-effect level (LOAEL) for non-neoplastic toxicity was 1000 ppm (equal to 213 mg/kg bw per day), based on significantly reduced body weight (> 10%) in females at all doses. The NOAEL for carcinogenicity was 8000 ppm (equal to 1444 mg/kg bw per day), the highest dose tested (Schilling et al., 1988c).

A supplementary 78-week study in mice was performed to determine a NOAEL for quinclorac in a chronic mouse study. Groups of mice (B6C3F1/CrlBr), 50 of each sex per group, received quinclorac (batch no. N57; purity 98.3%) at a dietary level of 0 or 250 ppm for 78 weeks. Achieved quinclorac intakes in the main groups were 0 and 42 mg/kg bw per day for males and 0 and 52 mg/kg bw per day for females, respectively. A satellite group of 10 mice of each sex was sacrificed after 26 weeks. Blood samples were taken during week 26 from the satellite group and week 78 from 10 animals of each sex per group for evaluation of haematological parameters. All main group animals received an extensive gross examination; no histopathological examinations were performed.

There were no effects on clinical signs or mortality. Survival was 90% or higher in all groups. Feed consumption was consistently lower in both sexes of treated mice during the first 6 months of the study and in females for the majority of the study. Body weights were similar in treated and control mice. There were no effects on haematology, organ weights or gross pathological findings.

The NOAEL was 250 ppm (equal to 52 mg/kg bw per day in females), the only dose tested (Schilling et al., 1988d).

Rats

Groups of 50 male and 50 female Wistar (Chbb=Thom) rats were given diets containing quinclorac (batch no. N55, purity 97.4%; and batch no. N57, purity 98.3%) at a dietary level of 0, 1000, 4000 or 8000 ppm for 2 years for evaluation of carcinogenic potential. Satellite groups received diets containing quinclorac at 0, 1000, 4000, 8000 or 12 000 ppm for evaluation of chronic toxicity over 2 years (20 rats of each sex per group) or 12 months (10 rats of each sex per group). Achieved intakes were 0, 55, 221 and 444 mg/kg bw per day for males and 0, 66, 262 and 529 mg/kg bw per day for females, respectively, in the carcinogenicity phase. In the satellite groups, mean test article intakes were reported as 0, 55, 221, 444 and 675 mg/kg bw per day for males and 0, 66, 262, 529 and 832 mg/kg bw per day per day for females, respectively.

Regular investigations of mortality, clinical signs, feed and water consumption, and body weight were performed. Ophthalmoscopy was performed on control and 12 000 ppm animals pretest and at 12 and 24 months. Blood samples for clinical chemistry and haematology were taken from satellite groups at 3, 6, 12, 18 and 24 months. All animals received a gross examination. A wide range of tissues from animals in the control and top-dose groups was examined histopathologically; liver, kidney, lung and gross lesions from low- and intermediate-dose group animals were also examined histopathologically. A supplementary investigation (Schilling, Maita & Hildebrand, 1991; Tobia & Mascianica, 1991) evaluated the pancreas from all animals in the main and satellite groups.

Survival was unaffected by quinclorac treatment and was greater than 65% in all carcinogenicity groups. Water consumption was routinely higher (~20%) in all groups receiving 8000 and 12 000 ppm, but the increase did not achieve statistical significance. Body weights were lower (~10%) in females in the 12 000 ppm group, attaining statistical significance between days 518 and 658 in the main group. The patterns of clinical signs, haematology results, clinical chemistry findings, and urine analysis results were similar in test and control groups. Relative kidney weights were increased by more than 10% in the 24-month satellite group, in males at 4000 ppm and above and in females at 8000 ppm; this was not reproduced in the main group, and statistical significance was not achieved (Table 12). The only renal lesion demonstrating a relationship to quinclorac was renal pelvis mineralization, which was increased in the main groups but not in the 24-month satellite groups. Overall, it is concluded that the renal findings do not present a coherent relationship to treatment and are not an adverse effect of quinclorac administration.

An increase in splenic haemangiosarcoma in top-dose males was not repeated in females and was not associated with any preneoplastic changes, but it was associated with an absence of haemangioma of the spleen; this is not considered to be an indication of a carcinogenic response to quinclorac. An increase in pancreatic acinar cell tumours (Table 12) was seen in top-dose males of the main and 24-month satellite groups, but this was not repeated in females; the incidence of hyperplasia was higher than the control incidence, but exhibited no dose–response relationship. Historical control

Table 12. Findings in the 24-month study of quinclorac in rats

Parameter	0 ppm	1 000 ppm	4 000 ppm	8 000 ppm	12 000 ppm
Males					
Body weight (g)					
12 months (satellite)	646	629	642	683	656
24 months (main)	679	694	715	702	–
24 months (satellite)	715	721	693	703	673
Main group (*n* = 50 rats)					
Total number of neoplasms	88	73	63	83	–
Spleen, haemangioma	2	0	1	0	–
Spleen, haemangiosarcoma	0	0	0	2	–
Pancreas, acinar cell adenoma	0	1	0	3	–
Pancreas, acinar cell adenocarcinoma	0	0	0	1	–
Pancreas, acinar cell hyperplasia	3	5	8	6	–
Pancreas, islet cell adenoma	1	1	1	0	–
Mineralization of renal pelvis					
Main	18	20	17	27	–
24-month satellite	5	8	11	10	5
Relative kidney weight (% of body weight)					
Main	0.61	0.63	0.59	0.62	–
24-month satellite	0.58	0.58	0.66	0.66	0.66
12-month satellite	0.54	0.55	0.54	0.53	0.55
Females					
Body weight (g)					
12 months (satellite)	374	350	354	344	335
24 months (main)	425	413	414	395	–
24 months (satellite)	401	394	401	397	366
Main group (*n* = 50 rats)					
Total number of neoplasms	86	90	84	79	–
Spleen, haemangiosarcoma	0	0	0	1	–
Pancreas, acinar cell adenoma	0	0	0	0	–
Pancreas, acinar cell adenocarcinoma	0	0	0	0	–
Pancreas, acinar cell hyperplasia	1	0	1	3	–
Pancreas, islet cell adenoma	0	0	0	1	–
Mineralization of renal pelvis					
Main	14	15	14	22	–
Satellite	2	2	2	4	3
Relative kidney weight (% of body weight)					
Main	0.71	0.72	0.78	0.76	–

Parameter	0 ppm	1 000 ppm	4 000 ppm	8 000 ppm	12 000 ppm
24-month satellite	0.77	0.80	0.80	0.85	0.84
12-month satellite	0.63	0.64	0.65	0.67	0.66

ppm: parts per million
Source: Schilling et al. (1988b)

data were supplied (see Tables A1 and A2 in Appendix 1): the mean incidence of acinar cell adenoma in males was 4.4% (range 0–18%); for adenocarcinoma, the mean incidence was 0.7% (range 0–5.1%). As the incidence of pancreatic tumours is within the pattern of the contemporary historical control data for the test facility, quinclorac is considered not to have carcinogenic potential in rats.

The NOAEL for general toxicity was 8000 ppm (equal to 529 mg/kg bw per day), based on lower body weights (~10%) in females at 12 000 ppm (equal to 832 mg/kg bw per day).

The NOAEL for carcinogenicity was 8000 ppm (equal to 444 mg/kg bw per day), the highest dose tested in the carcinogenicity segment (Schilling et al., 1988b).

2.4 Genotoxicity

(a) In vitro studies

Quinclorac was tested for genotoxicity in an adequate range of assays. Quinclorac was not genotoxic in an Ames test with batch no. N55, but a positive result was seen with batch no. N15, which was reported to contain impurities with genotoxic potential. Supplemental gene mutation assays with *Escherichia coli* were negative. Negative results were seen in a rec assay in *Bacillus subtilis.* Positive results were produced in a cytogenicity assay in human lymphocytes at high concentrations (see Table A3 in Appendix 1). Inconsistent results were seen in a gene mutation assay in Chinese hamster ovary cells; negative results were seen in the first assay, but a positive result was seen in the second assay in the presence of metabolic activation (see Table A4 in Appendix 4; see also Table 13).

(b) In vivo studies

Quinclorac was not genotoxic in assays for micronucleus induction in mouse bone marrow and unscheduled DNA synthesis in rat liver (Table 13).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Groups of Wistar (Chbb=Thom (SPF)) rats (24 of each sex per group) received diets containing quinclorac (batch no. N55 III, purity 97.38%; and batch no. N57 III/2, purity 98.29%) at 0, 1000, 4000 or 12 000 ppm. Achieved intakes were reported to be approximately 0, 96, 381 and 1180 mg/kg bw per day at 0, 1000, 4000 and 12 000 ppm, respectively. There were two litters in the first generation and one in the second. Parents were mated 1:1 after approximately 10 weeks of exposure to quinclorac for the F_{1a} and F_{2a} litters. For the F_{1b} mating, parents were paired 10 days after weaning of the F_{1a} pups. Litters were not culled on day 4 postpartum. Clinical signs, body weights, feed consumption, mating parameters, gestation and delivery parameters, pup survival, and physical and behavioural development (ear and eye opening, grip strength and pupillary reflex) were recorded. No evaluation of date of sexual maturation was included in the protocol. A gross necropsy examination was performed on all pups not selected for mating. Further examinations were performed on any pups found dead or dying or showing macroscopic changes. All parental animals were necropsied after weaning of their offspring and subjected to pathological examination.

Table 13. Genotoxicity studies with quinclorac

Test	Target	Concentration or dose tested	Purity (%)	Results	Reference
In vitro					
Gene mutations in bacteria	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA 1537	20–5 000 µg/plate (±S9)	Purity not stated (batch no. N55)	Negative ±S9	Gelbke & Engelhardt (1985)
Gene mutations in bacteria	*Escherichia coli* WP2uvrA	20–5 000 µg/plate (±S9)	Purity not stated (batch no. N55)	Negative ±S9	Gelbke & Engelhardt (1986c)
Gene mutations in bacteria	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	20–5 000 µg/plate (±S9)	98.3	Negative ±S9	Hoffmann & Engelhardt (1988)
Gene mutations in bacteria	*S. typhimurium* strain TA1535	20–5 000 µg/plate (±S9)	Purity not stated (batch no. N15)	Positive ±S9[a]	Gelbke & Engelhardt (1985)
Gene mutations in bacteria	*Bacillus subtilis* H17 and M45 (rec assay)	1–10 000 µg/plate (±S9)	Purity not stated (batch no. N57)	Negative ±S9	Hoorn (1987)
Gene mutations in mammalian cells	Chinese hamster ovary cells (HPRT)	46, 100, 215, 464, 1 000 and 2 150 µg/mL (±S9)	97.4	Equivocal +S9 Negative −S9	Jaeckh & Hoffmann (1990)
Chromosomal aberrations	Human lymphocytes	250, 500 and 1 000 µg/mL (±S9)	96.5	Positive ±S9	Gelbke & Engelhardt (1986a)
In vivo					
Micronucleus test	Male and female NMRI mice (bone marrow)	500, 1 000 and 2 000 mg/kg bw	96.5	Negative	Gelbke & Engelhardt (1986b)
Unscheduled DNA synthesis	Male Wistar rats (liver)	100 and 1 000 mg/kg bw	97.4	Negative	Fautz & Voelkner (1991)

bw: body weight; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] At cytotoxic concentrations.

There was no effect on mating, fertility, pregnancy outcome, litter size or pup survival (Table 14). Significant reductions in maternal body weight gain were seen in males and in females during pregnancy and lactation at 12 000 ppm in the F_1 matings. Pup body weights were similar to control values at birth, but were significantly lower by day 21 at 12 000 ppm in all generations (Table 14). Examinations of pups and parental animals did not identify any adverse effects of quinclorac administration. Attainment of physical developmental markers and behavioural results were similar in all groups of pups. Top-dose female, but not male, parents had an increased incidence of nephritis in both generations, but a reduction in calcification in F_1 parents (Table 15). There were no adverse findings in the reproductive organs.

The NOAEL for reproductive toxicity was 12 000 ppm (equivalent to 1180 mg/kg bw per day), the highest dose tested.

Table 14. Litter size and pup weights in rats exposed to quinclorac in the diet

	0 ppm		1 000 ppm		4 000 ppm		12 000 ppm	
	Day 0	**Day 21**	**Day 0**	**Day 21**	**Day 0**	**Day 21**	**Day 0**	**Day 21**
Pups alive/litter								
F_{1a}	12.4	12.0	12.1	11.5	12.5	12.1	12.4	11.1
F_{1b}	14.0	13.6	13.8	12.9	14.1	13.8	13.1	12.8
F_{2a}	12.5	11.7	13.2	12.4	11.4	11.1	11.7	10.7
Pup weight (g), males								
F_{1a}	6.1	45	6.3	46	6.2	43	5.9	33**
F_{1b}	6.0	43	6.1	45	6.2	41	6.0	35**
F_{2a}	6.1	48	6.0	47	6.2	47	5.7	37**
Pup weight (g), females								
F_{1a}	5.8	43	6.0	44	5.9	41	5.6	32**
F_{1b}	5.7	41	5.9	43	5.8	39	5.8	35**
F_{2a}	5.9	46	5.6	45	5.8	46	5.5*	37**

F_1: first filial generation; F_2: second filial generation; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Hellwig et al. (1988b)

Table 15. Renal pathology findings in female parental rats exposed to quinclorac

Parameter	**0 ppm**	**1 000 ppm**	**4 000 ppm**	**12 000 ppm**
F_0 females				
Interstitial nephritis	4	1	4	16
Calcification (cortex and medulla)	21	21	18	20
F_1 females				
Interstitial nephritis	6	5	4	17
Calcification (cortex and medulla)	23	19	11	1

F_0: parental generation; F_1: first filial generation; ppm: parts per million
Source: Hellwig et al. (1988b)

The NOAEL for parental toxicity was 4000 ppm (equivalent to 381 mg/kg bw per day), based on an increase in the incidence of interstitial nephritis in females at 12 000 ppm (equivalent to 1180 mg/kg bw per day).

The NOAEL for offspring toxicity was 4000 ppm (equivalent to 381 mg/kg bw per day), based on reduced pup weights during lactation at 12 000 ppm (equivalent to 1180 mg/kg bw per day) (Hellwig et al., 1988b).

(b) Developmental toxicity

Rats

Groups of 25 female Wistar rats received quinclorac (batch no. N32; purity 96.5%) by gavage in 0.5% carboxymethyl cellulose in distilled water at a dose of 0, 24.4, 146 or 438 mg/kg bw per day on days 6–15 of gestation. Dams were sacrificed on day 20, and the uterine contents were removed

and examined. Viable fetuses were enumerated and examined for external, visceral and skeletal abnormalities.

In the 438 mg/kg bw per day dose group, two dams died, and another had to be sacrificed during the treatment period. The general state of health of these animals deteriorated during treatment; at necropsy, severe ulcerations of the glandular stomach were evident. In this test group, a significant decrease in feed consumption and a marked increase in water intake were observed during the dosing phase, as well as a slight body weight loss at the beginning of the treatment period (Table 16). The body weight deficit is of a similar magnitude to the reduction in feed consumption. In the 24.4 and 146 mg/kg bw per day dose groups, there were no biologically relevant changes in clinical signs, feed intake, water intake, body weight or macroscopic findings. A statistically significant increase in water consumption (25%) was noted at 146 mg/kg bw per day on days 7–8 of gestation, but not subsequently.

There were no effects on number of viable fetuses or the incidence of fetal abnormalities or variations (Table 17). Fetal weights were slightly higher in treated groups, but this is considered to be of no biological relevance.

The maternal NOAEL was 146 mg/kg bw per day, on the basis of mortality, severe ulcerations of the glandular stomach, reduced feed consumption and increased water consumption at 438 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 438 mg/kg bw per day, the highest dose tested (Hellwig, 1987).

Table 16. Feed consumption, water intake and body weight data in a developmental toxicity study of quinclorac in rats

Parameter	0 mg/kg bw per day	24.4 mg/kg bw per day	146 mg/kg bw per day	438 mg/kg bw per day
Feed consumption (g)				
Days 7–8	48	46	49	42**
Days 9–10	50	48	52	43**
Days 11–13	80	76	79	71*
Days 18–20	88	85	88	87
Water intake (g)				
Days 7–8	50	50	63**	83**
Days 9–10	53	51	58	89**
Days 11–13	92	91	95	137**
Days 18–20	369	355	369	417**
Body weight gain (g)				
Days 6–8	7.4	6.2	7.0	−2.6
Days 8–10	11.5	11.6	12.8	8.6
Days 0–20	145	140	150	140
Gravid uterus weight (g)	70	68	77	71

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Hellwig (1987)

Table 17. Fetal data in a developmental toxicity study of quinclorac in rats

Observation	No. of fetuses / No. of litters affected			
	0 mg/kg bw per day	**24.4 mg/kg bw per day**	**146 mg/kg bw per day**	**438 mg/kg bw per day**
Fetal weight (g)	3.27	3.32*	3.33*	3.29*
No. of fetuses / no. of litters evaluated	321 / 25	297 / 22	307 / 23	259 / 20
Total skeletal anomalies	7 / 4	9 / 9	7 / 6	3 / 3
Total visceral anomalies	1 / 1	1 / 1	1 / 1	3 / 3
Total skeletal variations	33 / 16	24 / 15	38 / 20	23 / 15

bw: body weight; *: $P < 0.05$
Source: Hellwig (1987)

Rabbits

Quinclorac (batch no. N 57 III/2; purity unspecified) was administered to groups of 15 pregnant Himalayan rabbits via gavage in 0.5% carboxymethyl cellulose at a dose level of 0, 70, 200 or 600 mg/kg bw per day on days 7–19 of gestation. On day 29 post-insemination, all animals were sacrificed, and the fetuses were removed by caesarean section. Uterine contents and the fetuses were examined, including soft tissue and skeletal evaluations.

Administration of quinclorac caused severe signs of maternal toxicity at 600 mg/kg bw per day and slight toxicity at 200 mg/kg bw per day. In the 600 mg/kg bw per day dose group, severely reduced feed consumption and reductions in body weight were recorded (Table 18). There were also clinical signs, including reduced or no defecation, diarrhoea, apathy and/or poor general state. Water consumption was similar to that of controls. There were six deaths in the top-dose group between days 14 and 21 of gestation, five of which were considered by the study report authors to be compound related; the remaining one was attributed to dosing error. At necropsy, the findings included a reduction in uterine weights, an increased number of dead implantations/reduced number of live fetuses and slightly reduced fetal weights. An increase in the proportion of fetuses with skeletal variations was not associated with a specific variation (Table 19). In the 200 mg/kg bw per day dose group, slightly reduced feed consumption by the does during the treatment period and a trend towards reduced feed consumption, body weight and body weight gain were observed (Table 18), but the pattern and magnitude were not considered to be biologically relevant. There were no developmental effects at 200 mg/kg bw per day (Table 19). In the 70 mg/kg bw per day dose group, there were no notable effects of quinclorac.

The NOAEL for maternal toxicity was 200 mg/kg bw per day, on the basis of mortality and body weight loss at 600 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 200 mg/kg bw per day, based on reduced number of viable fetuses, reduced fetal weight and an increase in fetuses with skeletal variations at 600 mg/kg bw per day (Hellwig, Hildebrand & Shirasu, 1988).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

Groups of Crl:WI(Han) rats (10 of each sex) received quinclorac (batch no. 479-480; purity 99.9%) at 0, 150, 500 or 1500 mg/kg bw by gavage in 1% carboxymethyl cellulose in water. Animals were subjected to a functional observational battery and assessments of locomotor activity 7 days prior to dosing, at 3–5 hours on the day of dosing and at 7 and 14 days post-dosing. The functional

Table 18. Feed intake and body weight data for does in a developmental toxicity study of quinclorac in rabbits

Parameter	0 mg/kg bw per day	70 mg/kg bw per day	200 mg/kg bw per day	600 mg/kg bw per day
Number of does with viable fetuses	14	13	13	6
Feed consumption (g/animal per day)				
Days 4–7	133	129	127	137
Days 7–9	122	117	100	44
Days 14–16	95	96	90	50
Days 23–25	114	114	113	125
Body weight (kg)				
Day 0	2.29	2.28	2.29	2.28
Day 29	2.63	2.62	2.59	2.49*
Body weight gain (g)				
Days 4–7	7.6	10.8	17.2	23.4
Days 7–9	12	8.6	−2.2	−127**
Days 14–16	28	37	26	−37**
Mean gravid uterine weight (g)	303	293	311	238*

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Hellwig, Hildebrand & Shirasu (1988)

Table 19. Fetal data from a developmental toxicity study of quinclorac in rabbits

	No. of fetuses / No. of litters affected			
Observation	**0 mg/kg bw per day**	**70 mg/kg bw per day**	**200 mg/kg bw per day**	**600 mg/kg bw per day**
No. of fetuses evaluated / no. of litters evaluated	75 / 13	69 / 13	75 / 13	35 / 6
Mean no. of live fetuses/litter	5.4	5.3	5.8	4.4
Mean pup weight (g)	42	41	40	39*
Total external abnormalities	1 / 1	1 / 1	1 / 1	2 / 1
Total soft tissue malformations	1 / 1	3 / 2	1 / 1	1 / 1
Total soft tissue variations	38 / 11	43 / 12	43 / 12	17 / 5
Total skeletal malformations	0	0	0	1 / 1
Total skeletal variations	9 / 6	13 / 8	7 / 5	11 / 6*
Total skeletal retardations	41 / 12	31 / 12	27 / 8*	23 / 6

bw: body weight; *: $P < 0.05$
Source: Hellwig, Hildebrand & Shirasu (1988)

observational battery included open-field, reflex, neuromuscular and physiological assessments. At sacrifice, half the animals were perfused with 2.5% buffered glutaraldehyde, a full postmortem was performed and samples of a range of nervous tissue and muscle were preserved in formaldehyde, processed and then examined microscopically.

Male rats of the 1500 mg/kg bw dose group showed impaired body weight gain at days 7 (35% deficit) and 14 (15% deficit). Clinical signs and functional observational battery changes were observed on the day of administration at the highest dose level of 1500 mg/kg bw. Motor activity was reduced, with a dose–response relationship for magnitude, on study day 0, 4–5 hours post-dosing, in both sexes of the 1500 mg/kg bw dose group and in males at 500 mg/kg bw. Although a statistically significant decrease was seen in males at 150 mg/kg bw, this group showed a consistently low activity pretest and on days 7 and 14 post-dosing (Table 20). The findings in the low-dose males are considered not to be treatment related. All findings were reversible and not observed on study days 7 and 14. There were no effects noted for neuropathology or brain weight determinations.

Table 20. Locomotor results in rats (10 per group) dosed with quinclorac (total beam breaks in 12 intervals of 5 minutes each)

	Mean number of beam interruptions							
	0 mg/kg bw		**150 mg/kg bw**		**500 mg/kg bw**		**1 500 mg/kg bw**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Day −7, pretest	3 939	3 665	3 028	3 727	3 631	3 753	3 979	3 398
(% control)			(77)	(102)	(92)	(102)	(101)	(93)
Day 0	3 554	3 826	2 622*	4 641	2 425*	3 125	1 807**	2 772*
(% control)			(74)	(121)	(63)	(82)	(51)	(72)
Day 7	3 419	4 793	2 929	5 037	3 340	3 967	3 467	4 012
(% control)			(86)	(105)	(98)	(83)	(101)	(84)
Day 14	3 717	4 563	2 862	6 736	3 751	3 699	3 748	5 129
(% control)			(77)	(148)	(101)	(81)	(101)	(112)

bw: body weight: F: female; M: male; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Buesen et al. (2012a)

The NOAEL was 150 mg/kg bw, based on reduced motor activity in males at 500 mg/kg bw (Buesen et al., 2012a).

A benchmark dose (BMD) assessment was performed, using the United States Environmental Protection Agency's Benchmark Dose Software (BMDS), a discriminating parameter of 1 standard deviation and a Hill model. Values for the total locomotor activity BMD and lower limit on the benchmark dose (BMDL) for males rats were 106 mg/kg bw and 33 mg/kg bw, respectively (Dammann, 2015).

Subchronic neurotoxicity

Groups of Crl:WI(Han) rats (10 of each sex) received quinclorac (batch no. 479-480; purity 99.9%) at a dietary concentration of 0, 1500, 5000 or 15 000 ppm over a period of 13 weeks. Achieved concentrations, stability and homogeneity were verified by analysis. Achieved test material intakes were 0, 96, 301 and 976 mg/kg bw per day for males and 0, 112, 368 and 1142 mg/kg bw per day for females, respectively. Routine examinations of feed consumption, body weight and clinical signs were performed. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Functional observational batteries and motor activity measurements were carried out on study days −7, 1, 22, 50 and 85. Five animals of each sex per test group were fixed by in situ perfusion and subjected to neuropathological examinations.

Body weight at termination was approximately 5% lower in both sexes at 15 000 ppm; this was not statistically significant and is not considered to be adverse. No mortality occurred during the

study, and no treatment-related clinical signs were observed. Functional observational batteries and motor activity measurements revealed no test material–related neurobehavioural effects at any concentration on study days 1, 22, 50 and 85. There were no effects on neuropathology or brain weight determination.

Under the conditions of this study, the NOAEL for neurotoxicity and general toxicity was 15 000 ppm (equal to 976 mg/kg bw per day), the highest dose tested (Buesen et al., 2012b).

(b) Immunotoxicity

Quinclorac (batch no. 479-480; purity 99.9%) was administered via the diet to groups of eight female C57BL/6J Rj mice at a dose level of 0, 500, 1500 or 5000 ppm over a period of 4 weeks for evaluation of immunotoxicity (sheep red blood cell [sRBC] immunoglobulin M [IgM] antibody titres, spleen and thymus weights and pathology). Quinclorac intakes were 0, 176, 439 and 1760 mg/kg bw per day, respectively. A positive control group received cyclophosphamide monohydrate (10 mg/kg bw per day). Clinical signs, body weight development, and feed and water consumption were monitored.

Clinical examination of the animals treated with the test material revealed no adverse treatment-related findings. There were no effects on the spleen or thymus. sRBC IgM titres were reduced slightly at the top dose level, but mean values were within the typical variation for the parameter (Table 21). The sensitivity of the assay was confirmed by significant findings in the positive control group.

Table 21. SRBC IgM antibody titres in mice receiving quinclorac in the diet for 4 weeks

Parameter	0 ppm	500 ppm	1 500 ppm	5 000 ppm
sRBC mean	3 134	2 884	2 922	2 359
SD	492	356	1 379	600
Range	2 168–3 564	2 263–3 386	493–4 605	1 175–3 070

ppm: parts per million; SD: standard deviation; sRBC: sheep red blood cells
Source: Buesen et al. (2010)

The NOAEL for immunotoxicity was 5000 ppm (equal to 1760 mg/kg bw per day), the highest dose tested (Buesen et al., 2010).

(c) Toxicity of metabolites

Studies of absorption, excretion and metabolism, acute oral toxicity and repeated-dose oral toxicity (90 days) have been performed on quinclorac methyl ester (BAS Reg. No. 161555), a plant metabolite of quinclorac.

Absorption, distribution and excretion

In an initial investigation, a single group of three female Wistar rats was dosed with [3-^{14}C]quinclorac methyl ester (specific activity 71.3 Bq/µg; radiochemical purity 94%) by gavage in 1:1 Tween:ethanol at 50 mg/kg bw. Urine was collected at 8, 24, 48, 72, 96 and 120 hours. Faeces were collected at 24, 48, 72, 96 and 120 hours. Samples were processed and analysed by LSC. In the first 24 hours, 89% of the dose was excreted in the urine (66%) and faeces (24%). In total, 68% of the dose was recovered in the urine, with 32% of the dose recovered in the faeces (Parker, 1998).

The absorption, distribution and excretion of [3-^{14}C]quinclorac methyl ester (batch no. 753-1101; radiochemical purity 99.8%) were investigated in male bile duct–cannulated Wistar rats following a gavage dose at 15 or 600 mg/kg bw. Samples of urine, faeces, bile and tissues were

processed and analysed by LSC. Recovery was low (87%) at 15 mg/kg bw; the majority of the radiolabel was excreted within 24 hours, with 51% of the administered dose in urine and 30% in bile. At 600 mg/kg bw, excretion was slower, predominantly between 24 and 48 hours post-dosing, with the majority in bile (51%) and lesser amounts in urine (32%) and faeces (13%). Less than 1% of the administered radiolabel was detected in the carcass at 72 hours (Fabian & Landsiedel, 2011; Fabian, 2012).

Biotransformation

Urine and faecal samples in the study by Parker (1998) were extracted and analysed by high-performance liquid chromatography (HPLC). Only one major peak was identified in the majority of urine samples, which corresponded to quinclorac (approximately 80% of the radiolabel in the 8-hour sample). A secondary peak identified in the 24-hour urine sample was identified as quinclorac glucuronide following glucuronidase treatment. The faecal samples contained a multitude of metabolites, each less than 1.4% of the administered dose, which were not identified.

The biotransformation of quinclorac methyl ester was investigated using samples from the study of Fabian & Landsiedel (2011). Pooled samples of bile, urine and faeces were extracted and analysed by HPLC. At the low dose (15 mg/kg bw), there was no detectable quinclorac methyl ester in bile or urine; in faeces, quinclorac methyl ester represented only 0.45% of the administered dose. The predominant metabolite was quinclorac, representing 50% of the administered dose. At 600 mg/kg bw, the methyl ester represented 12% of the administered dose, and quinclorac, 17% (Thiaener, Glaessgen & Deppermann, 2011). Additional investigations of the bile samples from the 600 mg/kg bw group, using techniques including liquid chromatography with tandem mass spectrometry and electrospray ionization, time-of-flight mass spectrometry), identified or characterized further metabolites representing approximately half the administered dose (their structures and levels are presented in Tables A5 and A6 in Appendix 1) (Thiaener, Glaessgen & Deppermann, 2012).

A metabolic pathway for quinclorac methyl ester has been proposed, involving one or more of these primary steps:

- demethylation;
- glutathione conjugation at the chlorine on position 7; and
- arene oxide formation.

This can be followed by:

- glucuronidation;
- degradation and transformation of the glutathione moiety;
- hydroxylation of the quinoline ring structure; and
- dimerization.

Acute toxicity

Two groups of three female Wistar rats were administered quinclorac methyl ester (batch no. L84-18; purity 99.6%) by gavage in 0.5% carboxymethyl cellulose at 2000 mg/kg bw. Clinical signs, including dyspnoea, poor general state and piloerection, were seen, but there were no deaths. The acute oral LD_{50} was greater than 2000 mg/kg bw (Cords & Lammer, 2010).

Short-term studies of toxicity

Groups of 10 male and 10 female Wistar (Crl:WI(Han)) rats were given diets containing quinclorac methyl ester (batch no. L84-18; purity 99.6%) at a dose level of 0, 2000, 4000 or 8000 ppm for 3 months. Achieved test article intakes were 0, 128, 252 and 518 mg/kg bw per day for males and 0, 145, 274 and 509 mg/kg bw per day for females, respectively. Clinical signs, body weight and feed consumption were monitored regularly. Samples for clinical chemistry and haematology were

taken on day 92. Urine analysis samples were obtained on day 90. Ophthalmoscopy was performed on animals in the control and top-dose groups pretest and on day 90. A functional observational battery, including motor activity assessments, was performed on day 85. All animals were subjected to gross pathological examination followed by a microscopic examination. Control and top-dose animals received a full microscopic examination; for the low- and intermediate-dose groups, only thyroids, liver, kidneys and gross lesions were examined. Additional staining techniques were used on selected kidney samples.

There were no mortalities or treatment-related effects in the functional observational battery, ophthalmoscopy, haematology or urine analysis results. Discoloured urine, possibly due to excretion of test material (a beige powder), was reported at 4000 and 8000 ppm (Table 22). Body weights were reduced from day 7 onwards (Table 22) in both sexes receiving 8000 ppm and from day 21 in males receiving 4000 ppm. Feed consumption was consistently reduced in the 4000 and 8000 ppm groups. A number of clinical chemistry parameters were altered in top-dose animals (Table 22). The increased gamma-glutamyltranspeptidase (GGT) activity in top-dose males is indicative of liver toxicity. Although the inorganic phosphorus levels exhibit a dose-related increase, all values are within the normal range seen in the test laboratory. Absolute liver weights were significantly increased in males in the high-dose group (117%) and in females in all treatment groups (110%, 111% and 117% at 2000, 4000 and 8000 ppm, respectively). Absolute kidney weight in low-dose males (109%) and absolute adrenal gland weight in low-dose females (114%) were significantly increased, but these findings were not reproduced at higher dose levels and therefore are considered unrelated to quinclorac administration. The mean relative weights of the liver of animals in all treatment groups and of the thyroid glands of males in all treatment groups were significantly increased (Table 22) and are regarded as treatment-related effects. The significantly increased relative weights of adrenal glands, brain, epididymides, kidneys, spleen, thymus and testes are considered secondary to the decreased terminal body weights and not adverse in isolation.

Gross lesions of the liver (dark coloration and enlarged) were seen in treated males, but with no clear dose–response relationship or histopathological correlations. Histopathological changes included minimal to slight hepatocellular hypertrophy in male and female animals of all treatment groups (Table 22). In the thyroid gland of all treatment groups, an increased incidence of minimal to slight follicular hypertrophy/hyperplasia was evident (Table 22). The kidneys of treated males showed a "nuclear crowding" of tubular epithelial cells at the corticomedullary junction, increasing in incidence and degree of severity with dose. Immunohistochemical staining demonstrated an early cellular injury in affected areas (positive with the antibody against kidney injury molecule [KIM] antigen), but no increased proliferation rates (proliferating cell nuclear antigen [PCNA] and Ki-67 staining). No staining for α_{2u}-globulin was performed. There were no specific investigations of hepatic microsomal enzyme activity or thyroid hormones.

The authors of the study report proposed that the findings seen at the low dose levels were not adverse and/or not relevant to humans:

- The increased liver weight and hyperplasia are adaptive and related to increased microsomal enzyme activity.
- The increased thyroid weights and hyperplasia/hypertrophy are secondary to enhanced thyroid hormone clearance as a result of increases in uridine diphosphate-glucuronosyltransferase (UGT) activity.
- The kidney lesions are seen only in males and therefore are related to rat-specific chronic progressive nephropathy.

However, there are no specific data to support these contentions, and the suggestion that they are not treatment-related adverse findings is not accepted.

A NOAEL cannot be identified for this study owing to the presence of increased relative liver weights and hepatocellular hypertrophy, increased relative thyroid gland weights and hypertrophy/hyperplasia, and "nuclear crowding" of the kidney at 2000 ppm (equal to 128 mg/kg bw per day), the lowest dose tested (Buesen et al., 2011).

Table 22. Findings in rats receiving quinclorac methyl ester for 90 days

	Males				Females			
Observation	**0 ppm**	**2 000 ppm**	**4 000 ppm**	**8 000 ppm**	**0 ppm**	**2 000 ppm**	**4 000 ppm**	**8 000 ppm**
Occurrence of observation (%)								
Discoloured urine	0	0	100	100	0	0	100	100
Clinical chemistry (group mean values)								
GGT (nkat/L)	0	0	1	11**	2	1	5	2
Cholesterol (mmol/L)	1.98	2.26	2.91	2.57	1.36	1.58	1.88**	2.20**
Triglycerides (mmol/L)	0.85	0.86	0.79	0.42**	0.44	0.42	0.47	0.95**
Total bilirubin (µmol/L)	1.54	1.3	1.32	1.22	1.97	1.71	1.70	1.50**
Inorganic phosphate (mmol/L)	1.53	1.67*	1.70*	1.73*	1.20	1.17	1.34	1.41
Body weights (group mean values)								
Terminal body weight (g)	407.4	390.8	351.7**	340.8**	220.2	213.6	205.9	192.3**
Relative organ weights (% of body weight)								
Adrenal glands	0.014	0.015*	0.018**	0.018**	0.033	0.039**	0.035	0.033
Brain	0.516	0.523	0.577*	0.591**	0.863	0.900	0.925*	0.983**
Epididymides	0.271	0.299	0.318**	0.329**	–	–	–	–
Kidneys	0.578	0.657**	0.641	0.706**	0.683	0.703	0.742	0.733
Liver	2.165	2.476**	2.765**	3.038**	2.209	2.512**	2.627**	2.968**
- % of control	100	114	128	140	100	114	119	134
Testes	0.839	0.951*	1.018**	1.038**	–	–	–	–
Thyroid glands	0.005	0.007**	0.007*	0.007**	0.007	0.009	0.008	0.009
Incidence of microscopic findings in liver								
Hypertrophy (total)	0	5	9	10	0	3	6	10
Hypertrophy, centrilobular								
- Grade 1	0	0	0	0	0	3	4	1
- Grade 2	0	0	0	0	0	0	0	1
Hypertrophy, centrilobular to intermediate								
- Grade 1	0	5	2	0	0	0	2	0

Observation	Males				Females			
	0 ppm	2 000 ppm	4 000 ppm	8 000 ppm	0 ppm	2 000 ppm	4 000 ppm	8 000 ppm
- Grade 2	0	0	7	6	0	0	0	9
Hypertrophy, diffuse (grade 2)	0	0	0	4	0	0	0	0
Incidence of microscopic findings in thyroid glands								
Hypertrophy/ hyperplasia follicular								
- Grade 1	1	1	5	1	0	1	0	2
- Grades 2–4	0	1	2	7	0	1	6	8
Incidence of microscopic findings in kidneys								
Nuclear crowding	0	9	9	10	0	0	0	0
- Grade 1	–	6	4	–	–	–	–	–
- Grade 2	–	3	3	4	–	–	–	–
- Grade 3	–	–	2	6	–	–	–	–

GGT: gamma-glutamyltranspeptidase; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Buesen et al. (2011)

The pattern of toxicity seen with the methyl ester is not the same as that seen with quinclorac; therefore, a comparison of relative toxic potency is not straightforward. A comparison of the 90-day studies of toxicity in rats for quinclorac and quinclorac methyl ester results in a ratio of 2.4 (302/128) between the NOAEL for quinclorac and the LOAEL for the ester and a ratio of 7.3 (930/128) between the LOAEL for quinclorac and the LOAEL for the ester. The main metabolic step of the methyl ester is demethylation to quinclorac, which suggests that some aspects of the toxicity of the methyl ester will have been addressed by studies with quinclorac. In acute oral toxicity studies, similar clinical signs (poor general state, dyspnoea, piloerection) were reported at a dose of 2000 mg/kg bw for the methyl ester and a similar dose of 1780 mg/kg bw used in the oral LD_{50} study with quinclorac (Grundler & Kirsch, 1983a). The Meeting concluded that quinclorac methyl ester was likely to be less than 10-fold more toxic than quinclorac.

Several quinclorac conjugates were identified as plant metabolites. No specific toxicity data were available on these conjugates, but a structure–activity relationship (SAR) analysis (DEREK) identified no alerts that were not also present for quinclorac. It is expected that these conjugates will readily hydrolyse to the parent in the gastrointestinal tract. Therefore, it is expected that quinclorac conjugates will be of lower or equivalent toxicity compared with quinclorac.

3. Observations in humans

The facility that produces quinclorac also produces the closely related compound quinmerac. The production facility for quinclorac was designed as a closed system and is controlled by automation, with all the gas/dust and wastewater collected and incinerated at the production site. Given the design of the plant, the likelihood of exposure is low.

Currently, there are 41 people who work in the quinclorac production plant, including seven who work in the analysis laboratory. There is an annual medical examination for all the employees. In addition to a general medical examination, some workers will have additional testing as determined by the chemicals they handle in their daily work. To date, there have been no reports of exposure-related abnormal medical examinations in employees in the quinclorac production plant (Riffle, 2015).

Comments

Biochemical aspects

The toxicokinetics and biotransformation of quinclorac were investigated in rats administered (2,3,4-^{14}C)-labelled quinclorac at a single dose of 15, 100, 600 or 1200 mg/kg bw by gavage or at 15 000 ppm in the diet (equivalent to 1200 mg/kg bw); 7 daily doses of 15 or 600 mg/kg bw per day by gavage or at 15 000 ppm in the diet (equivalent to 1200 mg/kg bw per day); or 14 daily doses of 15 mg/kg bw per day of unlabelled quinclorac followed by a single labelled dose of 15 mg/kg bw. Absorption was rapid, with maximal blood concentrations achieved between 0.25 and 1 hour for single doses of 600 mg/kg bw and below. The extent of oral absorption was high (> 90%) at all dose levels, based on urinary and biliary data, with some of the biliary component being reabsorbed. Quinclorac was widely distributed in the body, with highest concentrations of radiolabel present in the blood, kidney and plasma. The labelled material was excreted primarily via urine (50–90% in 24 hours). Clearance from the blood was slower following repeated dosing with 600 mg/kg bw and a single dose of 1200 mg/kg bw, resulting in non-proportionate increases in AUC with dose. Absorbed quinclorac was metabolized to only a limited extent, with unchanged parent compound representing approximately 80% of the excreted radiolabel. The major biotransformation product was quinclorac–glucuronide conjugate, at approximately 5% of the administered dose. The excretion pattern, tissue distribution of radioactivity and/or metabolite profile were similar across administered dose levels and with single or repeated administration (Hawkins et al., 1986, 1987).

Toxicological data

Quinclorac was of low acute toxicity in rats via the oral route (LD_{50} = 2680 mg/kg bw) (Grundler & Kirsch, 1983a) or dermal route (LD_{50} > 2000 mg/kg bw) (Grundler & Kirsch, 1983b) and by inhalation (LC_{50} > 5.15 mg/L air) (Klimisch, 1986). Quinclorac was not irritating to the skin of rabbits (Grundler & Kirsch, 1983c), but was transiently and mildly irritating to the eyes of rabbits (Grundler & Kirsch, 1983d). Modern material of a high purity (99.4%) was not a skin sensitizer in guinea-pigs (Gamer & Leibold, 2005d), but a positive result was seen with older, less pure (97.4%) quinclorac (Kieczka, 1986).

In repeated-dose toxicity studies in mice, rats and dogs, the predominant effect was reduced body weight gain, often associated with reductions in feed consumption. The only organ showing consistency of effects was the kidney, with increases in organ weight and histopathological changes (e.g. interstitial nephritis) at high dose levels.

In a 90-day study of toxicity in mice, dietary concentrations of quinclorac were 0, 4000, 8000 and 16 000 ppm (equal to 0, 1001, 1992 and 4555 mg/kg bw per day for males and 0, 1466, 2735 and 5953 mg/kg bw per day for females, respectively). The NOAEL was 4000 ppm (equal to 1001 mg/kg bw per day), based on increases in blood urea levels and water consumption at 8000 ppm (equal to 1992 mg/kg bw per day). Slight changes in body weight and mean red blood cell volume were considered not to be adverse (Kuehborth et al., 1988).

In a subsequent 90-day dietary study of toxicity in mice administered 500 ppm quinclorac (equal to 85 mg/kg bw per day for males and 130 mg/kg bw per day for females), there were no treatment-related effects; the NOAEL was 500 ppm (equal to 85 mg/kg bw per day), the only dose tested (Schilling et al., 1988a).

In a 90-day study of toxicity in rats, dietary concentrations of quinclorac were 0, 1000, 4000 and 12 000 ppm (equal to 0, 77, 302 and 930 mg/kg bw per day for males and 0, 87, 358 and 1035 mg/kg bw per day for females, respectively). The NOAEL was 4000 ppm (equal to 302 mg/kg bw per day), on the basis of a range of clinical chemistry and haematology changes in both sexes and urothelial hyperplasia and interstitial nephritis in males at 12 000 ppm (equal to 930 mg/kg bw per day) (Kuehborth, Deckardt & Hildebrand, 1986).

In a 28-day dietary study in which dogs were administered quinclorac at 0, 1000, 3000, 9000 or 27 000 ppm (equal to 0, 31, 95, 278 and 912 mg/kg bw per day for males and 0, 36, 108, 315 and 956 mg/kg bw per day for females, respectively), the NOAEL was 9000 ppm (equal to 278 mg/kg bw per day), based on body weight loss and kidney lesions at 27 000 ppm (equal to 912 mg/kg bw per day) (Hellwig et al., 1985).

In a 1-year study in dogs in which quinclorac was administered in the diet at 0, 1000, 4000 or 12 000 ppm (equal to 0, 35, 139 and 490 mg/kg bw per day for males and 0, 35, 141 and 472 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 35 mg/kg bw per day), on the basis of an increase in relative kidney weight in males at 4000 ppm (equal to 139 mg/kg bw per day) (Hellwig et al., 1988a).

In a 78-week toxicity and carcinogenicity study in mice, dietary concentrations of quinclorac were 0, 1000, 4000 and 8000 ppm (equal to 0, 170, 711 and 1444 mg/kg bw per day for males and 0, 213, 869 and 1828 mg/kg bw per day for females, respectively). No NOAEL could be identified, as reductions in body weight were observed in females at all doses. Quinclorac did not produce any increase in the incidences of benign or malignant tumours (Schilling et al., 1988c).

A subsequent 78-week toxicity study in mice used a single dietary level of 250 ppm (equal to 42 mg/kg bw per day for males and 52 mg/kg bw per day for females). There were no adverse effects (Schilling et al., 1988d).

The Meeting concluded that the overall NOAEL for the 78-week toxicity studies in mice was 250 ppm (equal to 52 mg/kg bw per day), based on reductions in body weight in females at 1000 ppm (equal to 213 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in rats, dietary concentrations of quinclorac were 0, 1000, 4000 and 8000 ppm (equal to 0, 55, 221 and 444 mg/kg bw per day for males and 0, 66, 262 and 529 mg/kg bw per day for females, respectively) for evaluation of carcinogenic potential. Satellite groups received diets containing quinclorac at 0, 1000, 4000, 8000 or 12 000 ppm (equal to 0, 55, 221, 444 and 675 mg/kg bw per day for males and 0, 66, 262, 529 and 832 mg/kg bw per day for females, respectively) for the evaluation of toxicity. The only significant effect was a decrease in the body weight of top-dose females in the satellite group. The NOAEL was 8000 ppm (equal to 529 mg/kg bw per day), on the basis of reductions in body weight in females at 12 000 ppm (equal to 832 mg/kg bw per day). Quinclorac did not increase the incidence of benign or malignant tumours (Schilling et al., 1988b).

The Meeting concluded that quinclorac is not carcinogenic in mice or rats.

Quinclorac was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. The majority of studies produced negative results. Positive results were seen at high concentrations in a cytogenicity assay in human lymphocytes (Gelbke & Engelhardt, 1986a). In vivo assays of bone marrow micronucleus induction (Gelbke & Engelhardt, 1986b) and unscheduled DNA synthesis in hepatocytes (Fautz & Voelkner, 1991) gave negative results.

The Meeting concluded that quinclorac is unlikely to be genotoxic in vivo.

In view of the fact that quinclorac is unlikely to be genotoxic in vivo and the absence of carcinogenicity in mice and rats, the Meeting concluded that quinclorac is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats, with two matings in the F_1 generation and one in the F_2 generation, dietary concentrations of quinclorac were 0, 1000, 4000 and

12 000 ppm (equivalent to mean intakes of 0, 96, 381 and 1180 mg/kg bw per day, respectively). The NOAEL for reproductive effects was 12 000 ppm (equivalent to 1180 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 4000 ppm (equivalent to 381 mg/kg bw per day), based on an increase in the incidence of interstitial nephritis at 12 000 ppm (equivalent to 1180 mg/kg bw per day) in females of both generations. The NOAEL for effects on offspring was 4000 ppm (equivalent to 381 mg/kg bw per day), based on reduced pup weight during lactation at 12 000 ppm (equivalent to 1180 mg/kg bw per day) (Hellwig et al., 1988b).

In a study of developmental toxicity in rats dosed with quinclorac at 0, 24.4, 146 or 438 mg/kg bw per day by gavage in 0.5% carboxymethyl cellulose, there were no effects on any measured fetal parameters. The NOAEL for maternal toxicity was 146 mg/kg bw per day, on the basis of deaths, reduced feed intake, increased water intake and severe ulceration of the glandular stomach at 438 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 438 mg/kg bw per day, the highest dose tested (Hellwig, 1987).

In a study of developmental toxicity in rabbits dosed with quinclorac at 0, 70, 200 or 600 mg/kg bw per day by gavage in 0.5% carboxymethyl cellulose, severe maternal toxicity, including death, was observed at 600 mg/kg bw per day. Live pup numbers were reduced at 600 mg/kg bw per day. At the top dose level, there was an increase in the number of pups with skeletal variations, although there was no significant increase in any specific variation. There was no increase in the number of pups with malformations. The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on mortality and body weight loss at 600 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 200 mg/kg bw per day, based on an increase in fetuses with skeletal variations, reduced numbers of viable fetuses and reduced fetal weights at 600 mg/kg bw per day (Hellwig, Hildebrand & Shirasu, 1988).

The Meeting concluded that quinclorac is not teratogenic in rats or rabbits.

The acute neurotoxicity of quinclorac was investigated in rats administered dose levels of 0, 150, 500 or 1500 mg/kg bw by gavage in 1% carboxymethyl cellulose. Dose-related reductions in locomotor activity were seen at 4–5 hours post-dosing, but not subsequently, in the mid- and high-dose groups. Motor activity reductions in males in the low-dose group were considered not to be treatment related, as the background activity in this group was consistently lower than in the other groups. There were no indications of neuropathy. The NOAEL was 150 mg/kg bw, based on reduced motor activity at 500 mg/kg bw (Buesen et al., 2012a).

In a subchronic (90-day) neurotoxicity study in rats, dietary concentrations of quinclorac were 0, 1500, 5000 and 15 000 ppm (equal to 0, 96, 301 and 976 mg/kg bw per day for males and 0, 112, 368 and 1142 mg/kg bw per day for females, respectively). No adverse effects were reported. The NOAEL for neurotoxicity was 15 000 ppm (equal to 976 mg/kg bw per day), the highest dose tested (Buesen et al., 2012b).

The reduced motor activity seen in the acute neurotoxicity study is a relatively general finding, not specific to a neurotoxic mode of action; there was no evidence of specific neurotoxic findings in other studies. The Meeting concluded that quinclorac is not neurotoxic.

In a 28-day immunotoxicity study in female mice, dietary concentrations were 0, 500, 1500 and 5000 ppm (equal to 0, 176, 439 and 1760 mg/kg bw per day, respectively). No adverse effects were reported. The NOAEL for immunotoxicity was 5000 ppm (equal to 1760 mg/kg bw per day), the highest dose tested (Buesen et al., 2010).

The Meeting concluded that quinclorac is not immunotoxic.

Biochemical and toxicological data on metabolites and/or degradates

[3-^{14}C]Quinclorac methyl ester, a plant metabolite, administered to rats at 15, 50 or 600 mg/kg bw by gavage was rapidly and extensively absorbed and excreted via urine and bile. The proportion of radiolabel in the bile increased from 30% at 15 mg/kg bw to 51% at 600 mg/kg bw, with

a corresponding decrease in radiolabel in urine (51% and 32%, respectively) (Fabian & Landsiedel, 2011). The initial steps in biotransformation involved extensive demethylation to release free quinclorac (approximately 50% of the administered dose) (Parker, 1998; Thiaener, Glaessgen & Deppermann, 2011). Other metabolic steps, identified from biliary metabolites, were arene oxide formation and conjugation with glutathione, with subsequent transformation of the glutathione moiety, hydroxylation of the quinoline structure and dimerization (Thiaener, Glaessgen & Deppermann, 2012).

Quinclorac methyl ester has a low acute oral toxicity to rats (LD_{50} > 2000 mg/kg bw); clinical signs (poor general state, dyspnoea and piloerection) were noted (Cords & Lammer, 2010).

In a repeated-dose study of toxicity in rats, quinclorac methyl ester was administered in the diet at 0, 2000, 4000 or 8000 ppm (equal to 0, 128, 252 and 518 mg/kg bw per day for males and 0, 145, 274 and 509 mg/kg bw per day for females, respectively) for 3 months. The main findings were reduced body weight, increased relative organ weights and histopathological changes of the liver, thyroid and kidney. A NOAEL could not be identified, as increased relative liver weights, hepatocellular hypertrophy, increased relative thyroid gland weights, thyroid hypertrophy/hyperplasia and "nuclear crowding" of the kidney were observed at 2000 ppm (equal to 128 mg/kg bw per day), the lowest dose tested (Buesen et al., 2011).

The pattern of toxicity seen with the methyl ester is not the same as that seen with quinclorac; therefore, a comparison of relative toxic potency is not straightforward. A comparison of the 90-day studies of toxicity in rats for quinclorac and quinclorac methyl ester results in a ratio of 2.4 (302/128) between the NOAEL for quinclorac and the LOAEL for the ester and a ratio of 7.3 (930/128) between the LOAEL for quinclorac and the LOAEL for the ester. The main metabolic step of the methyl ester is demethylation to quinclorac, which suggests that some aspects of the toxicity of the methyl ester will have been addressed by studies with quinclorac. In acute oral toxicity studies, similar clinical signs (poor general state, dyspnoea, piloerection) were reported at a dose of 2000 mg/kg bw for the methyl ester and a similar dose of 1780 mg/kg bw used in the oral LD_{50} study with quinclorac (Grundler & Kirsch, 1983a). The Meeting concluded that quinclorac methyl ester was likely to be less than 10-fold more toxic than quinclorac.

Several quinclorac conjugates were identified as plant metabolites. No specific toxicity data were available on these conjugates, but a SAR analysis identified no alerts that were not also present for quinclorac. The Meeting concluded that these conjugates were likely to be of lower or equivalent toxicity compared with the parent compound, because they are expected to be readily hydrolysed to the parent in the gastrointestinal tract.

Human data

No adverse effects have been reported in quinclorac production and formulation plant workers, and no significant effects have been reported in exposed users of quinclorac-based products (Riffle, 2015).

The Meeting concluded that the existing database on quinclorac 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 quinclorac of 0–0.4 mg/kg bw, on the basis of the NOAEL of 35 mg/kg bw per day for increased relative kidney weights from the 1-year dog study. A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) for quinclorac of 2 mg/kg bw, on the basis of the NOAEL of 150 mg/kg bw per day for reductions in motor activity in the acute neurotoxicity study in rats. A safety factor of 100 was applied.

The plant metabolite quinclorac methyl ester is not found in rats administered quinclorac. The main metabolic step for the methyl ester in rats is demethylation to quinclorac. Similar clinical signs were seen at similar doses in the acute oral toxicity studies with quinclorac and the methyl ester. In a 90-day study of toxicity in rats, the methyl ester produced a pattern of liver, kidney and thyroid effects that differed from that seen in the equivalent study with quinclorac, and the LOAEL for the methyl ester was below the NOAEL for quinclorac.

The Meeting concluded that the methyl ester is likely to be less than 10-fold more toxic than quinclorac, that a 10-fold potency factor should be applied to the residue levels for use in both the acute and chronic dietary exposure estimates for quinclorac and that these should be added to the acute and chronic dietary exposures for quinclorac and compared with the ARfD and ADI for quinclorac, respectively.

The Meeting concluded that the quinclorac conjugates were of no greater toxicity than the parent.

Both the ADI and ARfD are established for the sum of quinclorac and its conjugates, and quinclorac methyl ester (×10), expressed as quinclorac.

Levels relevant to risk assessment of quinclorac and quinclorac methyl ester

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week studies of toxicity and carcinogenicity[a,b]	Toxicity	250 ppm, equal to 52 mg/kg bw per day	1 000 ppm, equal to 213 mg/kg bw per day
		Carcinogenicity	8 000 ppm, equal to 1 444 mg/kg bw per day[c]	–
Rat	Acute neurotoxicity study[d]	Neurotoxicity	150 mg/kg bw	500 mg/kg bw
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	8 000 ppm, equal to 529 mg/kg bw per day	12 000 ppm, equal to 832 mg/kg bw per day
		Carcinogenicity	8 000 ppm, equal to 444 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	12 000 ppm, equivalent to 1 180 mg/kg bw per day[c]	–
		Parental toxicity	4 000 ppm, equivalent to 381 mg/kg bw per day	12 000 ppm, equivalent to 1 180 mg/kg bw per day
		Offspring toxicity	4 000 ppm, equivalent to 381 mg/kg bw per day	12 000 ppm, equivalent to 1 180 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	146 mg/kg bw per day	438 mg/kg bw per day
		Embryo and fetal toxicity	438 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	200 mg/kg bw per day	600 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Embryo and fetal toxicity	200 mg/kg bw per day	600 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	1 000 ppm, equal to 35 mg/kg bw per day	4 000 ppm, equal to 139 mg/kg bw per day
Metabolite: Quinclorac methyl ester				
Rat	Ninety-day study of toxicity	Toxicity	–	2 000 ppm, equal to 128 mg/kg bw per day[e]

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

Estimate of acceptable daily intake (ADI) for the sum of quinclorac and its conjugates, and quinclorac methyl ester (×10), expressed as quinclorac

0–0.4 mg/kg bw

Estimate of acute reference dose (ARfD) for the sum of quinclorac and its conjugates, and quinclorac methyl ester (×10), expressed as quinclorac

2 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 quinclorac

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} = 0.25–1 h) and extensive (> 90%)
Dermal absorption	No data
Distribution	Widely distributed; highest levels in blood, kidney and plasma
Potential for accumulation	None
Rate and extent of excretion	Rapid (up to 90% excreted in urine within 24 h)
Metabolism in animals	Limited; 80% excreted unchanged; some glucuronidation
Toxicologically significant compounds in animals and plants	Quinclorac; quinclorac methyl ester; conjugates
Acute toxicity	
Rat, LD_{50}, oral	2 680 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.15 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Mildly, transiently irritating

Guinea-pig, dermal sensitization	Negative (maximization test)
Short-term studies of toxicity	
Target/critical effect	Reduced body weights; increased kidney weight, interstitial nephritis, urothelial hyperplasia
Lowest relevant oral NOAEL	35 mg/kg bw per day (12 months; dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (rabbit; highest dose tested)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight
Lowest relevant NOAEL	52 mg/kg bw per day (mouse)
Carcinogenicity	Not carcinogenic in mice or rats[a]
Genotoxicity	
	Unlikely to be genotoxic in vivo[a]
Reproductive toxicity	
Target/critical effect	No effects on reproduction; reduced pup weight during lactation
Lowest relevant parental NOAEL	381 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	381 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	1 180 mg/kg bw per day (rat; highest dose tested)
Developmental toxicity	
Target/critical effect	No effects in rats; reduction in viable fetuses, decreased fetal weight, increase in skeletal variations (rabbit)
Lowest relevant maternal NOAEL	146 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	200 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	150 mg/kg bw (decreased motor activity; rat)
Subchronic neurotoxicity NOAEL	976 mg/kg bw per day (rat; highest dose tested)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 760 mg/kg bw per day (mouse; highest dose tested)
Studies on toxicologically relevant metabolites	*Quinclorac methyl ester* Rapidly and extensively absorbed. Initial step in metabolism is demethylation to quinclorac. Acute oral LD_{50} > 2 000 mg/kg bw LOAEL in 90-day rat study = 128 mg/kg bw per day (lowest dose tested), based on kidney, liver and thyroid effects
Medical data	
	No adverse effects reported in humans

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

Summary

	Value	Study	Safety factor
ADI	0–0.4 mg/kg bw	One-year toxicity study (dog)	100
ARfD	2 mg/kg bw	Acute neurotoxicity study (rat)	100

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Appendix 1. Supplementary tables

Table A1. Historical control data for pancreatic lesions in rats: hyperplasia

Study	No. observed per sex	Males		Females	
		Frequency	%	Frequency	%
8116	50	0	0.0	0	0.0
8240	50	4	8.0	0	0.0
8241	50	0	0.0	0	0.0
8249	50	13	26.0	0	0.0
8345	50	0	0.0	0	0.0
8352	50	6	12.0	0	0.0
8424	98/100	8	8.2	3	3.0
8425	20	0	0.0	0	0.0
8519	50	3	6.0	1	2.0
8519	20	1	5.0	1	5.0
8583	20	0	0.0	0	0.0
8584	50	0	0.0	0	0.0
8591	20	0	0.0	1	5.0
8592	50	0	0.0	0	0.0
8604	50	4	8.0	1	2.0
87046	20	1	5.0	0	0.0

Study	No. observed per sex	Males		Females	
		Frequency	%	Frequency	%
87047	50	1	2.0	0	0.0
Sum	748/750	41	80.2	7	17.0
Mean	–	–	4.7	–	1.1
Median	–	–	2.0	–	0.0

Source: Schilling et al. (1988); Riffle (2015)

Table A2. Historical control data for pancreatic lesions in rats: adenoma and adenocarcinoma

Study	No. observed per sex	Males				Females			
		Adenoma		Adenocarcinoma		Adenoma		Adenocarcinoma	
		Frequency	%	Frequency	%	Frequency	%	Frequency	%
8116	50	0	0.0	0	0.0	1	2.0	1	0.0
8240	50	0	0.0	0	0.0	1	2.0	0	0.0
8241	50	4	8.0	0	0.0	0	0.0	0	0.0
8249	50	2	4.0	0	0.0	0	0.0	0	0.0
8345	50	9	18.0	1	2.0	4	8.0	0	0.0
8352	50	0	0.0	0	0.0	0	0.0	0	0.0
8424	98/100	9	9.2	5	5.1	1	1.0	0	0.0
8425	20	0	0.0	0	0.0	0	0.0	0	0.0
8519	50	0	0.0	0	0.0	0	0.0	0	0.0
8519	20	0	0.0	1	5.0	0	0.0	0	0.0
8583	20	1	5.0	0	0.0	0	0.0	0	0.0
8584	50	4	8.0	0	0.0	1	2.0	0	0.0
8591	20	1	5.0	0	0.0	0	0.0	0	0.0
8592	50	1	2.0	0	0.0	0	0.0	0	0.0
8604	50	8	16.0	0	0.0	0	0.0	0	0.0
87046	20	0	0.0	0	0.0	0	0.0	0	0.0
87047	50	0	0.0	0	0.0	0	0.0	0	0.0
Sum	748/750	39	–	7	–	8	–	1	–
Mean	–	–	4.4	–	0.7	–	0.9	–	0.1
Median	–	–	5.0	–	0.0	–	0.0	–	0.0

Source: Schilling et al. (1988); Riffle (2015)

Table A3. Cytogenetics assay in human lymphocytes

	Without S9 mix						With S9 mix					
	0 µg/L	DMSO	250 µg/L	500 µg/L	1 000 µg/L	MMC	0 µg/L	DMSO	500 µg/L	1 000 µg/L	2 000 µg/L	CP
Analysed metaphases	200	200	200	200	200	100	200	200	200	200	200	100
Aberrant	5	5	13	19	29	62	11	7	10	14	21	27

	Without S9 mix						With S9 mix					
	0 µg/L	DMSO	250 µg/L	500 µg/L	1 000 µg/L	MMC	0 µg/L	DMSO	500 µg/L	1 000 µg/L	2 000 µg/L	CP
metaphases including gaps												
Aberrant metaphases excluding gaps	1	2	3	3	18	58	2	1	2	3	8	19
Metaphases with exchanges	–	1	–	–	–	26	–	–	1		4	4
Polyploidy	1	1	2	2	1	–	4	–	1	1	10	1

CP: cyclophosphamide; DMSO: dimethyl sulfoxide; MMC: mitomycin C; S9: 9000 × *g* supernatant fraction from rat liver homogenate
Source: Gelbke & Engelhardt (1986)

Table A4. Gene mutation in mammalian cell gene mutation (second assay), with metabolic activation

Concentration (mg/mL)	Mutagenicity (number of colonies 7 days after seeding)					MR	MR		Cytotoxicity[a]			
							a	b	a		b	
									CE	%	CE	%
0	0	0	0	0	0	0	0	0	206/195	100.25	195/217	103.00
DMSO	1	0	0	1	1	2	2.27	2.08	196/156	88.00	190/195	96.25
0.0464	0	0	0	0	0	0	0	0	188/204	98.00	160/193	88.25
0.1	0	0	0	0	0	0	0	0	201/199	100.00	210/219	107.25
0.215	0	0	0	0	0	0	0	0	146/165	77.75	195/209	101.00
0.464	1	0	1	1	1	2.67	3.33	2.73	153/167	80.00	195/196	97.75
1.0	1	1	1	2	3	5.33	8.89	5.32	117/123	60.00	205/196	100.25
2.15	0	0	0	0	0	0	0	0	0/0	0	0	0
MCA 0.01	18	13	23	19	20	62.00	64.08	64.42	210/177	96.75	191/194	96.25

CE: cloning efficiency; DMSO: dimethyl sulfoxide; MCA: methylcholanthrene, positive control; MR: mutation rate
[a] "a" is for cytotoxicity based on cloning efficiency at 18–20 hours post-exposure; "b" is for cytotoxicity based on cloning efficiency at the end of the expression period.
Source: Jaeckh & Hoffmann (1990)

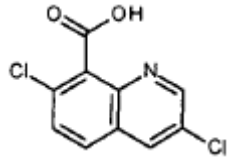
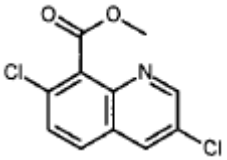
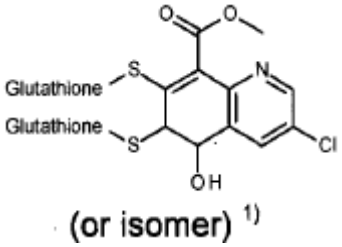
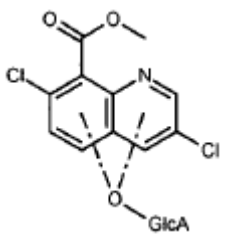
Table A5. Metabolites found in the bile of rats administered quinclorac methyl ester (600 mg/kg bw)

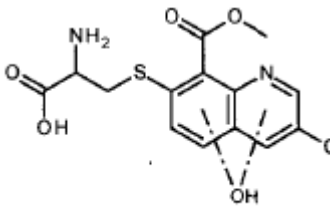
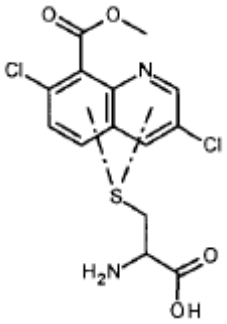
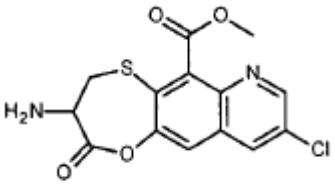
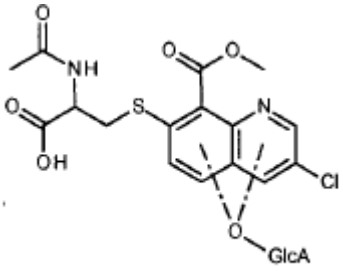
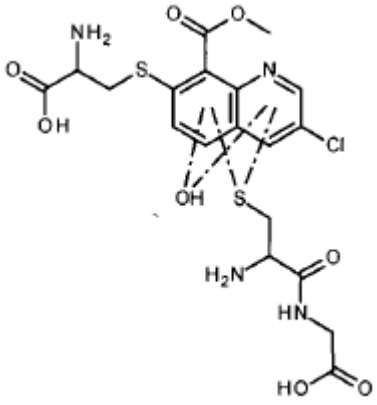
Designation of metabolite / peak (structures are in Table A6)	% TRR	% of dose
TRR (sample no. Lab0001)	100.00	46.56
Identified		
SES16382	5.69	2.65
SES16466 / SES16468 / SES16470	6.53	3.04

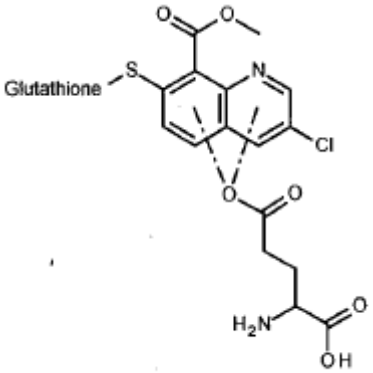
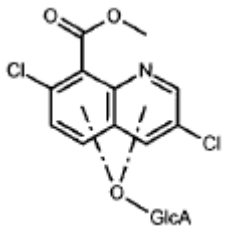
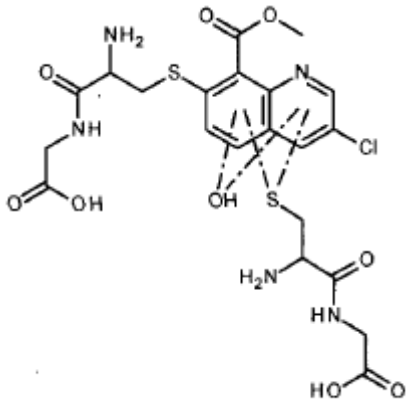
Designation of metabolite / peak (structures are in Table A6)	% TRR	% of dose
SES16442 / SES16458 / SES16454	21.47	10.00
SES16450 / SES16448 / SES16456	15.67	7.30
SES16438 and its isomer SES16452 / SES 16446 / MW = 443	5.81	2.70
Reg. No. 150732 (BAS 514 H)	1.61	0.75
Total identified	56.78	26.44
Characterized		
MW = 824 (dimer of SES16458), $C_{32}H_{30}Cl_2N_6O_{12}S_2$	6.84	3.19
28 further HPLC peaks (each below or equal to 1.88% of the dose)	36.38	16.94
Total characterized	43.22	20.12
Total identified and/or characterized	**100.00**	**46.56**

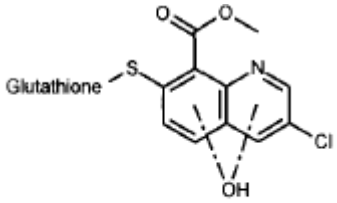
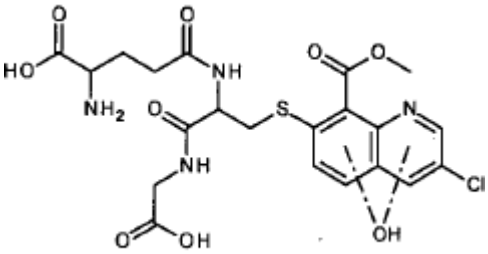
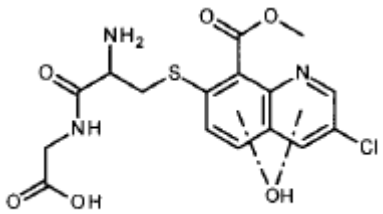
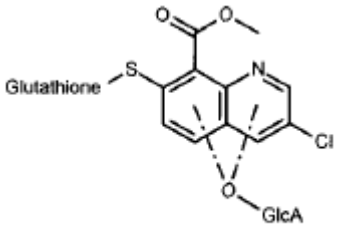
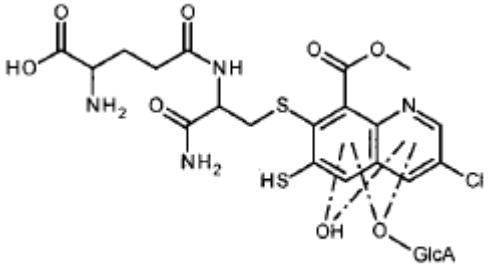
bw: body weight; HPLC: high-performance liquid chromatography; MW: molecular weight; TRR: total radioactive residue (in bile)
Source: Thiaener, Glaessgen & Deppermann (2012)

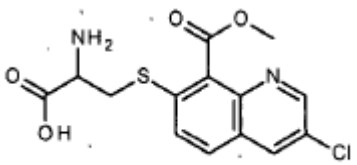
Table A6. Metabolites of ^{14}C-labelled quinclorac methyl ester identified or characterized in bile after oral administration to male rats

Metabolite code	Molecular formula	Nominal mass	Sample no.	Structural formula
SES1556 BAS 514 H Reg. No. 150732	$C_{10}H_5Cl_2NO_2$	241		
SES218 BH 514-Me Reg. No. 161555	$C_{11}H_7Cl_2NO_2$	255		
SES16382	$C_{31}H_{40}ClN_7O_{15}S_2$	849	Lab0005	 (or isomer) [1)]
SES16438	$C_{17}H_{15}Cl_2NO_9$	447	Lab0007 and Lab0017	

Metabolite code	Molecular formula	Nominal mass	Sample no.	Structural formula
SES16440	$C_{14}H_{13}ClN_2O_5S$	356	Lab0017	 (or isomer) [1)]
SES16442	$C_{14}H_{12}Cl_2N_2O_4S$	374	Lab0007 and Lab0017	
SES16444	$C_{14}H_{11}ClN_2O_4S$	338	Lab0017	 (or isomer) [1)]
SES16446	$C_{22}H_{23}ClN_2O_{12}S$	574	Lab0007 and Lab0017	 (or isomer) [1)]
SES16448	$C_{19}H_{21}ClN_4O_8S_2$	532	Lab0007 and Lab0017	 (or isomer) [1)]

Metabolite code	Molecular formula	Nominal mass	Sample no.	Structural formula
SES16450	$C_{26}H_{30}ClN_5O_{12}S$	671	Lab0007 and Lab0017	 (or isomer) [1)]
SES16452	$C_{17}H_{15}Cl_2NO_9$	447	Lab0017	
SES16454	$C_{21}H_{24}ClN_5O_9S_2$	589	Lab0007 and Lab0017	 (or isomer) [1)]

Metabolite code	Molecular formula	Nominal mass	Sample no.	Structural formula
SES16456	$C_{21}H_{23}ClN_4O_9S$	542	Lab0007 and Lab0017	 (or isomer) [1)] i. e.  (or isomer) [1)]
SES16458	$C_{16}H_{16}ClN_3O_6S$	413	Lab0007 and Lab0017	 (or isomer) [1)]
SES16466	$C_{27}H_{31}ClN_4O_{15}S$	718	Lab0006	 (or isomer) [1)]
SES16468	$C_{25}H_{29}ClN_4O_{14}S_2$	708	Lab0006	 (or isomer) [1)]

Metabolite code	Molecular formula	Nominal mass	Sample no.	Structural formula
SES16470	$C_{14}H_{13}ClN_2O_4S$	340	Lab0006	 (or isomer) [1)]
–	$C_{32}H_{30}Cl_2N_6O_{12}S_2$	824	Lab0008	Oxidative dimer of SES16458
–	–	443	Lab0007	

[1)] Positions of metabolically introduced substituents were not determined.
Source: Thiaener et al. (2012)

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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 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.
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This volume contains toxicological monographs that were prepared by the 2015 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Geneva on 15–24 September 2015.

The monographs in this volume summarize the safety data on 10 pesticides that could leave residues in food commodities. These pesticides are abamectin, acetochlor, cyazofamid, ethephon, flonicamid, flumioxazin, flupyradifurone, lufenuron, penconazole and quinclorac. 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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